

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA



TESIS DOCTORAL

Síntesis y evaluación de heterociclos activos para el diagnóstico precoz y tratamiento de enfermedades cerebrales

Synthesis and evaluation of active heterocycles for the early diagnosis and treatment of brain diseases

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

María Gracia Baquero Gálvez

Directores

Francisco Sánchez Sancho
Aurelio García Csäky
Aikaterini Lala

Madrid

UNIVERSIDAD COMPLUTENSE DE MADRID

Facultad de Farmacia



Tesis Doctoral

*SÍNTESIS Y EVALUACIÓN DE HETEROCICLOS ACTIVOS PARA EL
DIAGNÓSTICO PRECOZ Y TRATAMIENTO DE ENFERMEDADES
CEREBRALES*

*SYNTHESIS AND EVALUATION OF ACTIVE HETEROCYCLES FOR THE
EARLY DIAGNOSIS AND TREATMENT OF BRAIN DISEASES*

Memoria para optar al Grado de Doctor presentada por

María Gracia Baquero Gálvez

Directores

Dr. Francisco Sánchez Sancho

Dr. Aurelio García Csäky

Dra. Aikaterini Lalatsa

**SYNTHESIS AND EVALUATION OF ACTIVE
HETEROCYCLES FOR THE EARLY DIAGNOSIS AND
TREATMENT OF BRAIN DISEASES**

SÍNTESIS Y EVALUACIÓN DE HETEROCICLOS ACTIVOS
PARA EL DIAGNÓSTICO PRECOZ Y TRATAMIENTO DE
ENFERMEDADES CEREBRALES



UNIVERSIDAD COMPLUTENSE
MADRID

by

MARIA GRACIA BAQUERO GÁLVEZ

Advisors:

Dr. Francisco Sánchez Sancho

Dr. Aurelio García Csäky

Dra. Aikaterini Lalatsa

School of Pharmacy

Year 2020

This doctoral thesis work has been supported by:



Obra Social "la Caixa"

A Nicola

Agradecimientos

Esta tesis comenzó a escribirse el 14 de marzo de 2020, día en el que se estableció en España el Estado de Alarma debido la pandemia mundial causada por SARS-CoV-2. Muchos recordarán ese día como el inicio de un largo periodo de incertidumbre y confinamiento. En cambio para mí fue el inicio del final de una etapa, el final de mi experiencia como estudiante de doctorado.

A lo largo de este camino tan lleno de baches, he tenido la suerte de contar con personas que me han ayudado, soportado y alentado para seguir adelante.

En primer lugar, quisiera agradecer a mis tres directores de tesis, Dr. Francisco Sánchez Sancho, Dr. Aurelio García Csäky y Dra. Katerina Lalatsa, su inestimable labor guiando este trabajo de tesis doctoral. Gracias a Aurelio, por darme su voto de confianza incluso en los momentos más difíciles del grupo, y por sus clases de heterociclos que, aunque él diga lo contrario, son interesantes y han sido de gran provecho para mí. Gracias a Katerina, por admitirme en su grupo y permitirme desarrollar mis habilidades más allá de la síntesis orgánica. Mi estancia en Portsmouth no solo ha permitido la finalización de esta tesis, sino también vivir una experiencia en el extranjero que estoy segura me abrirá muchas puertas en el futuro. Por último y no menos importe, a Paco, porque con él empezó este camino. Gracias por tu disponibilidad, tu paciencia, incansable sentido del humor y optimismo. Después de casi 4 años bajo su dirección, me llevo conmigo los buenos ratos de trabajo en el laboratorio, todos los trucos de químico experimentado que no vienen en la bibliografía y un amigo.

En segundo lugar, un sentido agradecimiento todos los colaboradores y profesores que de muchas maneras diferentes han aportado su granito de arena. Gracias a la Dra. Laura Lagartera y Ricardo por su gran ayuda con SPR; al Dr. Luis García y la Dra. Mercedes Delgado, al profesor John McGeehan y especialmente al Dr. Chenyi Wu, por su ayuda y paciencia resolviendo mis interminables dudas de enzimología. A Maite y Elisa del servicio de RMN, a Guada, Felipe y Dani del servicio de HPLC/MS.

Un especial agradecimiento a mis amigos y compañeros dentro y fuera del laboratorio. A Silvia, por toda su ayuda y consejos; a mi compañeros Ana, Clara, Martín, Alberto, Andrea, Jesús y Carlos. A todos mis compañeros del IQM. A Diego, que siempre estuvo ahí para echarnos un cable y un rotavapor siempre que hiciera falta. A mis compañeros de Portsmouth, a Santina, a Nadine. A Tosca y Prospero, por su gran ayuda y consejos en los ensayos celulares. A Marta y María, que siempre han estado y estarán a mi lado.

A la Fundación la Caixa, por concederme una fantástica beca para poder continuar mis estudios y por permitirme conocer a todos mis amigos y compañeros becarios. Todos y cada uno de ellos son personas fantásticas y admirables, y - al contrario de lo que todos pensábamos cuando nos vimos por primera vez - unos *nerds* con mucho juego.

A Teresa y Marco, por cuidarme como si fuese una hija, sobre todo en este último periodo.

Gracias a mis padres y a mi hermana, por su incondicional apoyo, por hacerme ser quien soy y creer en lo que hago. Si hoy estoy aquí es gracias a vosotros. Esta tesis también lleva vuestro nombre.

Ahora llega la parte más compleja, pues debo poner en pocas líneas mi eterno agradecimiento a mi marido. Gracias Nicola por ser un hombre en el que apoyarme, por escucharme, aconsejarme y por sacar lo mejor mí misma. Me siento muy afortunada porque nunca me he sentido sola: a mi lado había una persona que comprende lo que hay detrás de un doctorado. Esta tesis ha sido una dura prueba para los dos, y la hemos superado.

Table of Contents

Agradecimientos.....	IV
Resumen.....	XI
Abstract.....	XIII
List of Tables.....	XV
List of Figures.....	XVI
List of Acronyms	XXV
CHAPTER 1.....	1
1.1 INTRODUCTION	2
1.1.1 Alzheimer's disease and tauopathies	2
Amyloid- β biogenesis and the amyloid cascade hypothesis	2
The genetics of Alzheimer's Disease:.....	6
Redefinition of the amyloid cascade	8
1.1.2 Tau Protein.....	8
Tau protein isoforms and biological function	8
Tau aggregation process	10
Tau and Amyloid β : "I need you - You need me"	16
1.1.3 Diagnosis of AD	17
Tau and Amyloid- β PET imaging	19
Tau PET radiotracers	19
1.2 HYPOTHESIS, AIM AND OBJECTIVES.....	24
1.3 RESULTS AND DISCUSSION.....	25
1.3.1 Introduction and background	25
Tau imaging and the requirements for tau PET-radiotracer design.....	25
Imidazo[1,2- <i>a</i>]pyridines	25
Synthetic methods for the preparation of imidazo[1,2- <i>a</i>]pyridines	26
Multicomponent Reactions	31
Intramolecular C-H amination	34
Oxidative coupling	35
1.3.2 Results and discussion	39
Synthesis of imidazo[1,2- <i>a</i>]pyridine derivatives.....	39

Synthesis of 2-aminopyridine derivatives.....	47
Cold ligands and radiosynthesis precursors	48
1.3.3 Tau Aggregation and Morphological Studies.....	50
1.3.4 Binding studies with Surface Plasmon Resonance	56
1.3.5 Synthesis and SPR studies on imidazo[2,1- <i>b</i>]thiazole derivatives.....	61
Previous work	61
Synthesis of imidazo[2,1- <i>b</i>]thiazole derivatives	63
1.3.6 Fluorescence-based affinity studies - Thioflavin T competition assay	65
ThT competition assay with insulin aggregates.....	65
Screening of imidazopyridine and imidazothiazole analogues with tau aggregates	73
1.3.7 Computer-assisted modelling studies	78
1.3.8 Comparison of ThT competitive assay and SPR interaction study: a theory supported by molecular modelling	81
1.3.9 Haemolysis assay	83
1.4 CONCLUSIONS	85
1.5 MATERIALS AND METHODS	86
1.5.1 Materials and Equipment	86
1.5.2 Synthesis of Imidazo[1,2- <i>a</i>]pyridine and Imidazo[2,1- <i>b</i>]thiazole Derivatives.....	88
Experimental procedures A-C for the synthesis of imidazo[1,2- <i>a</i>]pyridine derivatives 1 and 9.	88
Experimental procedure C: Heck's reaction	99
Experimental procedure D for the synthesis of 2-aminopyridines derivatives.....	100
Experimental procedure E for the synthesis of cold ligand 1n:.....	103
Experimental procedure F for the synthesis of 6-arylimidazo[2,1- <i>b</i>]thiazole derivatives, 14.	104
1.5.3 Thioflavin T Competition Assays with Amyloid Proteins	106
1.5.4 Morphological Studies	109
Atomic Force Microscopy (AFM)	109
1.5.5 Surface Plasmon Resonance (SPR).....	109
1.5.6 Modelling Studies	110
Docking simulations.....	110
Re-scoring methodology.....	110

1.5.7	<i>Ex Vivo Red Blood Cell Haemolysis Assay</i>	110
1.5.8	Statistics	111
CHAPTER 2		112
2.1	INTRODUCTION	113
2.1.1	Cancer and the Warburg effect	113
2.1.2	Human lactate dehydrogenase.....	116
	<i>h</i> LDH isoforms	116
	The catalytic activity of LDH.....	117
	LDH and metabolic reprogramming in cancer	119
	Hypoxia as an Obstacle to Medical Treatment of Cancer	120
	Taking advantage of Tumour Hypoxia: LDH-A inhibition	120
2.1.3	Lactate dehydrogenase A inhibitors	121
	Gossypol and its derivatives	121
	Naphthoic acids.....	122
	Piperidinone derivatives	122
	Pyrazole and indole derivatives	123
	Pyrazolidine derivatives	124
2.1.4	Enzymology: a short introduction.....	126
	The rapid equilibrium model of enzyme kinetics	126
	The steady state model of enzyme kinetics.....	129
	The significance of K_m – Interpretation of the Michaelis-Menten equation.....	132
	What value do K_m add to the understanding of the enzyme under study?	133
	“Apparent” Michaelis-Menten constant, K_m'	133
2.2	HYPOTHESIS, AIM AND OBJECTIVES.....	135
2.3	RESULTS AND DISCUSSION.....	136
2.3.1	Biologically active quinones.....	136
2.3.2	Synthesis of Quinone-Amino acid Conjugates.....	137
	Structural elucidation of compound 17x	139
	Summary of the synthesised candidate LDH inhibitors.....	145
2.3.3	Assessment of the Inhibitory Activity of Quinone Activity of Quinone-Amino Acid Conjugates	146
	Screening with Surface Plasmon Resonance (SPR).....	146

Kinetic studies with SPR	148
Enzymatic assays	152
Screening of inhibitory activity of quinone-amino acid hybrids on enzymatic assays	152
Determination of the inhibition constant (K_i)	154
Experimental obtaining of K_i	156
Effect of inhibitors in binding isotherms of pyruvate and NADH	158
Different substitutions modulate the inhibitory potency	161
Determination of the IC_{50}	163
2.3.4 K_D , K_i and IC_{50}	165
2.3.5 Antiproliferative Assays	166
2.4 CONCLUSIONS	168
2.5 MATERIALS AND METHODS	169
2.5.1 Materials and Equipment	169
2.5.2 Synthesis and characterization of Quinone-Amino Acid Conjugates	171
2.5.3 Enzymatic assays	177
Determination of Michaelis-Menten constants:	177
Screening of quinone - amino acids hybrids as LDH-A inhibitors.	180
Determination of the half maximal inhibitory concentration (IC_{50})	181
Determination of the inhibition constant (K_i)	182
2.5.4 Surface Plasmon Resonance (SPR) Studies	182
2.5.5 Cell Culture and Cytotoxicity Tests	183
The Sulforhodamine B assay	183
CHAPTER 3	185
3.1 INTRODUCTION	186
3.1.1 Gliomas and their classification	186
3.1.2 Glioblastoma Multiforme	187
3.1.3 New approaches for the treatment of glioblastoma: Endocrine targeted therapy	191
Tyrosyl ⁵ palmitoyl GnRH (TPGnRH) nanofibers as a targeted endocrine GBM therapy	192
3.1.4 microRNA as treatment of cancers	194
3.1.5 microRNA-21	195
Role of miRNA-21 in GBM	196
Targeted molecular pathways by miR-21 in GBM	197

How does miR-21 maintains its expression over the time?	200
3.1.6 Cancer stem cells and miRNAs.....	200
miR-205	201
3.1.7 11PS04, TMZ and other strategies for cancer stem cells	201
3.2 HYPOTHESIS, AIM AND OBJETIVES.....	205
3.3 RESULTS AND DISCUSSION.....	206
3.3.1 11PS04 and 11PS04 – TMZ loaded TPGnRH Nanofibers Characterization.....	206
Particle size and zeta potential measurements. Quantification of loading using HPLC.....	206
Transmission electron microscopy (TEM) and Atomic Force Microscopy (AFM).....	207
3.3.2 Antitumoral Activity of 11PS04, Temozolomide and TPGnRH	210
11PS04 and Temozolomide	210
The Triple-Treatment.....	211
3.3.3 ANTITUMOURAL ACTIVITY OF LOADED TPGnRH NANOFIBERS.....	214
11PS04 – TMZ loaded nanofibers	214
Combining 11PS04 loaded nanofibers with TMZ.....	216
Doubling the dose of 11PS04.....	218
3.3.4 Discussion.....	220
TPGnRH	221
TPGnRH and TMZ.....	222
TPGnRH + 11PS04	222
TPGnRH + TMZ + 11PS04	226
3.4 CONCLUSIONS	227
3.5 MATERIALS AND METHODS	228
3.5.1 Materials and Equipment	228
3.5.2 Experimental procedures for the synthesis of 11PS04 ³⁵¹	230
(S,Z)-tert-Butyl 4-(3-methoxy-3-oxoprop-1-en-1-yl)-2,2-dimethyloxazolidine-3-carboxylate (2)	230
(S)-tert-Butyl (6-oxo-3,6-dihydro-2H-pyran-3-yl)carbamate (3).....	230
(S)-3-Propoxy-N-(6-oxo-3,6-dihydro-2H-pyran-3-yl)benzamide (4)	231
(3aR,7aS)-2-(3-Propoxyphenyl)-7,7a-dihydro-3aH-pyrano[3,4-d]oxazol-6(4H)-one (5; 11PS04)	231
3.5.3 Solid Phase Peptide Synthesis (SPPS)	233

Synthesis of Tyrosyl-Palmitoyl-Gonadotropin Releasing Hormone (TPGnRH)	233
Solid phase peptide synthesis of TPGnRH	233
3.5.4 Peptide Characterization	237
HPLC	237
Fourier-transform infrared (FTIR) spectroscopy.....	237
3.5.5 Formulation of 11PS04 and TMZ into TPGnRH nanofibers	238
3.5.6 Characterization of Formulations	239
Particle size and zeta potential measurements.....	239
Transmission electron microscopy (TEM).....	239
Atomic Force Microscopy (AFM)	239
Quantification of loading using HPLC – Drug loading and encapsulation efficiency.....	240
3.5.7 Antiproliferative Assays in U87 MG cells	241
Cell culture and cell seeding	241
Experiments design.....	241
Cell metabolic activity.....	249
3.5.8 Statistics	249
REFERENCES.....	250
SUPPLEMENTARY INFORMATION	276

Resumen

Síntesis y evaluación de heterociclos activos para el diagnóstico precoz y tratamiento de enfermedades cerebrales

De acuerdo con la Fundación Americana del Cerebro, “las enfermedades cerebrales se manifiestan de muy distintas maneras, y reciben diferentes denominaciones”. Efectivamente, las enfermedades del cerebro presentan una alta incidencia en la población, afectando 1 de cada 6 personas Enel mundo, lo que supone un gran reto para la sociedad tanto desde un punto de vista socioeconómico como sanitario.

El presente trabajo de tesis doctoral se ha centrado en desarrollo de heterociclos para el diagnóstico y tratamiento de enfermedades cerebrales, en particular, Enfermedad de Alzheimer (EA) y glioblastoma multiforme (GBM). EA es una enfermedad neurodegenerativa perteneciente a un grupo de patologías denominadas tauopatías, que se caracterizan por los depósitos anormales de proteína tau en el cerebro, en forma de agregados de la misma, ubicados en el interior de las neuronas y denominados ovillos neurofibrilares. El mayor problema que presenta actualmente EA es su diagnóstico, pues el daño cerebral se produce años antes de la aparición de los primeros síntomas. Es por ello que existe la necesidad de desarrollar potentes herramientas para el diagnóstico fiable y precoz, que permitirá a su vez incrementar el éxito de cualquier terapia destinada a tratar EA. GBM, por su parte, es un tipo de tumor cerebral perteneciente al grupo de los astrocitomas y el más agresivo entre los de su clase. El mayor reto para la terapia de GBM es la presencia de la barrera hematoencefálica (BBB), que limita el paso del 98% de los nuevos tratamientos y reduce las concentraciones efectivas de fármaco que llegan al cerebro. El tratamiento standard para GMB (protocolo Stupp) comprende cirugía, radiación y quimioterapia. Sin embargo, este protocolo presenta altos niveles de recurrencia tras la intervención y, una esperanza de vida de 15 meses. Esta falta de efectividad corrobora el hecho de que GBM es una enfermedad muy compleja y heterogénea, que necesita el desarrollo de nuevas más sofisticadas terapias capaces de atravesar la BBB que permitan tratarla eficazmente.

La tomografía por emisión de positrones (PET) es una técnica de imagen médica de diagnóstico ampliamente extendida que permite la visualización y monitorización de las funciones de tejidos y órganos mediante el uso de radiofármacos o radiotrazadores. En el presente estudio se plantearon objetivos que incluyeron la síntesis y caracterización de nuevos heterociclos con afinidad por agregados de proteína tau, baja citotoxicidad e idoneidad para ser transformados en radiotrazadores para administración parental. Por último, plantear posibles rutas de síntesis para convertir estas moléculas en radiotrazadores. Así, en esta primera parte del trabajo dedicada a EA y tauopatías, se han desarrollado satisfactoriamente familias de compuestos pertenecientes al grupo de las imidazo[2,1-*a*]piridinas e imidazo[2,1-*b*]tiazoles capaces de unirse a agregados de proteína tau, además de no presentar citotoxicidad frente a células del hematocrito. Se desarrolló además una ruta sintética para la obtención de radiotrazadores marcados con flúor radioactivo. Estos resultados abren el camino a la utilización de estas nuevas familias de heterociclos identificadas como

potenciales nuevos radiotrazadores selectivos ^{18}F -PET para el diagnóstico precoz de este tipo de enfermedades.

La segunda parte de esta tesis doctoral está dedicada a los tumores cerebrales, en particular al tratamiento de GBM. Para ello, se han planteado dos aproximaciones diferentes. Por un lado, la inhibición del enzima LDH-A humana mediante heterociclos con actividad inhibidora de LDH-A. Por otro lado, la utilización de terapias dirigidas basadas en nanopéptidos para “cargar y lanzar” compuestos citotóxicos activos frente a células tumorales.

Para el desarrollo de inhibidores de LDH-A humana, se planteó la realización de un screening de una biblioteca de compuestos del grupo de investigación, mediante el uso de las técnicas de Resonancia de Plasmón de Superficie (*Surface Plasmon Resonance*; SPR) y ensayos enzimáticos en disolución. El screening señaló a los conjugados quinona-aminoácido como potenciales inhibidores de LDH-A, por lo que se sintetizaron y caracterizaron más análogos que probaron su óptima actividad tanto en SPR como en los ensayos enzimáticos. Una vez identificados los compuestos activos frente a LDH-A, el siguiente objetivo fue la caracterización de dichos compuestos mediante la determinación de las constantes cinéticas K_i y K_D , así como evaluar su eficacia antitumoral (IC_{50}) en células de glioblastoma (U87 MG). Los ensayos celulares mostraron resultados más modestos, pero definitivamente nos abren paso para el desarrollo de inhibidores de LDH-A más potentes y efectivos.

El último capítulo del presente trabajo de tesis doctoral tenía como objetivo el empleo de terapias dirigidas para el tratamiento de GBM con 11PS04, una molécula innovadora capaz de modificar y restaurar a niveles fisiológicos la transcripción de microRNAs (miRNA) que se encuentran alterados en procesos patológicos como el cáncer. Tal y como ha sido descrito previamente por nuestro grupo de investigación, 11PS04 puede actuar como agente sensibilizante de células tumorales, e incrementar el efecto citotóxico de TMZ, un fármaco actualmente indicado para el tratamiento de GBM, pero que presenta un gran nivel de resistencia en pacientes tratados con el protocolo Stupp. Por ello, 11PS04 ha sido formulado en el interior de un péptido denominado TPGnRH, el cual ha sido desarrollado por el equipo de la Dra. Lalatsa a partir de la hormona liberadora de gonadotropina (GnRH). TPGnRH es capaz de atravesar la barrera hematoencefálica y dirigirse específicamente a las células de GBM, que sobreexpresan GnRH. La formulación de 11PS04 con TPGnRH fue caracterizada en cuanto a morfología y grado de encapsulación, así como su actividad frente a células de glioblastoma humano en presencia y ausencia de TMZ. La formulación permitió reducir las dosis necesarias efectivas de cada componente sin sacrificar el efecto antiproliferativo deseado. Se puede concluir, por tanto, que la terapia combinada desarrollada tiene un gran potencial farmacológico para el tratamiento de GBM.

Abstract

Synthesis and evaluation of active heterocycles for early diagnosis and treatment of brain diseases.

According to the American Brain Foundation: “Brain disease comes in many different forms, and goes by many different names”. It affects 1 out of 6 people worldwide, which entail important challenges of all societies from a medical and socio-economic perspective.

This work is focused in developing novel heterocyclic molecules for the early diagnosis and treatment of brain diseases and particularly we focused on Alzheimer’s disease and Glioblastoma multiforme (GBM). AD is a well-known neurodegenerative disease, and belongs to a family of tauopathies, which are characterized by the deposition of aberrant tau protein aggregates forming neurofibrillary tangles inside the neurons. Early and accurate diagnosis is critical in treatment of AD but remains an unmet challenge as development of potent diagnostic tools for an early and reliable diagnose will increase the success of any applied therapy for the treatment of AD. A major challenge in GBM therapy and diagnosis remains the presence of the blood-brain barrier which limits 98% of novel compounds from reaching the brain in theragnostic concentrations. The presence of the BBB lies behind the poor treatment and short time between survival and diagnosis of GBM, the most prominent and malignant tumour astrocytoma subtype in adults. GBM therapy involves surgery, radiation and chemotherapy (usually temozolomide) (Stupp protocol) which can only increase time from diagnosis to death by 15 months. Thus, only therapies able to cross the BBB and address the tumour heterogeneity are likely to improve therapeutic outcome and ideally improve survival and quality of life.

Positron Emission Tomography (PET) is an imaging technique widely used that allows functional imaging of biological processes of tissues and organs by using radiopharmaceuticals. In this work we aim to synthesise and characterise new heterocycles with high affinity for the tau protein aggregates and low cytotoxicity and haemotoxicity that can be easily converted into radiotracers for parenteral administration. Hence, in this first part of the study devoted to AD and tauopathies, several families of compounds containing the imidazo[2,1-*a*]pyridine and imidazo[2,1-*b*]thiazole scaffolds which bind tau proteins have been developed. Additionally, we have established a workable synthetic route to obtain radio-fluorine-labelled tracers. These results open the door for the utilization of these families of heterocycles as potential new selective radiotracers ^{18}F -PET for the early diagnose of tauopathies such as AD.

The second part of this doctoral thesis work was focused on developing heterocycles with inhibitory activity against the lactate dehydrogenase A (LDH-A) for the treatment of GBM. LDH-A inhibitors were tested for efficacy in U87MG GBM cells for efficacy. A screening library of compounds from our research group were tested as LDH-A inhibitors using surface plasmon resonance (SPR) and enzymatic assays. Screening revealed quinone-amino acid conjugates as the most promising family of compounds, and more analogues from this family were synthesised and screened on SPR and on enzymatic assays. The most potent LDH-A inhibitors against LDH-A enzyme were selected and the

kinetic constants K_i and K_D were determined. Their antitumoral efficacy (IC_{50}) in human glioblastoma cells (U87 MG) was also assessed. Quinone-amino acid hybrids showed moderate to excellent K_i values, although their physicochemical properties were not optimal for membrane permeability as discreet results were obtained on glioblastoma cells. These finding demonstrate the potential of this family of compound and constitute a good starting point for the development of LDH-A inhibitors with higher potency and effectiveness.

The final chapter of the present work was focused in the targeted delivery of a novel compound, 11PS04, that modifies and restores the physiological levels of microRNAs (miRNAs) that are altered in conditions such as GBM. 11PS04 acts as a sensitising agent for GBM cells and potentiate the cytotoxic effect of temozolomide (TMZ). Patients treated with the Stupp protocol, show high level of resistance to TMZ. In this work, we have loaded 11PS04 in brain permeable peptide loaded nanofibers prepared from a gonadotrophin-releasing hormone (GnRH) amphiphile developed by Dr Lalatsa's group to protect it from degradation, enable its permeability across the blood-brain barrier and target it to GBM cells known to overexpress the GnRH receptor towards a targeted nanomedicine for the treatment of GBM. The formulation was characterised in terms of loading and morphology and assessed for efficacy in GBM cells in the presence and absence of TMZ in clinically relevant and clinically ineffective concentrations. Loading 11PS04 into nanofibers (TPGnRH) allowed the reduction of the effective concentration of each component needed without sacrificing the desired antiproliferative effect which also allowed the efficacy of subclinical TMZ concentrations when used in combination. Thus, the combined treatment elicited powerful antiproliferative results and could constitute a potential strategy for the treatment of GBM.

List of Tables

Table 1 - Chemical structures of boronic acids (entries 1-5) and alpha-haloketones (entries 6-9) utilised in the present study.	41
Table 2 - Reagents and conditions explored for the preparation of intermediates 4a-d and 12a . Key: DME, dimethoxyethane. Reactions were carried out in a microwave reactor.	42
Table 3 – Method A, step A2. Reagents and conditions for the preparation of imidazo[1,2- <i>a</i>]pyridine analogues 1a-i	43
Table 4 - Method B, step B1. Reagents and conditions for the synthesis of intermediates 6a-c via condensation of 4- and 5-halo-2-aminopyridines and α -haloketones.	45
Table 5 – Method B, step B2. Reagents and experimental conditions for the synthesis of imidazo[1,2- <i>a</i>]pyridines 1i-l	46
Table 6 - Major (X) and minor (Y) axis length (μm) of tau assemblies (Figure 20) measured after 13 days of incubation.	53
Table 7 – Optimization test of ThT: protein ratio. Tests were done in triplicates. Data is shown as mean $\pm$ SD.	72
Table 8 – HINT scores obtained after rescoring docking results of tracer candidates compared with a known tau radiotracer, RO6924963.	79
Table 9 - Summary table of reagents utilised.	86
Table 10 - Stock solutions and volume employed in the ThT competition assay.	106
Table 11 - Synthesis of quinone- <i>L</i> -tryptophan conjugates (17a-e) via Michael addition and re-oxidation.	137
Table 12 - K_D values obtained in SPR for quinone-amino acids hybrids.	150
Table 13 - K_i for LDH-A - inhibition data in the presence of quinone-amino acid hybrids 17a-h and 17x . Mixed-model inhibition fit (GraphPad Prism 8 software) was applied for the obtention of K_i and alpha values (α). Mean $\pm$ SD (n=3).	157
Table 14 - Half maximal inhibitory concentration (IC_{50}) of quinone-hybrids.	164
Table 15 - Summary of results obtained with quinone derivatives by SPR and enzymatic assays.	165
Table 16 - Cytotoxic effect on U87-MG cells after 48 h of treatment with quinone-amino acid hybrids, as determined by the SRB assay. Mean $\pm$ SD (n=6).	166
Table 17 - Volumes used in IC_{50} and K_i calculations.	181
Table 18 - The half-life (minutes) of Glu-GnRH and TPGnRH in 50% plasma or brain or liver homogenate.	193
Table 19 – Average particle size (nm), peaks (% by volume), Z potential and drug loading of 11PS04-loaded and 11PS04-TMZ-loadend TPGnRH formulations. Peak (%) noted by volume. Mean $\pm$ SD (n=3).	206
Table 20 - Half maximal inhibitory concentration (IC_{50}) of TPGnRH alone, TPGnRH with 250 μM of TMZ and TPGnRH with TMZ (250 μM) and 11PS04 (5 μM) after 6 days of treatment.	213
Table 21 - Summary table of reagents utilised.	228
Table 22 - HPLC gradient method for TPGnRH.	237
Table 23 - Gradient HPLC method for analysis of 11PS04, TMZ and TPGnRH formulations.	240

List of Figures

Figure 1 - Amyloid precursor protein (APP) processing and Aβ biogenesis. Adapted from reference 11.	4
Figure 2 - The mechanism of Aβ toxicity. Key: AD, Alzheimer's disease; FAD, familial AD; ROS, reactive oxidative species.	5
Figure 3 - APP mutations. APP transmembrane protein (grey string) and key residues (grey spheres) that affect β -secretase processing of APP in humans. Image reproduced from reference 17.	7
Figure 4 - Schematic representation of tau gene, mRNA and the six tau protein isoforms. Adapted from reference 38.	9
Figure 5 - Tau protein (yellow filament) helps to maintain the microtubule integrity in the neuronal axon. Upon dysregulation of the phosphorylation-dephosphorylation control system, tau can progress into toxic paired helical filaments (PHFs) and straight filaments (SPs) species that ultimately aggregate forming NFTs. Taken from reference 37.	11
Figure 6 - Example of a β-sheet conformation of one single molecule of Tau⁴⁴¹. Arrows in different colours indicate the different β -strands that connect one another to conform the β -sheet structure. In the case of Tau protein, each β -sheet presents differences in height of 10 Å. Picture taken from reference 46.	11
Figure 7 - (A) 3D view of two antiparallel β-sheets that forms a protofilament. (B) Hierarchy of atomic-resolution motifs involved in the self-assembly or amyloid fibrils. Picture taken from refence 50. .	12
Figure 8 - A) Sequence alignment of the four microtubule-binding repeats (R1-R4) of Tau protein. Each of the eight β -strands observed are coloured from blue to red. R1 and R2 may form an additional, less-ordered β -sheet which is indicated with grey dashed underlines. B) C-shaped - protofilament core unit - representation of the secondary structure elements in three successive rungs. Pictures taken from reference 46.	13
Figure 9 - Cross-sections of the PHF and SF structures. Cryo-EM structural assignments of Pair Helicoidal Filaments (PHF), A and Straight Filaments (SFs), B . Red arrows indicate additional densities in contact. Red squares highlight the interface between the two protofilaments. Taken from reference 53.	14
Figure 10 - (A) 2N4R tau sequence and ultrastructure in tau filaments. N-terminus (γ EFE ₉ is represented by a blue dot). The microtubule-binding repeats are labelled R1-4. (B) Radom packing of all six different isoforms of tau. (C and D) Schematic representation of full-length tau filaments, with the β-sheet region bold of PHFs (C) and SPs (D), colour scheme following (C). The hypothesized "backbiting" interaction is shown between the γEFE₉ motif (blue dot) and the filament core. Images taken from Fitzpatrick et al. ⁴⁶	15
Figure 11 - Schematic representation of Alzheimer's disease pathology and progression. (A) The first pathological event is the production of the A β 42 peptide, which is yielded by the cleaving activity of two enzymes, β -secretase and γ -secretase, of protein APP. (B, C) Extracellular A β 42 peptide forms A β 42 oligomers and initiates A β plaque deposition. The A β plaque activates microglial defence system, which surround the plaque and recruit astrocytes. Initially, this response is neuroprotective	

by forming a barrier between the plaques and the surrounding neurons, diminishing the impact A β -induced neurotoxicity. (D) As the disease progresses, the A β plaque deposition reaches a threshold forming the dense-core plaques that are associate with dystrophic neurites and influences tau pathogenesis. In late-stage AD, disease-associated microglia may facilitate tau pathogenesis by phagocytosing tau protein and secreting neurotoxic factors, thus promoting a neurotoxic inflammatory response. (E) Tau pathology spreads to connect brain regions, which drives the neurodegenerative process. Reproduced from reference 14.	16
Figure 12 - Annihilation between a positron and an electron (left) producing 511keV photons that are registered by the circular gamma ray detector in the PET camera. Picture taken from reference ⁶¹ .18	18
Figure 13 - Chemical structures of the first generation of tau tracers.....	20
Figure 14 - Enantiomers <i>R</i> and <i>S</i> of ¹⁸ F-THK5117.	21
Figure 15 – Chemical structures of second generation of tau tracers.	22
Figure 16 - Imidazo[1,2- <i>a</i>]pyridine-based marketed drugs.....	26
Figure 17 – General chemical structure of imidazo[1,2- <i>a</i>]pyridine derivatives presented in this work.	39
Figure 18 - TEM images of tau assembly into spherical nucleation units (SNUs) and their linear assembly. Tau was incubated for 8 days at 37 °C. A drop with tau aggregates was placed in a copper coated grid and negatively stained with uranyl acetate aqueous solution (2% w/v) for 30 seconds. Presence of SNUs -highlighted by a white circle-, and short fibres formed by multiple SNUs linked together is detected in the 8 th day of incubation.	51
Figure 19 - AFM imaging of Tau 441 assemblies into granular oligomers and mature tau fibrils. 5 μ L of aggregation buffer containing tau aggregates was placed in the surface of a mica disk and analysed with tapping mode AFM (A – zoom n. 1, and B – zoom n. 2).	52
Figure 20 - AFM imaging of Tau 441 assemblies into granular oligomers and mature tau fibrils - Enlarged section from Figure 19.B. Different tau aggregates were selected for elucidating their aggregation state (granular tau oligomers or fibrils) according to their major-to-minor axis ratio. ...	53
Figure 21 - Tau fibrils twist profile. The backbone of the fibre (B) was plotted (C) as the registered height (Y) versus the traversed distance by the cantilever. Data was processed with Gwyddion software and plotted on Excel.	54
Figure 22 - TEM imaging of tau fibrils. Granular tau oligomers (a) and PHFs (b) were visible after 13 days of incubation at 37 °C.	55
Figure 23 - Schematic representation of amine coupling (A) in SPR. (B) Experimental set up of an SPR experiment. Binding events provoke changes in the resonance angle ($\delta\theta$) of refracted light when the analyte, flowing through the channel, binds to the immobilized ligand and increases in density at the sensor chip. (C) Typical shape of a sensorgram after one run or analysis cycle. Resonance Units (Y axis) are represented as function of time (X axis). Bars below the sensorgram curve represent type of solutions that pass over the sensor surface. Picture <i>B</i> has been taken from reference ¹⁴⁶ and picture <i>C</i> has been taken from the Sensor Surface Handbook (Biacore TM).	56
Figure 24 – Binding data obtained by SPR with immobilized tau aggregates on sensor chip CM5. Sensorgrams were obtained after exposing imidazo[1,2- <i>a</i>]pyridines (Sensorgrams A and B) to immobilized tau aggregates. Compounds were injected at 100 μ M.	58

Figure 25 – (A) Chemical structures of radiotracer T808, compound 1c and benzothiazole-based compound (internal control) and their sensorgrams with binding affinities for tau aggregates (B) . Compounds were injected at 100 μ M.	59
Figure 26 - Chemical structures of one tau radiotracer (A) and a radioligand for amyloid- β plaques (B) . ^{68,147}	60
Figure 27 - Binding data obtained by SPR with immobilized tau aggregates on sensor chip CM5. Sensorgrams were obtained after exposing imidazo[2,1- <i>b</i>]thiazole analogues to immobilized tau aggregates. Compounds were injected at 100 μ M.....	64
Figure 28 - (a) Chemical structure of Thioflavin T dye. (b) Changes in ThT emission fluorescence spectrum upon binding to amyloid fibrils. Picture taken from reference 160.....	65
Figure 29 - Binding affinity of imidazo[1,2-<i>a</i>]pyridines-based compounds expressed as remaining ThT fluorescence (%). The lower the percentage value, the higher the affinity of competitor for tau aggregates. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing control samples (no competitor added) with test samples. One-Way ANOVA with Dunnett’s post-hoc test. Mean $\pm$ SD (n=6).	68
Figure 30 - Chemical structure of imidazo[1,2- <i>a</i>]pyridine derivatives 1b-1e	68
Figure 31 - Chemical structures of imidazo[1,2- <i>a</i>]pyridine derivatives 1d, 1f, 1g and 1k	69
Figure 32 – Chemical structures of compounds imidazopyridine derivatives 1a, 9 and 1l and the N-N-dimethyl-2-aminopyridine analogue 12b	69
Figure 33 – Chemical structures (top) and binding affinity (bottom) of imidazo[2,1-<i>b</i>]thiazole-based analogues for insulin aggregates, expressed as remaining ThT fluorescence (%). Binding affinity is expressed as remaining ThT fluorescence (%), the lower the percentage value, the higher the affinity of competitor for tau aggregates. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing control samples (no competitor added) with test samples. One-Way ANOVA with Dunnett’s post-hoc test. Mean $\pm$ SD (n=6).....	70
Figure 34 - ThT competition assay results. Binding affinity of 12b compound and benzothiazole (positive control) expressed as remaining ThT fluorescence (%). Chemical structures of 12b (top) and benzothiazole (bottom) are shown on the left. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are relative to control values. P values <i>no significant</i> ns> 0.05, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, indicate multiple comparisons between groups. One-Way ANOVA with Dunnett’s post-hoc and Tukey’s Post-hoc test. Mean $\pm$ SD (n=3).....	73
Figure 35 - Binding affinity of compounds for tau aggregates expressed as remaining ThT fluorescence (%). Student’s t-test. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are shown for competitors relative to control. P values > 0.05 were statistically <i>no significant</i> (ns). Comparisons between compounds were analysed by One-way ANOVA and Tukey’s post hoc test. Mean $\pm$ SD (n=3).	74
Figure 36 – Chemical structures of compounds 1g, 1m, 1f, 1k and 12b	75
Figure 37 – (A) Binding affinity of compounds for tau aggregates expressed as remaining ThT fluorescence (%). Molar ratio tau aggregates: compound was 1:10. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are shown for competitors relative to control (Student’s t test). P values > 0.05 were statistically <i>no significant</i> (ns). Student’s t-test Mean $\pm$ SD (n=3). (B) Chemical structures of tested compounds.	76

Figure 38 - Tau protofibril structure showing the various high-affinity binding sites S1-S4.....	78
Figure 39 - Structure of selected compounds (1b , 1c , 12b and RO6924963) for molecular docking simulations. RO6924963 is a known tau selective radiotracer used as reference in this study. ⁶⁸	79
Figure 40 - Haemolysis (%) of selected imidazopyridines, imidazothiazole and pyridine derivatives, at 50 nM (left) and 50 μ M (right). Compounds were incubated with RBC for one hour at 37 $^{\circ}$ C. RBC treated with Triton 1% represented the 100% haemolysis.	83
Figure 41 - Glycolysis is the first route in the breakdown of glucose to extract energy for cellular metabolism. Glycolysis is the source of metabolic intermediates that are used in parallel metabolic pathways, such as the pentose phosphate pathway (yellow) and the glycogenesis (light blue pathway).	114
Figure 42 - Proteins (squared) up-regulated by HIF-1 α and their effect on cell metabolism in hypoxic environment. Key: ECM: extracellular matrix. Adapted from reference 191.	115
Figure 43 - LDH activity and isoforms. (A) LDH catalyses the redox interconversion between pyruvate and lactate with NADH as cofactor. (B) human LDH subunits H and M. (C) The functional LDH is a tetramer containing different ratios of the M and H subunits, which associate randomly. The five isozymes of LDH are shown.	117
Figure 44 - Hydride and proton transfer occurring in the LDH active site. The cofactor NADH binds first and prepares the binding site for pyruvate binding. Arg109 polarises the carbonyl residue of pyruvate, which facilitates the hydride transfer between NADH and pyruvate, and the proton transfer from His195 and the substrate. Taken from reference 191.	118
Figure 45 - Gossypol chemical structure.	121
Figure 46 – Structure of naphthoic acid derivative FX-11.	122
Figure 47 - Examples of piperidone-based LDHA inhibitors.	122
Figure 48 - Structures of NHI-2 and its glucose-conjugated analogue.	123
Figure 49 - General chemical structure of pyrazole (left) and indole derivatives (right).	123
Figure 50 - Example of pyrazolidine analogue with LDH inhibitory activity.	124
Figure 51 - Pyrazolylthiazole derivative active as LDHA inhibitor.	124
Figure 52 - 4-aminoquinoline derivative.	125
Figure 53 - Chemical structure of the <i>h</i> LDH5 inhibitor AZ-33.	125
Figure 54 - Example of nicotinic acid derivative developed as LDH-A inhibitor.	125
Figure 55 - Enzyme activity rates measured at different substrate concentrations. Data are fitted by a non-linear regression. Taken from reference 234.	127
Figure 56 - Langmuir equation (Equation 2.7) and its representation (right graph, the Langmuir isotherm) for the formation of the binary complex, RL. Taken from reference 234.	128
Figure 57 - Saturating curve representing the variation of the reaction rate of an enzyme as function of substrate concentration [S]. Michaelis-Menten constant (K_M) and V_{max} values are also indicated. At the beginning of the reaction, there is a relative straight line and then, the velocity starts to decrease and approach asymptotically to the V_{max} value.	132
Figure 58 - Substrate titration of steady state velocity for LDH-A in the presence of an inhibitor at varying concentrations.	134

Figure 59- Chemical structures of some quinone-base compounds with different physiological and pharmacological activities.	136
Figure 60 - Quinone-based fragments used in this study: the 1,4-naphthoquinone, 15a , and the 1,4-anthraquinone, 15b	137
Figure 61: ¹ H NMR spectrum of crude product 17x , the deuterated solvent of choice was DMSO. This spectrum was acquired in a 400 MHz Bruker instrument.	140
Figure 62- Base-peak ion chromatogram (BPI) of compound 17x	141
Figure 63 - Low energy (A) and high energy spectrum (B) of the compound 17x in ESI+.	142
Figure 64 - Low energy spectrum of compound 17x showing its [M+Na] ⁺ and [M+K] ⁺ adducts.	142
Figure 65- First hypothesised chemical structure for compound 17x	143
Figure 66 - Proposed chemical structure of compound 17x	144
Figure 67 - Quinone-amino acids hybrids proposed in the present study as candidates for LDH-A inhibitors.	145
Figure 68 - SPR Sensorgrams for quinone-amino acids hybrids and NHI-2 binding to the surface with immobilised LDH-A.	147
Figure 69 – SPR sensorgrams and kinetic fitted curves (black lines) of synthesised compounds. Sensorgrams were obtained using dilution series of the tested compounds (5-100 μM). Curve fit and error (Chi ²) values were obtained with Biacore Software.	149
Figure 70 – Typical curve shape of a SPR sensorgram. Taken from reference ¹⁴⁶	150
Figure 71 - Schematic representation of the NADH competition assay. In this experiment, the natural substrate, pyruvate (P), is added at high concentrations, so the inhibitor (illustrated as red circles and diamond figures) is forced to compete with NADH (N) for binding the NADH site.	152
Figure 72 - Measurement of inhibition (%) of enzymatic activity of LDH-A in the presence of quinone-amino acid hybrids (17a-h and 17x) at 125 μM. Results are reported as mean ± SD (n=3).	153
Figure 73 - Equilibrium scheme for enzyme turnover in the presence and absence of a reversible inhibitor. K _D is the dissociation constant for dissociation of the ES complex, k _{cat} expresses the overall rate of reaction after the ES complex formation. Alpha quantifies the effect of inhibitor on K _D and K _i and ultimately, the alteration substrate- enzyme and inhibitor enzyme affinity. Taken from Copeland, 2000. ²⁴⁸	155
Figure 74 - Michaelis-Menten curves corresponding to NADH and pyruvate. K _m NADH= 20.7 μM; K _m pyruvate = 118.7 μM. Data is reported as the mean of 3 independent experiments.	156
Figure 75 - Michaelis-Menten curves of LDH-A in the absence (black line) or in the presence of compounds (coloured line). Quinone derivatives were tested at a range of concentrations (2-80 μM). Curves were obtained with GraphPad Prism software.	160
Figure 76 - Structure of LDH inhibitors bearing the OH/COOH pharmacophore and proposed pharmacophore quinone-amino acid conjugates. Key: 3-hydroxyisoxazole-4-carboxylic acid (HICA) and 4-hydroxy-1,2,5-thiadiazole-3-carboxylic acid (HTCA).	161
Figure 77 - Chemical structure of quinone-tryptophan conjugates. K _i values are shown for the NADH site.	162

Figure 78 - UV-visible absorbance spectra of some quinone-amino acid hybrids and NADH at 100 μM. OD values were acquired from 200 to 600 nm, although only the important window of values is shown.....	180
Figure 79 - Schematic algorithm for classification of gliomas. Not only the histology but also molecular genetic features are nowadays considered for classifying gliomas. IDH: isocitrate dehydrogenase; NOS: not otherwise specified.	187
Figure 80 - Chemical structures of Mitozolomide (A) and Temozolomide (B).....	188
Figure 81- Scheme of TMZ pH-dependent conversion into its active form. Adapted reference ²⁸⁵	189
Figure 82 - Signalling pathways involved after the activation of the GnRH receptor and their effects on glioblastoma cells. Key: dashed arrows: indirect activation. Solid arrows: direct activation. Solid line with the transversal line: direct blocking.....	192
Figure 83 - Molecular structure of Tyrosyl⁵ palmitoyl GnRH (TPGnRH) peptide.	193
Figure 84 - Transcription and translation processes for peptide biosynthesis on cells. Adapted from https://www.cancer.gov/	194
Figure 85 - Biosynthesis of miR-21. The course followed by miR-21 transcript is indicated with arrows. Mature miR-21, coupled with RNA-induced silencing complex (RISC), binds target mRNA in the cytoplasm and provokes translational block and (or) mRNA cleavage. Picture adapted from 326 and prepared with Servier Medical Art (https://smart.servier.com/).	196
Figure 86 - Scheme of miR-21 signalling pathways for carcinogens and feedback regulation. As a result of the direct repression on targeted mRNA, many signalling pathways involved in cell proliferation, invasiveness and metastasis are altered and initiates carcinogenesis phenomena in the cell. Key: miR, microRNA; pri-mRNA, primary mRNA; pre-mRNA, precursor mRNA; AP-1, activated protein-1; NFIB, nuclear factor I/B; Maspin, mammary serine protease inhibitor; PDCD4, programmed cell death protein 4; TPM1, tropomyosin 1; RECK, reversion-induced cystine-rich protein with Kazal motifs; TIMP3, tissue inhibitor of metalloproteinases 3; MMP, matrix metalloproteinase; PTEN, phosphatase and tensin homologue; c-Myb transcription factor; XIAP, X chromosome-linked inhibitor of apoptosis; STAT3, signal transducer and activator of transcription 3. Picture made using Servier Medical Art (https://smart.servier.com/).	199
Figure 87 - 11PS04 regulates the expression of known targets of miR-21 and miR-205 in MCF-7 and U87 cells. Regulation of proteins that are downstream targets of miR-21 and miR-205: PDCD4 and E-Cadherin, respectively. Exposure to 11PS04 (5 μ M, 24h exposure time) led to the transcriptional regulation of miR-21 (A) PDCD4 (B) miR- 205 and ZEB1. Transcription was compared with the endogenous control: ACTB ribosomal RNA. The accumulation of the miR-21 and miR-205 target proteins in extracts of treated MCF-7 and U87 cells was measured in Western blots, PDCD4 (C) and E-Cadherin (D) respectively (mean $\pm$ SEM; n = 3; *P < 0.05; **P < 0.01; ***P < 0.005 (Student's t-test). Taken from reference 351.	202
Figure 88 - (A) Cell viability of U87 MG cells after 72 h of treatment with Temozolomide (TMZ). Cells were pre-treated for 48 h with 11PS04. MTT test (n = 3): *p < 0.05, **p < 0.01, ***p < 0.001 relative to the vehicle pre- treated; #p < 0.05, ##p < 0.01, ###p < 0.001 relative to vehicle cells. (B) Sphere frequencies from U87 single-cell suspensions are plotted relative to the number of cells seeded per	

well, in the presence of 11PS04 (5 μ M), TMZ (100 μ M) or 11PS04 (5 μ M) plus TMZ (100 μ M) during the formation of the spheres. Solid lines represent the frequency estimation and the dashed lines the 95% confidence intervals. The right panel shows the frequency of spheres produced by U87 cells pre-treated with 11PS04 (5 μ M) or the vehicle alone, and then exposed to 11PS04 (5 μ M), TMZ (100 μ M) or 11PS04 (5 μ M) plus TMZ (100 μ M) during the formation of the spheres. The controls are the cells pre-treated and treated during sphere formation with the vehicle alone. **(C)** The frequency of initiating cancer stem cells was calculated using the ELDA platform. Taken from reference 351. ...204

Figure 89 - HPLC chromatogram at 254 nm of TMZ and 11PS04-loaded TPGnRH nanofibers showing the peaks of TMZ, 11PS04 and TPGnRH at 3.294, 8.607 and 14.819 minutes respectively. 207

Figure 90 - Negatively stained TEM images of the 11PS04-TMZ-loaded TPGnRH **(A,B)** and 11PS04-loaded TPGnRH corresponding to F1 and F3 **(C,D)**. Formulations were diluted (1:100 v/v) in 0.01 M of PBS (pH 7.4) and negatively stained with 2% uranyl acetate prior to their visualization in TEM. 208

Figure 91 - **AFM image of 11PS04-loaded TPGnRH nanofibers (Formulation 1)**. AFM images were analysed) using Gwyddion software.....209

Figure 92 - **Cell viability of U87 MG cells treated with Temozolomide without (black bars) or with 11PS04 (white bars)**. U87 MG cells were seeded at 1400 cells/cm² in complete medium and allowed to attach and grow for 3 days. Cells were treated with 11PS04 every two days for four days. Treatment with TMZ was added on the 4th day alone or in combination with 11PS04. Cell metabolic activity was measured at the end of the assay by the MTT test. Statistical comparisons among black bars: P values *p< 0.05, **p< 0.01, ***p< 0.001 relative to non-treated cells (0 μ M of TMZ); comparisons among white bars: P values #p< 0.05, ##p< 0.01, ###p< 0.001 relative to treated cells with 5 μ M of 11PS04 and 0 μ M of TMZ. Two-way ANOVA with Dunnett's post hoc test. Mean $\pm$ SD (n=3). 210

Figure 93 - **Cell viability of U-87 MG cells during the triple-treatment**. U87-MG cells were seeded at 1400 cells/cm² and allowed to attach and grow for 3 days. Cells were treated with TPGnRH (7-70 μ M) every two days for 6 days in every test performed. Treatment with only TPGnRH is represented by black bars, treatment with TMZ and TPGnRH in grey colour, and the triple-treatment with 11PS04, TMZ and TPGnRH in white colour. Cell metabolic activity was evaluated by the MTT assay at day 2 (TPGnRH alone, A), at day 4 (TPGnRH with or without 11PS04, B), and day 6 (TPGnRH with or without 11PS04 and two different doses of TMZ (TMZ at 100 μ M (C) and 250 μ M (D))). P values *p< 0.05, **p< 0.01, ***p< 0.001 comparing treated cells with untreated cells. P values #p< 0.05, ##p< 0.01, ###p< 0.001 indicated for multiple comparisons between groups. Two-Way ANOVA with Dunnett's post-hoc and Tukey's Post-hoc. Mean $\pm$ SD (n=3). See protocol 2 on materials and methods for more details.....213

Figure 94 - **Cell viability (%) of U87MG cells after treatment with 11PS04 and TMZ-loaded nanofibers as determined by the MTT assay**. U87-MG cells were seeded at 1400 cells/cm² and allowed to attach and grow for 3 days. P values *p< 0.05, **p< 0.01, ***p< 0.001 comparing treated cells with untreated cells, and multiple comparisons between groups were done. Two-Way ANOVA with Dunnett's post-hoc and Tukey's Post-hoc. Mean $\pm$ SD (n=4). Key Formulation 1 included 11PS04-loaded nanofibers; formulation 2 contained 11PS04-TMZ-loaded nanofibers. Treatment A corresponds to TPGnRH dosed alone. Treatment B corresponds to TPGnRH, 11PS04 and TMZ

externally added. Treatment C corresponds to TPGnRH, 11PS04 and TMZ formulated. Treatment D corresponds to TMZ alone. Treatment E corresponds to 11PS04 and TMZ. 215

Figure 95 – Evaluation of cell viability (%) of U87 MG cells by MTT test, after the addition of 11PS04 loaded into TPGnRH nanofibers formulation, with TMZ externally added. U87-MG cells were seeded at 1400 cells/cm² and allowed to attach and grow for 3 days and received treatments every 2 days for 6 days. Results are shown for day 2 (A), day 4 (B) and day 6 (C). P values *ns*: no significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001 are relative to untreated cells (vehicle), One-Way ANOVA with Dunnett's post hoc test. One-Way ANOVA with Tukey's post hoc test was run for multiple comparisons between groups, #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001. Mean ± SD (n=3).....217

Figure 96 - Cell viability of U87MG cells at day 4 after their exposure to 10 μM of 11PS04 in the presence and absence of TPGnRH nanofibers. U87-MG cells were seeded at 1400 cells/cm² and allowed to attach and grow for 3 days. Cells were pre-treated with TPGnRH (15 μM) for two days prior to adding 11PS04 (Protocol 5, Figure 108). P values **p* < 0.05, ***p* < 0.01, ****p* < 0.001 are relative to untreated cells (vehicle), One-Way ANOVA with Dunnett's post hoc test. Mean ± SD (n=3). 218

Figure 97 - Cell viability of U87MG (%) on the 6th day. P values **p* < 0.05, ***p* < 0.01, ****p* < 0.001 are relative to untreated cells (vehicle), One-Way ANOVA with Dunnett's. Mean ± SD (n=3). Tukey's post hoc test for multiple comparisons between groups (# *p* < 0.05). Mean ± SD (n=3). 219

Figure 98 - Molecular pathways induced by TPGnRH-11PS04 and TMZ treatment(s). 11PS04, TPGnRH and TMZ combine their effect upon their interaction with the GnRH-R (in the case of TPGnRH and 11PS04-loaded peptide), or with channel proteins in the cellular membrane. TPGnRH effects are coloured in deep purple. TPGnRH binds to GnRH-R, which is coupled to a Gαi protein, and inhibits the adenylate cyclase provoking a decrease in cAMP levels with subsequent antiproliferation and apoptosis. Dashed purple arrows indicate the hypothesised mechanism of action of TPGnRH at low concentrations. TMZ hydrolyses and form MTIC which alkylates guanine bases of DNA and activates caspase-3 for a global apoptotic effect. TMZ mechanism is indicated by red arrows. 11PS04 reduce the intracellular levels of miR-21 and increases PDCD4 expression upon miR-21 downregulation. Whether if this effect is due to the inhibition of the pro-transcription factor STAT3 or by direct interaction with pre-miR-21 it has not been elucidated yet. Signalling pathways that can be beneficiate indirectly by miR-21 downregulation after 11PS04 treatment are indicated in blue dashed arrows. Key: cAMP cyclic AMP, PKA protein kinase A, CREB, cAMP response element binding; RECK, reversion-induced cysteine-rich protein with Kazal motifs; TIMP3, tissue inhibitor of metalloproteinases 3; PDCD 4, programmed cell death protein 4; PTEN, phosphatase and tensin homologue; Akt, Protein kinase B; PIP2, phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate, PIP3. Picture made with Servier Medical Arts. 225

Figure 99 - Schematic representation of the synthesis of 11PS04. Adapted from reference ³⁵¹. 232

Figure 100 - Chemical structure of TPGnRH peptide (Glu-His-Trp-Ser-Tyr⁵-(O-palmitoyl)-Gly-Leu-Arg-Pro-Gly-CO-NH₂)......233

Figure 101 - Schematic representation Fmoc/tBu method for peptide synthesis......233

Figure 102 - Schematic SPPS synthesis of tyrosyl-palmitoyl gonadotropin releasing hormone peptide (TPGnRH). a. 20% v/v piperidine/DMF, twice. b1-b10. Corresponding Fmoc-aa (4.2 eq.),

HBTU (4 eq.) dissolved in NMM, 30', twice. b1 Fmoc-Gly-OH, b2 - Fmoc-Pro-OH, b3 . Fmoc-Arg(Pbf)-OH, b4 . Fmoc-Leu-OH, b5 . Fmoc-Gly-OH, b6 . Fmoc-Tyr(2ClTrt)-OH, b7 . Fmoc-Ser-(tBu)-OH, b8 . Fmoc-Trp(Boc)-OH, b9 . Fmoc-His(Trt)-OH, and b10 . Fmoc-Glu(tBu). c . TFA: TIS: DCM (5:5:5 v/v/v), 5' (x4). d . Palmitic acid N-hydroxysuccinimide ester (8eq.) and EtN ₃ (24.2eq) in DMF, 24h e . TFA:TIS:H ₂ O (95:2.5:2.5) for 4 hours, then dry under vacuum. f . Precipitation and extraction with cold diethyl ether and freeze drying.	236
Figure 103 - FTIR spectra of TPGnRH.....	238
Figure 104 - Schematic representation of TMZ and 11PS04 assay . After 3 days growing, the media were removed and substituted with fresh media. On day 2, the media were again removed and substituted with fresh media, this time containing 5 µM of 11PS04. Two days after (day 4), the media were changed and TMZ was added in a range of concentrations (25-500 µM) in the presence or absence of a new dose of 11PS04. Cell metabolic activity was measured by the MTT test on day 6.	242
Figure 105 - Schematic representation of Triple Treatment assay . Cells were treated with TPGnRH (7-70 µM) every two days for 6 days in every test performed. Treatment with only TPGnRH is represented by black bars, treatment with TMZ and TPGnRH in grey colour, and the <i>triple-treatment</i> with 11PS04, TMZ and TPGnRH in white colour. Cell viability was assessed by the MTT assay.	244
Figure 106 - Schematic representation of assay with 11PS04 loaded nanofibers (Formulation 1) and 11PS04-TMZ loaded nanofibers (Formulation 2) . Cells were treated for 2 days with TPGnRH at 40 µM. Formulation 1 (TPGnRH 40 µM; 11PS04 5 µM) was added on the second day and, two days after, media were removed and refreshed with a new dose of formulation 2 (TPGnRH 40 µM; 11PS04 5 µM, TMZ 20 µM). Cell viability was assessed by the MTT assay.....	245
Figure 107 - Schematic representation of the applied treatments with 11PS04-loaded nanofibers (Formulation 3) and TMZ . Treatments started at day 0 with periodical refreshment of the medium and addition of the corresponding treatments every two days. TPGnRH was added at a final concentration of 15 µM, 11PS04 at 5 µM and TMZ 175 µM. Cell viability was measured by the MTT assay every 2 days.....	247
Figure 108 - Schematic representation of the addition protocol followed in assay 5 . Vehicle contains 0.5% (v/v) of DMSO in PBS. The final concentration of each treatment was 15 µM and 5 µM for TPGnRH and 11PS04, respectively.....	248

List of Acronyms

AcOEt	Ethyl Acetate
DCM	Dichloromethane
MeOH	MeOH
AD	Alzheimer's Disease
ADAM	A Disintegrin and Metalloprotease protein
AFM	Atomic Force Microscopy
AICD	Amyloid Intracellular Domain
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
Aβ	Amyloid- β
BBB	Blood Brain Barrier
CD14	Cluster of differentiation 14
CD36	Cluster of differentiation 36
CD47	Cluster of differentiation 47
CHCl₃	Chloroform
CK	Creatine Kinase
Cryo-EM	Cryo-Electron Microscopy
Cu(OAc)₂	Copper acetate
Cu(OTf)₂	Copper triflate
CuCl	Copper (I) chloride
CuI	Copper Iodide
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EtOH	Ethanol
FAD	Familia Alzheimer's Disease
FeCl₂	Iron (II) chloride
FeCl₃	Iron (III) chloride
GC	Gas Chromatography
GMB	Glioblastoma multiforme
GS	Glutamine Synthetase
HINT	Hydrophatic INTERations
HPLC	High Performance Liquid Chromatography

IBX	2-Iodobenzoic acid
K₂CO₃	Potassium carbonate
MAPT	Microtubule-Associated-Protein Tau
MBD	Microtubule Binding Domain
MgCl₂	Magnesium chloride
Na₂CO₃	Sodium carbonate
NaHCO₃	Sodium bicarbonate
NFTs	neurofibrillary tangles
PET	Positron Emission Tomography
Ph(OH)OTs	[Hydroxy(tosyloxy)]benzene
PHF	paired helical filament
SNUs	spherical nucleation units
SPECT	single photon emission computed tomography
SPR	Surface plasmon resonance
TEM	Transmission Electron Microscopy
ThS	Thioflavin S
ThT	Thioflavin T
TLR2	Toll-like Receptors 2
TLR4	Toll-like Receptors 4
TLR6	Toll-like Receptors 6
TPGnRH	Tyrosyl ⁵ Palmitoyl Gonadotropin-Releasing Hormone
ZnI₂	Zinc iodide
ATP	Adenosine triphosphate
NADH	nicotinamide adenine dinucleotide
GTP	Guanosine triphosphate
CoA	acetyl-coenzyme A
FADH₂	Flavin adenine dinucleotide
LDH	Lactate dehydrogenase
OxPhos	Oxidation-phosphorylation
TMZ	Temozolomide
ROS	Reactive oxygen species
<i>pf</i>LDH	Plasmodium falciparum LDH
Et₃N	Triethyl amine
HPLC-MS	Coupled HPLC- Mass Spectrometry
NMR	Nuclear Magnetic Resonance

IM-HRMS	Ion Mobility-High Resolution Mass Spectrometry
BPI	Base-peak ion chromatogram
RUs	Resonance units
K_i	Inhibition constant
K_D	Dissociation constant
IC₅₀	Half maximal inhibitory concentration
SRB	Sulforhodamine B
Gy	Gray
U87 MG	Uppsala 87 Malignant Glioma cell line
mRNA	Messenger RNA
miRNA	Micro RNA
RNA	Ribonucleic acid
pri-mRNA	primary mRNA
pre-mRNA	precursor mRNA
AP-1	Activated protein-1
NFIB	Nuclear factor I/B
Maspin	Mammary serine protease inhibitor
PDCD4	Programmed Cell Death Protein 4
MMP	Matrix Metalloproteinase
PTEN	Phosphatase and tensin homologue
RECK	Reversion-induced cystine-rich protein with Kazal motifs
STAT3	Signal transducer and activator of transcription 3
CSCs	Cancer stem cells
TNF- α	Tumoral Necrosis Factor α
ZEB1	Zinc finger E-box binding homeobox 1
TIMP3	Tissue inhibitor of metalloproteinases 3
11PS04	((3aR,7aS)-2-(3-propoxyphenyl)- 7,7a-dihydro-3aH-pyrano[3,4-d]oxazol-6(4H)-one)
TPM1	tropomyosin 1
MCF-7	Michigan Cancer Foundation-7 cell line

CHAPTER I

Synthesis and evaluation of novel heterocycles with differential affinity for tau protein

1.1 INTRODUCTION

1.1.1 Alzheimer's disease and tauopathies

Progressive dysfunction and death of nerve cells remain the hallmark of neurodegenerative diseases in human beings. It causes ataxias (problems with movements) or dementias (problems with mental functioning). In 2015, dementia affected 47 million people worldwide, a figure that it is predicted to increase by 2030 to 75 million and 132 by 2050.¹ Alzheimer's Disease (AD), one of the most representative examples of dementia and may contribute with 60-70% of cases. The Global action plan on the public health response to dementia (World Health Organisation, WHO) states AD represents one of the most important challenges of ageing societies from a medical and socio-economic perspective.² In Spain, the prevalence for this disease among elderly people between 85-89 years is 20,1%, and 39,2% among those older than 90 years.³

AD, named in honour of Alois Alzheimer, belongs to a family of conditions known as tauopathies. Clinically, biochemically and morphologically, tauopathies involve the deposition of aberrant tau protein (tubulin associated unit), also known as microtubule-associated-protein tau (MAPT), in the brain.⁴ Molecular lesions start many years before the apparition of clinical symptoms in tauopathies, which makes early detection difficult.⁵ Tauopathies differ according to the affected brain regions and the isomeric forms of aggregated tau.⁶ There is also well-established evidence for disease-specific spatial patterns of tau accumulation. For example in AD, the earliest aggregation of tau is preferentially found in the transentorhinal cortex, before extending widely to the medial and inferior temporal lobe, the parietal regions and the posterior cingulate cortex.⁷ This hierarchical pattern is different from the midbrain and frontostriatal accumulation found in PSP and CBS, respectively.^{8,9} However, AD can be clinically defined by two distinct features; the first involves the above intraneural accumulation of aberrant tau protein, and the second involves β -amyloidosis which clinically manifests as extraneuronal amyloid β (A β) deposits known as senile plaques.¹⁰

Amyloid- β biogenesis and the amyloid cascade hypothesis

Until recently, A β accumulation has been considered the distinct morphological hallmark of early onset of AD and as well as to be an activator to induce the sequential lesion events induced by the aggregation of tau in the so-called "amyloid cascade hypothesis".¹¹

AD neuropathological features include extracellular senile plaques and the intracellular neurofibrillary tangles (NFTs).¹² The formation of senile plaques starts with the accumulation of aggregated, non-fibrillar, fibrillar or oligomeric A β peptide. In AD is possible to find two types of A β plaques: diffuse and dense-core plaques. Differences between them stem from their staining properties with the dyes Thioflavin S (Th S) and Congo Red, that are specific for the β -pleated sheet conformation.^{12,13} Diffuse A β plaques are Th S negative and are commonly present in the brains of cognitively healthy adults. By contrast, the dense-core A β plaques are made of A β fibrils, which are Th S positive and are mostly found in AD patients. Dense-core plaques are associated with neurotoxicity as they frequently appear surrounded by synaptic loss, microglia and reactive astrocytes. The dense-core plaques also correlate with dystrophic neurites, which are defects in

neuronal axons or dendritic processes that contain tau paired helical filament (PHF), also referred to as neuritic plaques.¹⁴

Amyloid- β biogenesis

A β is a peptide composed by 39-43 amino acids. The most important A β isoforms are A β_{1-40} and A β_{1-42} , the latter in higher percentage concentration in AD patients and are more prone to aggregate.¹¹

A β is produced from amyloid precursor protein (APP). APP is a highly conserved large transmembrane type-I protein, which can be sequentially cleaved by either α -secretase or β -secretase (also known as BACE-1) to yield sAPP α and sAPP β peptides. α -secretase is responsible for the non-amyloidogenic processing of APP, whereas β -secretase initiates the formation of A β peptide (Figure 1). Under physiological conditions, 90% of APP is cleaved by the α -secretase.¹⁴

α -secretase is part of a large group of proteolytic proteins referred to as ADAM (a disintegrin and metalloprotease domain). The APP cleavage site for α -secretase is very close to the cell membrane surface and occurs between Lys-16 and Leu-17 (based on A β -peptide numbering), thus clearly disrupting the release of full length A β peptide. Therefore, α -secretase activity affords two fragments: the nonamyloidogenic and soluble APP α fragment, and CTF83 (a soluble carboxyterminal fragment). CTF83 is further processed by γ -secretase, which generates the small non-toxic p3 peptide (Figure 1).¹⁵ The γ -secretase is a multi-subunit complex composed of four transmembrane proteins: presenilin, nicastrin, Pen2, and Aph1.¹⁶

The amyloidogenic processing pathway is initiated by β -secretase cleavage between Met – Asp residues of APP, affording the soluble APP β and a CTF99 fragment (Figure 1). The latter is targeted by γ -secretase at distinctly different sites than the CTF83. γ -secretase generates different A β peptide isoforms, 38–43 amino acids in length, and an amyloid intracellular domain (AICD). Under non-pathological conditions, the predominant isoform is the A β_{40} peptide whereas in AD there is an increase of the A β_{42} peptide, which displays a higher propensity to aggregate.¹⁷

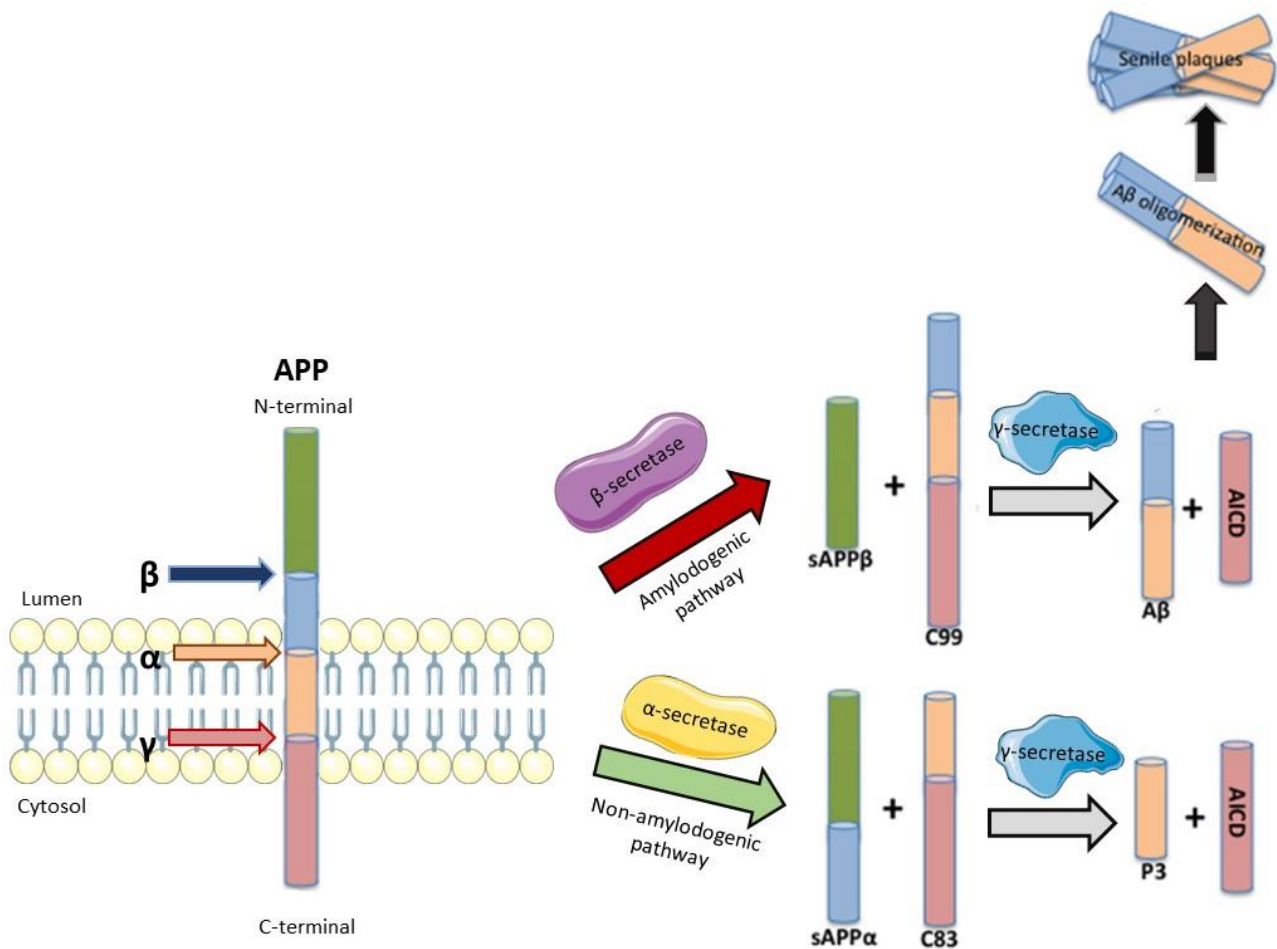


Figure 1 - Amyloid precursor protein (APP) processing and Aβ biogenesis. Adapted from reference 11.

Aβ₄₂ peptides can associate together more readily, forming larger structures called oligomers. Further accumulation produces insoluble fibrils, which then aggregate into the characteristic plaques found in Alzheimer's disease. These plaques are often surrounded by a halo of soluble oligomers. Aβ removal is dependent on the lysosome degradation system and proteolysis.

The amyloid cascade hypothesis

While aggregation features of Aβ₄₀ and Aβ₄₂ are identical, the aggregation kinetics differ as the additional amino acids in Aβ₄₂ increase the peptide's hydrophobic C-terminal end and its rate of aggregation. Accumulating Aβ leads to Aβ oligomerization, widely considered the most neurotoxic aggregation-state of Aβ. Maturation of these oligomers generates individual filaments that come together to form the fibrils, which adopt the antiparallel cross-β-sheet conformation, attributed to amyloid-type aggregates.

The aggregation of Aβ might also triggers free radical formation, such as reactive oxidative species (ROS) which rapidly react with several moieties of proteins and lipids. Protein oxidation might cause harm to the membrane integrity or damage the sensitivity to oxidative modification of the enzymes

such as glutamine synthetase (GS) and creatine kinase (CK), which are critical to neuronal function.¹⁸ Lipids peroxidation can afford 4-hydroxy-2-nonenal (HNE) and 2-propenal (acrolein), which are toxic products that migrate to different parts of the neurons to cause multiple deleterious alterations of cellular function. For instance, loss of Ca^{2+} homeostasis, inhibition of ion-motive ATPases and glial cell sodium-dependent glutamate and disruption of signalling pathways, are reported all of which are associated with neuronal death.^{19–21}

Furthermore, A β aggregation alters the kinase/phosphatase activity, such as GSK-3 β protein kinase, that leads to the Tau protein hyperphosphorylated, which initiates tau pathology ending with the formation of neurofibrillary tangles (NFTs).²²

Additionally, continuous A β aggregation or sustained elevation of A β would cause a chronic response of the innate immune system by activating microglia through some immunological receptors such as Toll-like Receptors 2 (TLR2), TLR4, TLR6, their coreceptors CD14, CD36, and CD47, which can probably destroy functional neurons by direct phagocytosis.^{23–25} Besides, it also generates inflammatory response, concomitantly releasing a lot of inflammation related mediators including complement factors, eicosanoids, chemokines, and proinflammatory cytokines, which can impair microglial clearance of A β .

All these events would ultimately lead to synaptic and neuronal dysfunction, microglia-mediated neuronal death and AD (Figure 2).

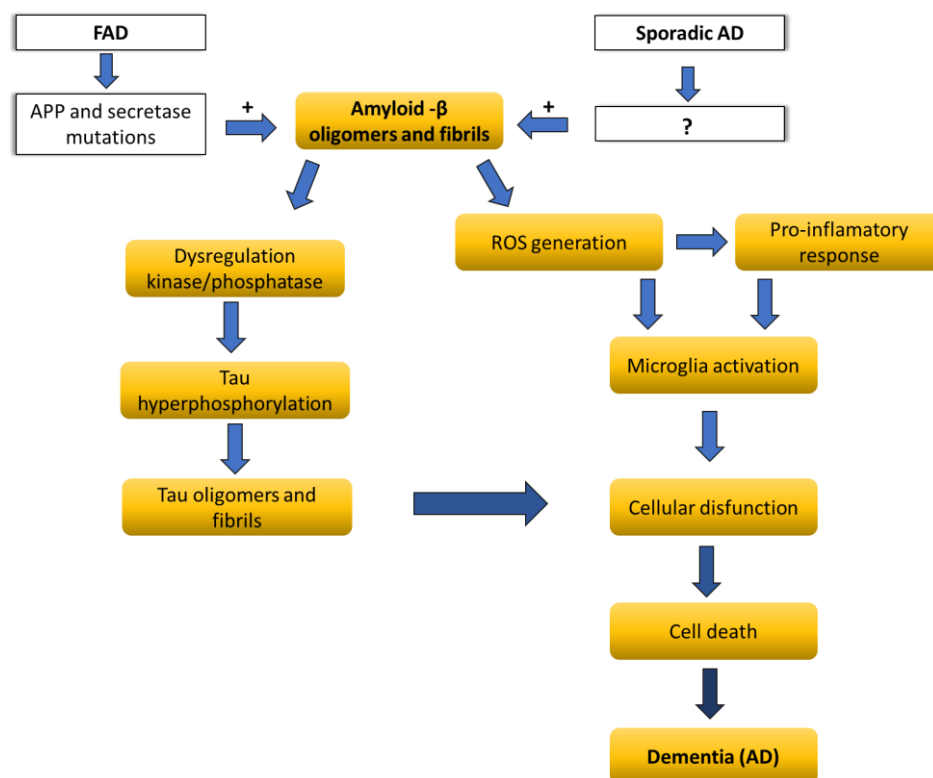


Figure 2 - The mechanism of A β toxicity. Key: AD, Alzheimer's disease; FAD, familiar AD; ROS, reactive oxidative species.

The genetics of Alzheimer's Disease:

AD is classified as familial AD (FAD) if the onset of the disease is related to the presence of mutations in different genes highly involved in the metabolism of A β peptide, or as sporadic AD, which include AD with unknown origin. The pathology of FAD is very similar to sporadic AD,²⁶ including the development of neurofibrillary tangles and microglial infiltration. This single observation is central to the concept that A β deposition is also the primary event in sporadic AD.

Secretases have been the target of multiple therapeutic strategies to reduce the burden of A β peptide, either by inhibition of β - and γ -secretases, or by activation of α -secretase. These strategies were reinforced thanks to the knowledge in the human genetics of AD.

There are over 200 autosomal dominant mutations in the genes for APP and presenilin, the active subunit of γ -secretase, that cause familial AD (FAD). These mutations unfailingly lead to either an increased A β ₄₂ : A β ₄₀ ratio or over-production of total A β . Remarkably, the FAD mutations in APP are found near the β - and γ -secretase cleavage sites and make APP a more efficient substrate for endoproteolysis by the secretases. Notably mutations are the K670N; M671L (Swedish) double mutation²⁷ and the A673V mutation²⁸ that are adjacent to the β -secretase cleavage site and cause FAD by increasing β -secretase processing and total A β production (Figure 3).

Rare APP locus duplications and duplication of the APP gene in Down syndrome/trisomy 21 also cause FAD, due to APP over-expression and total A β production. Furthermore, the epsilon 4 allele (ϵ 4) of apolipoprotein E (ApoE) is the major genetic risk factor for late-onset AD (LOAD). In humans, there are three common alleles of the APOE gene (ApoE2, ApoE3, and ApoE4). Strong evidence supports that ApoE protein influences amyloid- β plaque deposition. ApoE4 carriers show an earlier age of onset for clinical dementia and these patients also present an increased amyloid plaque burden, which is in alignment with the amyloid cascade hypothesis.^{29,30}

α -secretase enzyme (ADAM10) has shown to harbour rare mutations in the prodomain that debilitate α -secretase activity, thereby causing increased β -secretase cleavage of APP, A β over-production, and LOAD.³¹ Two more mutations were identified in 1996, in the genes that encode the γ -secretase proteins presenilin 1 (PSEN1) and presenilin 2 (PSEN2), which condition the location at which γ -secretase cuts APP.³² The mutations favour the production of longer variants of amyloid- β that clump together more readily.

In the same way, there are known genetic variants that protect against AD, such as the A673T coding substitution in the APP gene. The A673T substitution occurs only two amino acids C-terminal to the β -secretase cleavage site (Figure 3) and is at the identical position as the A673V mutation that causes FAD.²⁸ However, opposite to A673V, APP carrying A673T is less efficiently cleaved by β - secretase, leading to a ~40% reduction in A β production *in vitro*. These results suggest that heterozygous carriers of the A673T mutation should have a decrease in A β generation, thus protecting them from AD.

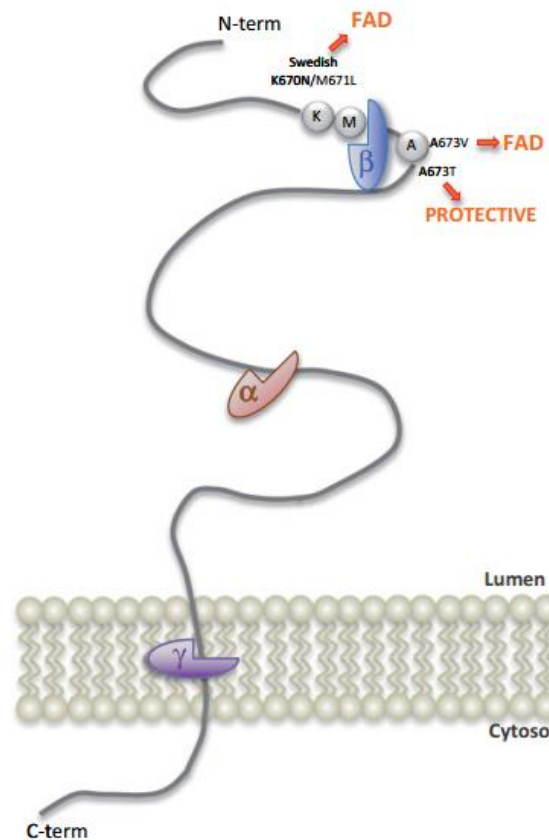


Figure 3 - APP mutations. APP transmembrane protein (grey string) and key residues (grey spheres) that affect β -secretase processing of APP in humans. Image reproduced from reference 17.

The A673T mutation served as proof-of-principle that modest inhibition of β -secretase cleavage of APP may prevent AD. In fact, the amyloid hypothesis gained strength since the discovery of the dominantly inherited mutations that are responsible for the familial forms of the disease.

For that reason and some others, secretase enzymes have been the object of multiple therapeutic strategies to reduce the burden of A β peptide, either by inhibition of β - and γ -secretases, or by activation of α -secretase.

The amyloid hypothesis for AD has guided research toward understanding the biological and pathological roles of A β and its precursor protein, monopolising the field over the past two decades. However, this dominance has led to research too focused on A β , developing therapeutic strategies, which aimed at either enhancing A β clearance and plaque burden or decreasing A β production and aggregation. To date, large clinical trials targeting A β has failed to meet their primary endpoints.³³ This situation raised the questionability of the approach in the amyloid cascade hypothesis. Several facts can be considered after the failing of these multiple clinical trials when targeting A β : first, therapies that went in phase III did not hit their primary objectives and did not effectively removed A β from the brain. Second, these therapies are being given at the wrong point in the progression of Alzheimer's disease, this is ~20-25 years after A β started accumulating, when it is quite probable

that other mechanism are driving disease.³⁴ Third, A β may be critical as initiator and catalyst element of a series of pathological events (which includes tau aggregation) in a time-dependent manner, that we ultimately known of as AD. Therefore, consistent with the amyloid hypothesis, A β may still be the trigger factor of the disease but targeting A β production or elimination alone might not be enough to interrupt downstream events once significant A β deposits have already taken place.

Redefinition of the amyloid cascade

Recent clinical studies, advances in imaging analysis, mouse models and biomarkers are now redefining this original hypothesis, as it is likely tau, A β and other physio-pathological mechanism such as inflammation, converge all together to finally lead to the development of AD.¹⁴ Furthermore, Brier and co-workers evaluated the relationship between cerebrospinal fluid measures, A β PET imaging, tau PET imaging and cognition in healthy control and mild AD patients. The study concluded that tau burden, and no A β burden, can reflect better the progression of the disease.³⁵ In contrast to A β PET imaging, tau PET could discern more efficiently between groups of patients classified according to their different global cognitive and functional performance (Clinical Dementia Rating scale, CDR). Same authors found that the severity of temporal lobe tau pathology is enough to predict the levels of cognitive dysfunction in early disease stages of AD, unlike A β plaque deposition. These findings reinforce the necessity of a new approach less A β -focused, that considers the role of tau pathology and other mechanisms such as inflammation as necessary events for AD progression.

1.1.2 Tau Protein

Tau protein isoforms and biological function

In human tissues, tau protein exists in 6 isoforms originated by alternative splicing (process that involves intron and exon elements rearrangement that alter the mRNA coding sequence, and conducts to different protein variants – isoforms – which may have different functions or properties³⁶ of the MAPT gene.³⁷ Exons 2, 3 and 10 suffer alternative splicing, whereas exons 4A, 6 and 8 are not expressed (Figure 4). Tau isoforms present four distinct regions identified as the N-terminal domain (N), the proline-rich domain (PRD), the microtubule-binding domain, with 18 amino acid imperfect repeats (R1, R2, R3 and R4), and the C-terminal domain. The length of the isoforms ranges from 352 to 441 amino acids and the six tau isoforms are differentiated depending on the number of N-terminal inserts (0, 1 or 2) and the number of repeats in the repeat region (3 or 4).³⁸

Thus, tau protein denomination is based on the difference in the N and R length, with 0N3R for the shortest and 2N4R for the longest isoform. 0N3R is found only in foetal brains, whereas the proportion of 3R and 4R proteins is practically equal in adult brains.

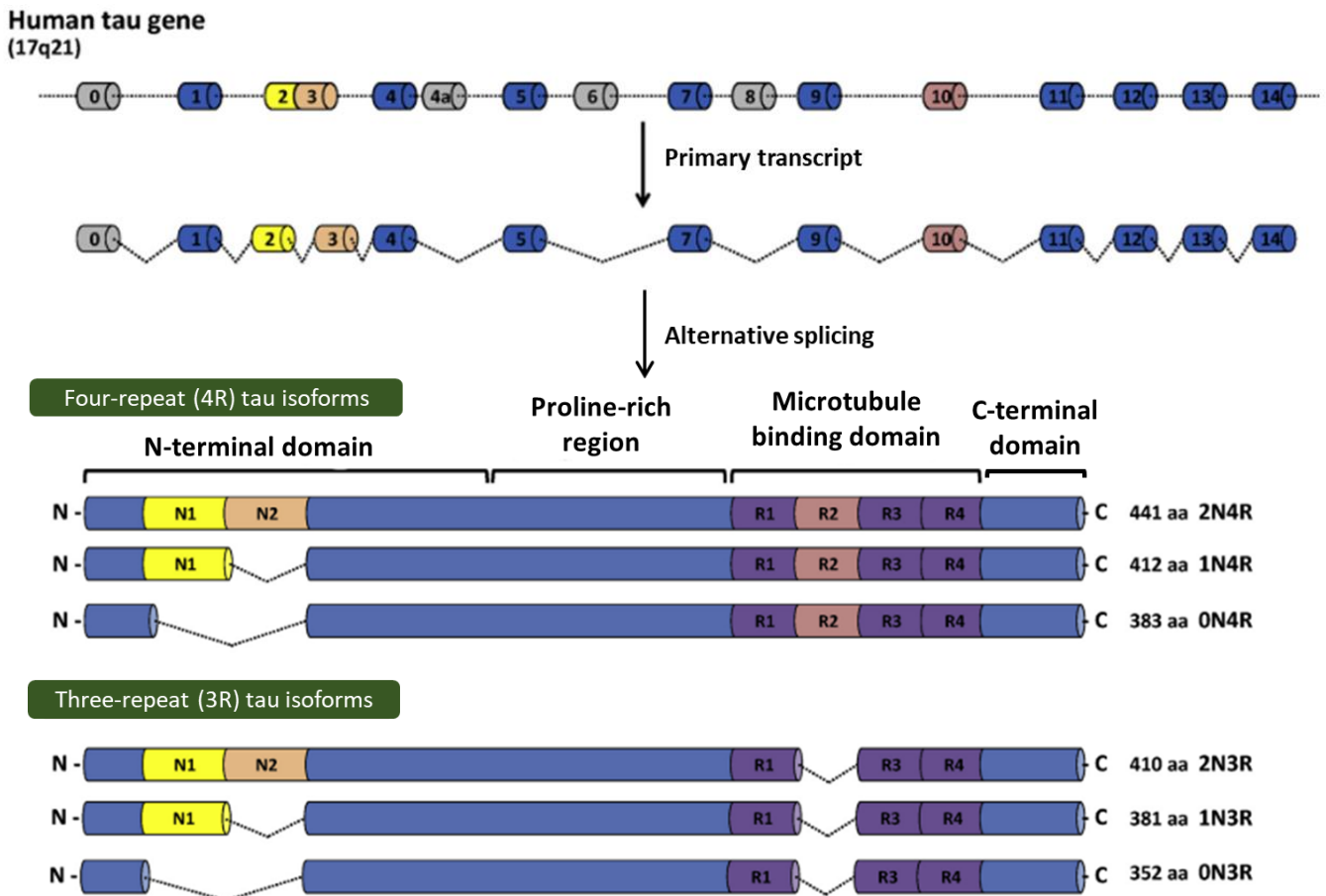


Figure 4 - Schematic representation of tau gene, mRNA and the six tau protein isoforms. Adapted from reference 38.

Tau protein is mostly associated with microtubules in the axon of neurons. Microtubules are the major constituent of the cytoskeleton, composed of a tubulin polymer (Figure 5). After translation, tau undergoes several post-translational modifications (e.g. phosphorylation, glycation, acetylation, methylation, etc), that will determine its functionality. Among all these modifications, phosphorylation is one of the most important. The 2N4R isoform presents up to 85 potential phosphorylation sites, normally occurring on serine (Ser), tyrosine (Tyr) and threonine (Thr) residues of the protein,³⁸ all observed to weaken the interaction with microtubules.³⁹ There is in fact a very dynamic phosphorylation by kinases and dephosphorylation by phosphatases activity which allow cell to control over the degree of tau proteins bind to microtubules. For example, the main kinases that phosphorylate serine/ tyrosine/ threonine residues include glycogen synthase kinase-3 β (GSK-3 β), extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK /MAPK), cyclic AMP-dependent protein kinase (PKA), cyclin- dependent kinase 5 (Cdk5), Ca²⁺ or calmodulin-dependent protein kinase II (CaMKII), Akt (or protein kinase B, PKB), protein kinase C (PKC) and tyrosine kinases such as the SRC family members LCK (lymphocyte- specific protein tyrosine kinase), FYN (proto-oncogene tyrosine-protein kinase) and SYK (spleen tyrosine kinase), and the ABL (a non-receptor tyrosine kinase) family members ARG and ABL1.^{40,41}

Regarding phosphatases, the main enzymes are phosphoprotein phosphatases (PPP) and metal-dependent protein phosphatases (PPM) families. The main phosphatase is protein phosphatase 2A (PP2A), which shows low activity in AD brains. It belongs to the PPP family and includes almost 70 % of overall tau phosphatase activity in the human brain.⁴²

Upon binding microtubules, the repeat region in tau protein interacts with tubulin. Interestingly, the 4R isoforms show stronger affinity than the 3R isoforms. When bound to microtubules, the 4R C-terminal domain is projected away from the microtubule core, probably due to coulombic repulsion, helping to maintain the proper inter-strand distance between microtubules in filaments.

Microtubules carry out more important functions, such as being transport highways for vesicles, proteins and organelles among cells. Thus, in neurons, tau plays an important role in axoplasmic flow by creating and maintaining the microtubule network. It has been observed that more than 80% of the available tau proteins are bound to microtubules at any given time, even if the average residence time of tau proteins on microtubules may be as short as 40 ms.³⁹

Although phosphorylation is critical for tau activity, it has been demonstrated that improper hyperphosphorylation at serine and threonine residues at sites within or immediately adjacent to the MBD (Microtubule Binding Domain) alters tau conformation leading to its detachment from microtubules, therefore inhibiting its activity.⁴¹ However, it is still a matter of debate and it remains to be proven that phosphorylation is the trigger for tau assembly in human diseases. Alternatively, a change in tau conformation as a result of other post-translational modifications above mentioned such as glycosylation, may contribute to hyperphosphorylation and aggregation.⁴⁰ Upon hyperphosphorylation, soluble phosphorylated tau proteins, also known as pre-fibrils, relocate to the neuronal body and accumulates. The neuronal depletion of functional tau protein results in destabilized microtubules, significantly blocking axonal transport and hindering the neuronal health maintenance. The process would explain the degraded neuronal synapses found in AD.³⁷

In summary, two mechanisms – a shift in tau protein kinase and phosphatase activities, and dissociation from microtubules – contribute to promote tau hyperphosphorylation, leading to neurofibrillary pathology and neurodegeneration.

Tau aggregation process

Neurofibrillary tangles (NFTs) are protein inclusions found in the brain of AD patients. Although they were the first histopathological elements identified for the post-mortem diagnosis of AD, NFTs constitute the last stage in tau pathology.¹⁰ NFTs are mostly composed of self-aggregated hyperphosphorylated tau structures, known as Pair Helicoidal Filaments (PHFs) and Straight Filaments (SFs) (Figure 5). It has been proposed that the real toxic species in tauopathies are tau oligomers (PHFs and SPs) and the formation of tau tangles as NFTs would represent a cellular defence mechanism, in order to sequester the toxic forms as tangles, until this mechanism fails and ultimately the cell dies.³⁹

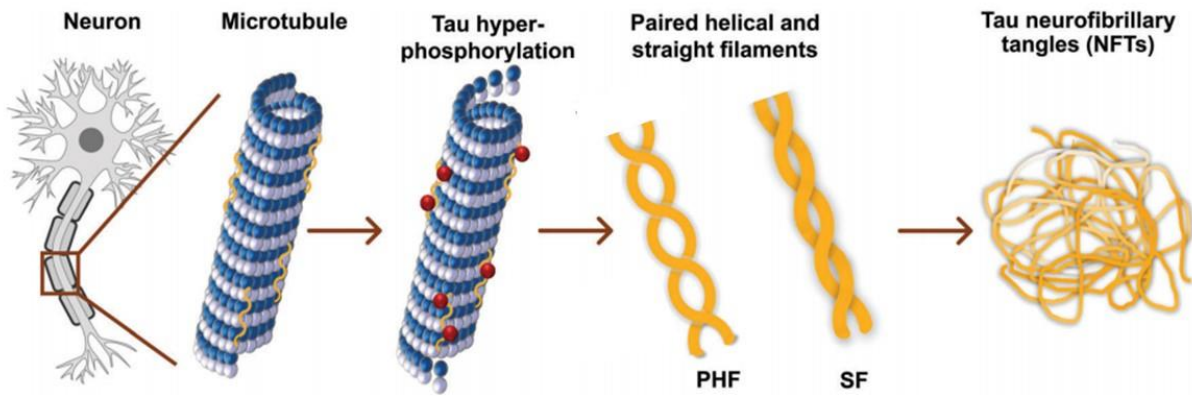


Figure 5 - Tau protein (yellow filament) helps to maintain the microtubule integrity in the neuronal axon. Upon dysregulation of the phosphorylation-dephosphorylation control system, tau can progress into toxic paired helical filaments (PHFs) and straight filaments (SPs) species that ultimately aggregate forming NFTs. Taken from reference 37.

Tau does not aggregate by itself.⁴³ The MBD, core of tau protein, is positively charged and that charge avoids intermolecular interactions of tau. These positively charged residues interact with negatively charged residues of tubulin. Under pathological conditions, tau is highly phosphorylated and neutral as the positive charge is neutralised by the negative charges of phosphorus residues, and this neutralization of charges in the MBD could induce detachment of tau from tubulin and allow tau-tau interactions.⁴³ Polyanionic compounds such as heparin or RNA promote aggregation of recombinant tau probably by also neutralizing the positive charges of tau.⁴⁴ Tau oligomers self-assemble and aggregate forming **cross- β amyloid fibrils**.

Monomers, oligomers and fibrils – The hierarchy of cross- β amyloid fibril

Proteins can adopt β -sheet conformation as secondary structure. As initially demonstrated by Berriman *et al.*, and later by Fitzpatrick and co-workers via Cryo-EM studies, tau protein aggregates forming β -sheets, which consist of β -strands connected laterally by hydrogen bonds between N-H of one strand with the C=O groups of opposite strands, inside the protein itself (Figure 6).^{45,46}

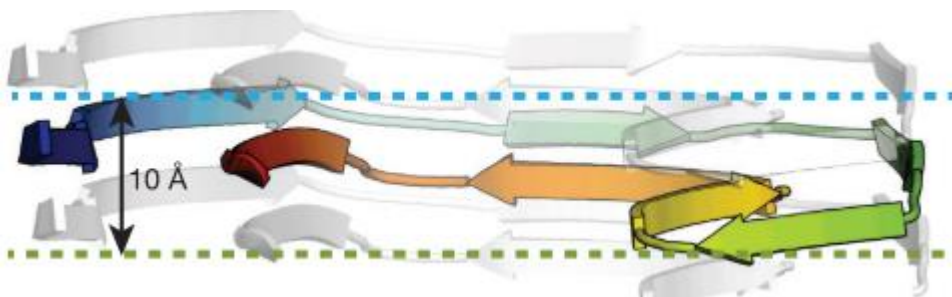


Figure 6 - Example of a β -sheet conformation of one single molecule of Tau⁴⁴¹. Arrows in different colours indicate the different β -strands that connect one another to conform the β -sheet structure.

In the case of Tau protein, each β -sheet presents differences in height of 10 Å. Picture taken from reference 46.

A wide variety of peptide of proteins can self-assemble into amyloid fibrils without any evidence of sequence similarity.^{47,48} Amyloid fibrils have many common characteristics, such as being 100-200 Å in diameter and a core structure composed of arrays of beta-sheets stack antiparallel one to another via 180° rotation, which running parallel to the long axis of the fibrils to create a two-sheet protofilament^{49,50} (Figure 7A). This pattern is called “cross- β ” structure and confers mechanical properties comparable to those of steel,⁵¹ and much greater than typical biological filaments such as microtubules, due to its highly ordered state and persistence lengths of the order of microns.⁵¹ Amyloid fibrils possess high kinetic and thermodynamic stabilities, as well as greater resistance to degradation by biological or chemical means.⁵²

Understanding the conversion of normally soluble functional proteins into the amyloid state is key for the study of many human disorders, such as AD or type II diabetes. Indeed, Fitzpatrick and co-workers observed that a hierarchical order of assembly is established in amyloid fibrils.⁵⁰

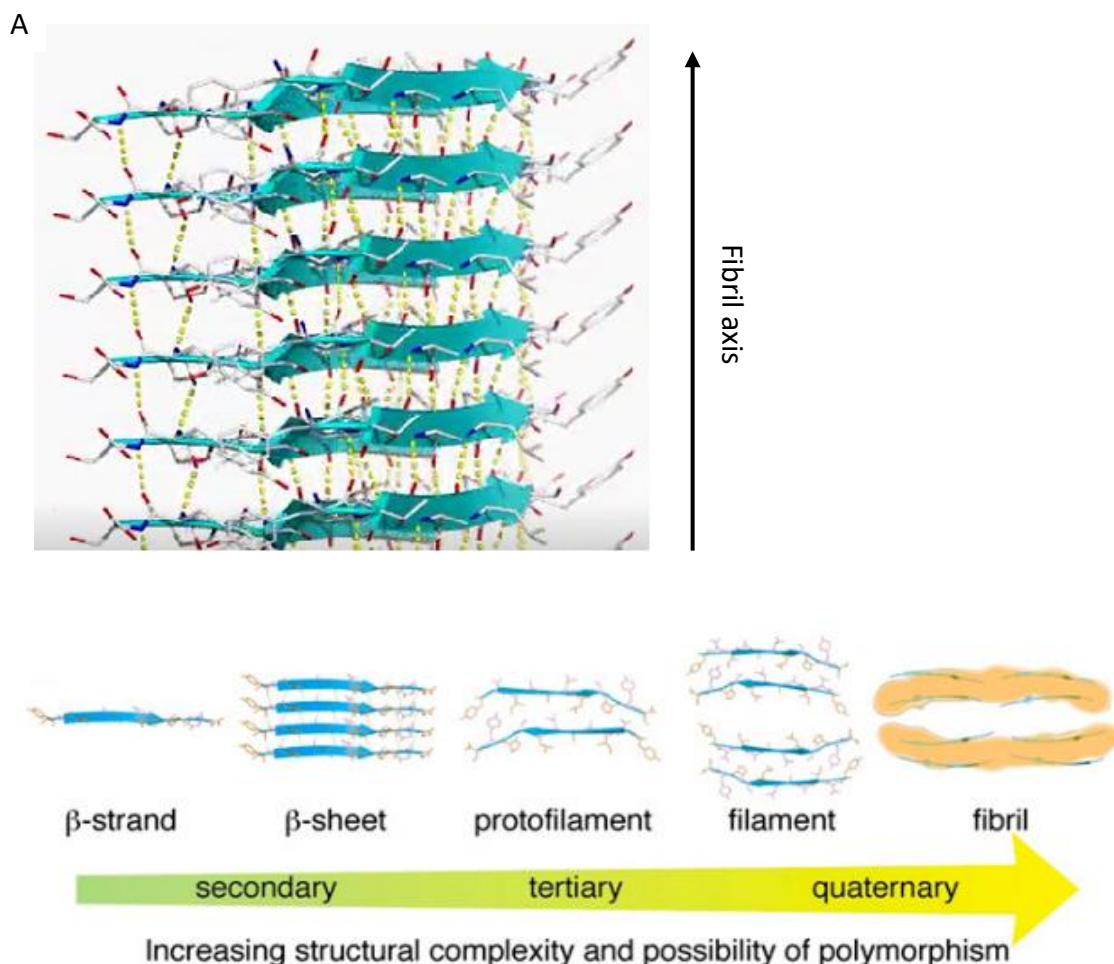


Figure 7 - (A) 3D view of two antiparallel β -sheets that forms a protofilament. **(B)** Hierarchy of atomic-resolution motifs involved in the self-assembly of amyloid fibrils. Picture taken from reference 50.

The next level is the assembly of protofilaments into filament, where two protofilaments face each other. As final step, a fibril is form by interactions between filaments interconnected in a head-to-tail fashion. A fibril can easily accommodate 2, 3 or 4 sets of filaments, forming mature fibrils of different size (Figure 7B).

Ultrastructural polymorphism is a common feature of amyloid fibrils.^{47,48} Human Tau protein presents polymorphism at the filament level of assembly: straight filaments (SPs) and Paired Helicoidal Filaments (PHFs).

SPs and PHFs structures

Tau inclusions on AD patients are made of PHFs and SFs. Analysis and modelling of the cryo-EM densities by Fitzpatrick *et al.* revealed that both PHFs and SFs filaments are composed of two protofilaments with C-shaped subunits comprising residues 306-378, and disordered N- and C termini forming the fuzzy coat (Figure 8). The β -sheet rich C-shaped cores consist of R3, R4 domains and the 10 following C-terminal amino-acids (Figure 8A) which form eight β -sheets (β 1-8) that run along the length of the protofilament (Figure 8B).⁴⁶

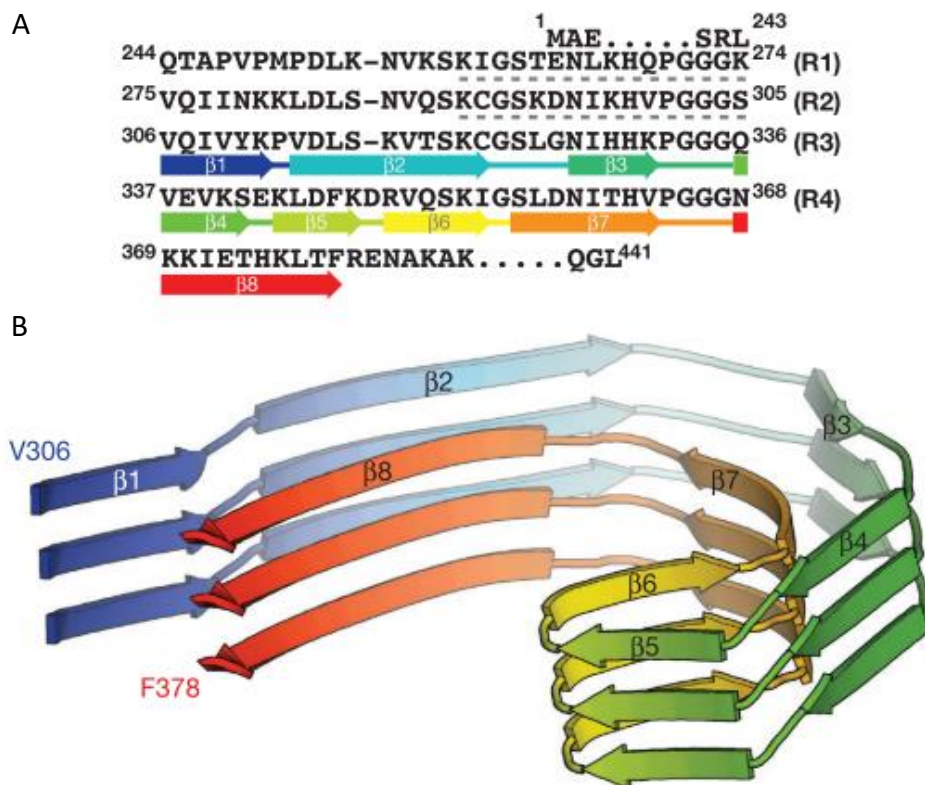


Figure 8 - A) Sequence alignment of the four microtubule-binding repeats (R1-R4) of Tau protein. Each of the eight β -strands observed are coloured from blue to red. R1 and R2 may form an additional, less-ordered β -sheet which is indicated with grey dashed underlines. **B)** C-shaped -

protofilament core unit - representation of the secondary structure elements in three successive rungs. Pictures taken from reference 46.

Differences in lateral contacts between the two protofilaments explains the ultrastructural polymorphism found between PHFs and SPs.

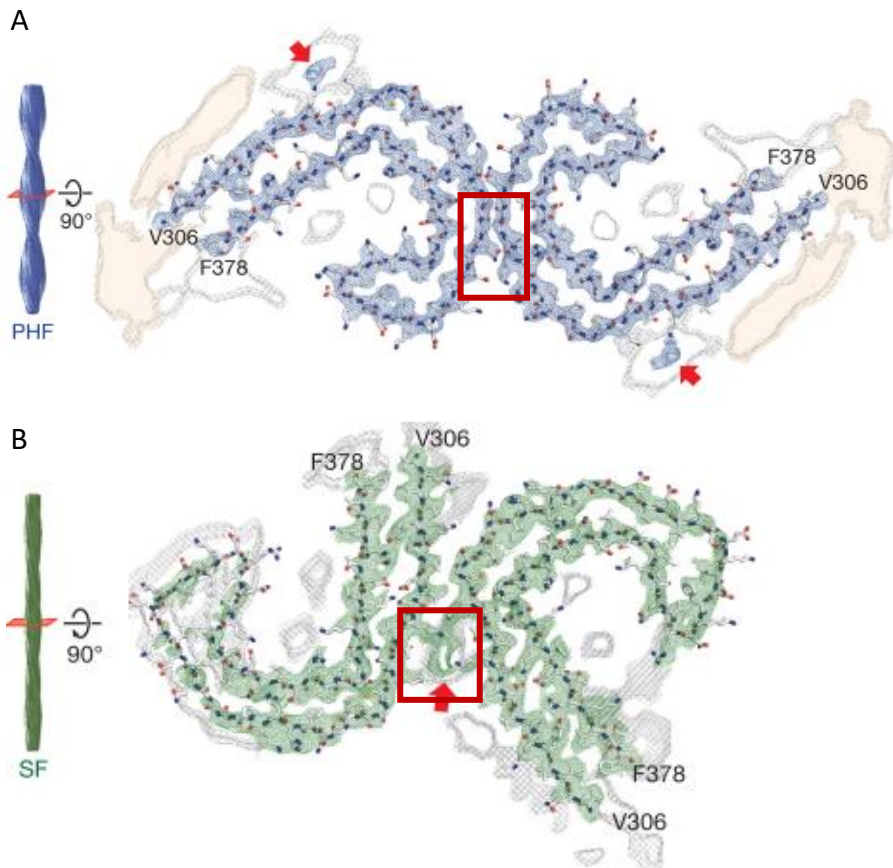


Figure 9 - Cross-sections of the PHF and SF structures. Cryo-EM structural assignments of Pair Helicoidal Filaments (PHF), **A** and Straight Filaments (SFs), **B**. Red arrows indicate additional densities in contact. Red squares highlight the interface between the two protofilaments. Taken from reference 53.

In PHFs, the two protofilaments form identical structures, whose interface is formed by the anti-parallel stacking of residues $_{332}\text{PGGGQ}_{336}$. The glycine tripeptide adopts a characteristic polyglycine II,⁵⁴ β -spiral structure that forms a hydrogen-bonding pattern both within and between the two protofibrils. The PHF interface is further stabilized by the formation of two hydrogen bonds between Q_{336} and the backbone carboxyl of K_{331} on the opposite protofilament.⁴⁶

In the case of SFs, the two protofilaments pack asymmetrically. The backbones of the two protofilaments are nearest each other between residues $_{321}\text{KCGS}_{324}$ of the first and $_{313}\text{VDLSK}_{317}$ of the second protofilament. However, these residues do not form hydrogen bonds or inter-protofilament salt bridges, nor the interaction seem to be mediated by hydrophobic packing. Fitzpatrick and co-workers⁴⁶ speculated that salt bridges between the negatively charged glutamates of the N terminus, (γEFE_9 motif), and the positively charged lysines of the core stabilize the interaction between the two

protofilaments (red arrow, Figure 9B). Similar additional density has been observed in PHFs, although it does not contribute to the protofilament interfaces (red arrows, Figure 9A).

In summary, Tau protein can assemble and form a core consisting of a pile of parallel β -sheets with C-shape,⁵⁵ whereas the amino- and carboxy-terminal regions of tau are disordered and project away from the core to form the fuzzy coat⁵⁶ (Figure 10A and 10B). The two polymorphs derived from the distinct lateral contact between protofibrils constitute the PHFs and SP. The former more symmetrical than the latter, since the N-terminal of each protofibril can interact with the core of the filament (Figure 10C).

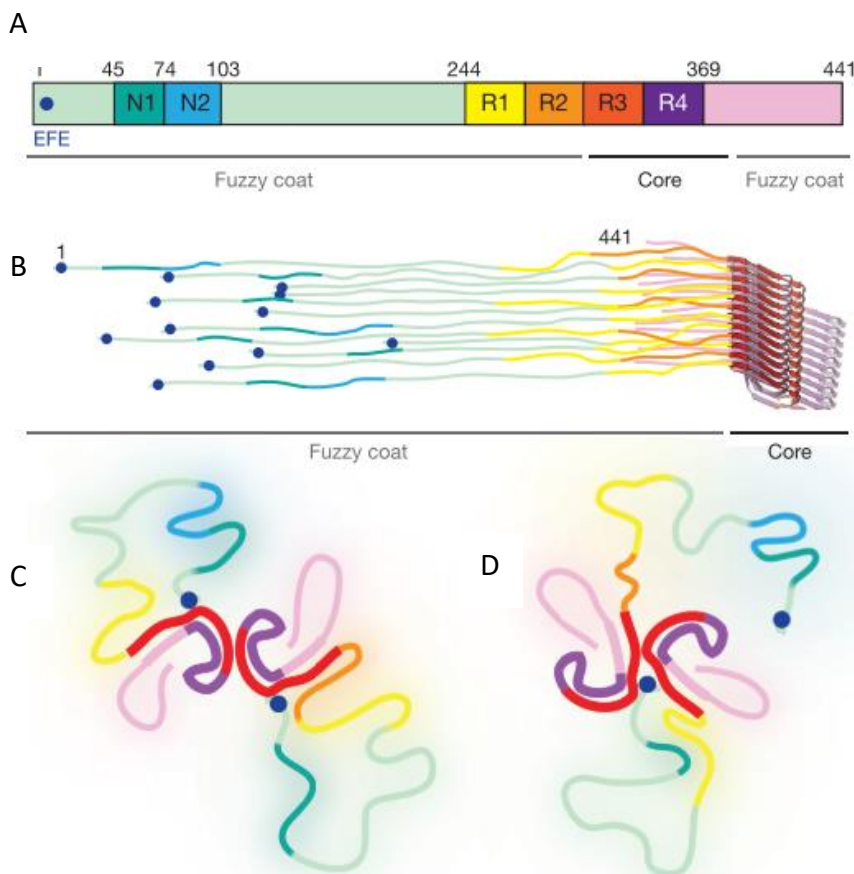


Figure 10 - (A) 2N4R tau sequence and ultrastructure in tau filaments. N-terminus (γ EFE $_9$ is represented by a blue dot). The microtubule-binding repeats are labelled R1-4. **(B)** Random packing of all six different isoforms of tau. **(C and D)** Schematic representation of full-length tau filaments, with the β -sheet region bold of PHFs **(C)** and SPs **(D)**, colour scheme following **(C)**. The hypothesized “backbiting” interaction is shown between the γ EFE $_9$ motif (blue dot) and the filament core. Images taken from Fitzpatrick et al.⁴⁶

Tau and Amyloid β : “I need you - You need me”

Even though tau pathology is more closely associated with cognitive decline and neuronal cell loss than $A\beta$ plaque deposition in AD, Braak and co-workers frequently found NFTs in the medial temporal lobe and brainstem of older subjects who are cognitively normal with no evidence of neurodegeneration. These findings suggest tau inclusions do not necessarily provoke AD.⁵⁷ On the other hand, brain regions with both $A\beta$ deposition and tau tangles present, have been found in patients with AD, which enforces the theory that only the combination of these two molecular pathologies are necessary for the AD degenerative process to occur.¹⁴

Brier and co-workers showed evidence suggesting $A\beta$ plaque burden aggravate tau pathology progression, as there is a strong correlation between the severity of tau pathology and $A\beta$ pathology in clinically demented AD patients.³⁵ The injection of synthetic $A\beta$ fibrils into transgenic mice overexpressing either human APP or human mutant tau induced tau pathology.^{14,58}

Therefore, AD is a complex disease characterized by a dual proteinopathy, which needs both amyloid and tau deposition to disease progression. Figure 11 summarise the pathology and progression in Alzheimer’s Disease:¹⁴

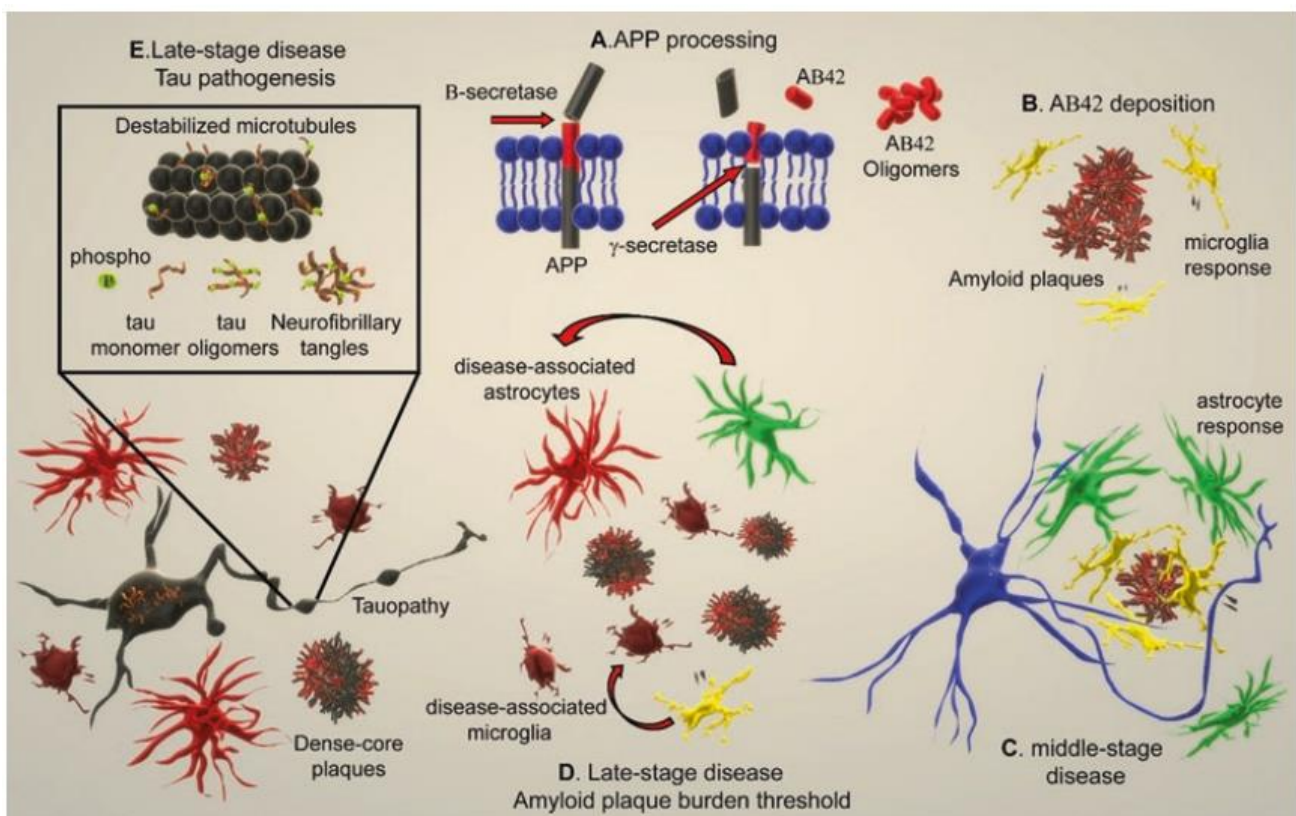


Figure 11 - Schematic representation of Alzheimer’s disease pathology and progression. (A) The first pathological event is the production of the $A\beta_{42}$ peptide, which is yielded by the cleaving activity of two enzymes, β -secretase and γ -secretase, of protein APP. (B, C) Extracellular $A\beta_{42}$ peptide forms $A\beta_{42}$ oligomers and initiates $A\beta$ plaque deposition. The $A\beta$ plaque activates microglial defence system, which surround the plaque and recruit astrocytes. Initially, this response is neuroprotective

by forming a barrier between the plaques and the surrounding neurons, diminishing the impact A β -induced neurotoxicity. (D) As the disease progresses, the A β plaque deposition reaches a threshold forming the dense-core plaques that are associated with dystrophic neurites and influences tau pathogenesis. In late-stage AD, disease-associated microglia may facilitate tau pathogenesis by phagocytosing tau protein and secreting neurotoxic factors, thus promoting a neurotoxic inflammatory response. (E) Tau pathology spreads to connect brain regions, which drives the neurodegenerative process. Reproduced from reference 14.

1.1.3 Diagnosis of AD

PET imaging

In AD, tau pathology is more closely linked to neuronal death than A β aggregation and is presumed to begin decades before the clinical onset of the disease.⁵⁹ Awareness of the importance of early diagnosis in neurodegenerative diseases such as AD, has risen in the last decade, and there is a growing interest in the development of new radiotracers capable of showing high selectivity for one of the two hallmarks on AD, especially on tau protein, due to its better correlation with the disease progression. Therefore, the possibility of visualizing tau lesions in living brains would provide information about AD progression from an asymptomatic early stage of the condition.

Nowadays, examples of very sensitive molecular imaging techniques are the radionuclide-based positron emission tomography (PET) and single photon emission computed tomography (SPECT).

PET is a medical radiodiagnosis technique effective for the obtention of functional images of biological processes, from molecular to body level, using specific imaging probes.⁶⁰ This technique is non-invasive, possess high sensitivity, good resolution, and enables accurate quantification of biochemical, physiological and pharmacological processes real-time. As molecular imaging technique, PET provides the physician with information on metabolic or molecular events, e.g. which is the drug efficacy in a precise biological system. By contrast, Computerized Tomography (CT) or Nuclear Magnetic Resonance (NMR) techniques provide structural imaging (anatomical images with information on the shape and size) of organs.

PET requires the use of imaging probes named radiotracers, which are drug-like organic molecules in which one of the atoms is radioactive, i.e. fragments spontaneously resulting in the emission of radiation. As its name indicates, PET requires that the radioactive decay takes place by emission of positrons.

After the administration of the radiotracer, the emission of radioactivity is measured from the outside (Figure 12). The tracer will reach its target and the positron generated by the natural decay of the positron-emitting radionuclide will collide with an electron (the antimatter of the positron) belonging to the patient. This collision (annihilation) generates two γ -ray photons (511 KeV), which depart from each other in the same direction and opposite sense. The external detection of the two

photons simultaneously emitted is key in the accuracy of PET technique, and allows the construction of three-dimensional maps of radioactivity by sections (computerized tomography).⁶¹

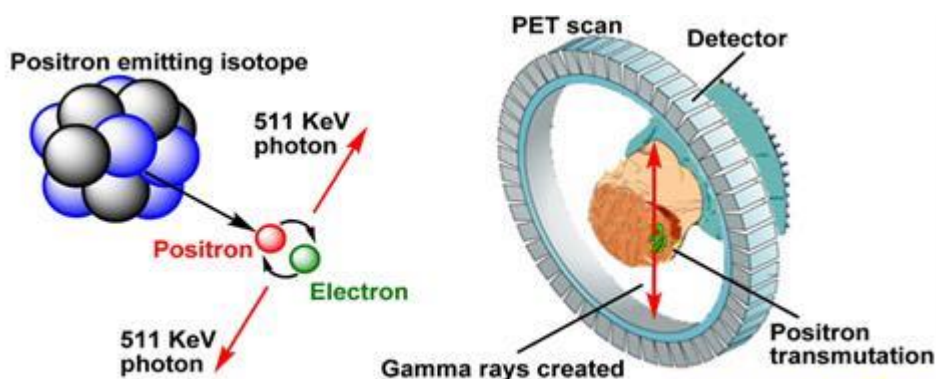


Figure 12 - Annihilation between a positron and an electron (left) producing 511keV photons that are registered by the circular gamma ray detector in the PET camera. Picture taken from reference ⁶¹.

PET employs positron-emitting isotopes of atoms found in bioorganic molecules such as carbon, nitrogen and oxygen, thus allowing the syntheses of radiopharmaceuticals that are chemically equal to their non-radioactive counterparts (apart from a negligible isotopic effect). The biological activity also remains the same. Fluorine-18 radiolabelling is commonly used due to its advantageous physical properties: it has the best imaging physical characteristics (due to low positron energy) and an optimal half-life ($t_{1/2} = 110$ min). Fluorine atom is not a common constituent of biomolecules but is frequently used for bio-isosteric replacements of hydrogen atoms or methyl groups. From a steric point of view, H-F swap causes minimum perturbations. However, the strong electron-withdrawing properties of F can alter electronic properties, lipophilicity or biological characteristics, sometimes improving the outcomes when compared to their nonfluorinated analogues.⁶⁰

More examples of PET radionuclides are ^{15}O ($t_{1/2} = 2$ min), ^{13}N ($t_{1/2} = 10$ min) and ^{11}C ($t_{1/2} = 20,3$ min), ^{94}Tc ($t_{1/2} = 52.5$ min) or ^{124}I ($t_{1/2} = 4.17$ d).

The choice of a radionuclide depends on its half-life, the aim of the study (for example, receptor binding equilibrium of antibody might be only obtained several days after the injection of the tracer, so long life radionuclides are used), the protein binding propensity and the clearance rate. Low binding of radioligand to plasma proteins is essential, since only free fraction of radiotracer will reach the target. The accepted free fraction of a radioligand in plasma (f_p) is ~ 0.1 .⁶² The ability to cross the blood brain barrier (BBB) is fundamental for radiopharmaceuticals targeting the brain. For instance, fluorodeoxyglucose (^{18}F -FDG), a widely used radiotracer in clinics, can be used to monitor the glucose metabolism in the brain after brain injury and it easily cross the BBB by means of GLUT transporters.⁶³ This mechanism guaranties the access to the targeted area.

Synthesis, isolation, and purification of radiopharmaceuticals is carried out in shielded hoods or “hot cells”, normally using automatic synthesis modules. Purification is performed under radioactive protection by semipreparative HPLC inside the hot cell, and characterization is carried out under

radioactive protection by HPLC or GC. This requires the availability of cold standards (non-radioactive compound) for reference.

Tau and Amyloid- β PET imaging

One of the main issues of AD was the accuracy of the diagnosis. Until recently, patients were diagnosed based on clinical tests (e.g. Mini-Cog test)⁶⁴ that were oriented to identify other neurological conditions. When any other disorder was dismissed, then the physician would diagnose AD. In fact, accurate diagnosis could only be performed via post-mortem analysis of patient's brains with suspected Alzheimer's disease.

However, the clinical phenotypes of patients with proteinopathy do not always allow the identification of the underlying cause of the condition, especially in early stages of the disease. By contrast, biochemical and imaging biomarkers can identify, even at asymptomatic stages, the hidden proteinopathy likely to cause the disease.⁶⁵ Imaging of tau biomarker in combination with new diagnostic criteria (such as levels of tau and A β in cerebrospinal fluid) can help stage disease progression, without being conditioned by clinical symptoms that are often late features (sometimes nonspecific, mainly at early stages) of AD.

Progress for tau tracers has considerably delayed behind its amyloid counterparts due to several challenges: first, tau is intracellular, thus requiring the ligand to cross the plasma cell membrane as well as the blood-brain barrier, imposing restrictions on molecular size and lipophilicity and second, are present in lower concentrations than amyloid- β aggregates, thus requiring tau ligands to have higher selectivity (20–50 fold affinity) for tau over amyloid- β deposits.⁶⁶ Until now, our understanding about the pathological behaviour and topography of tau deposition has only been based on post-mortem and cerebrospinal fluid studies. Having evidence in the progression of the disease in patients can provide evidence to demonstrate tau as a central driver of downstream neurodegenerative processes and cognitive decline.⁶

Tau PET radiotracers

In recent years, significant advances have occurred in tau tracers starting from the development of first class PET tracers (namely ¹⁸F-FDDNP, ¹⁸F-Flortaucipir or ¹⁸F-T807, ¹⁸F-T808, ¹⁸F-THK-5351, ¹⁸F-THK-523) and moving to second generation tau-tracers (namely, ¹⁸F-RO69558948, ¹⁸F-MK-6240, ¹⁸F-PI2620 and ¹⁸F-PM-PBB3) which are under development.^{65,67–70}

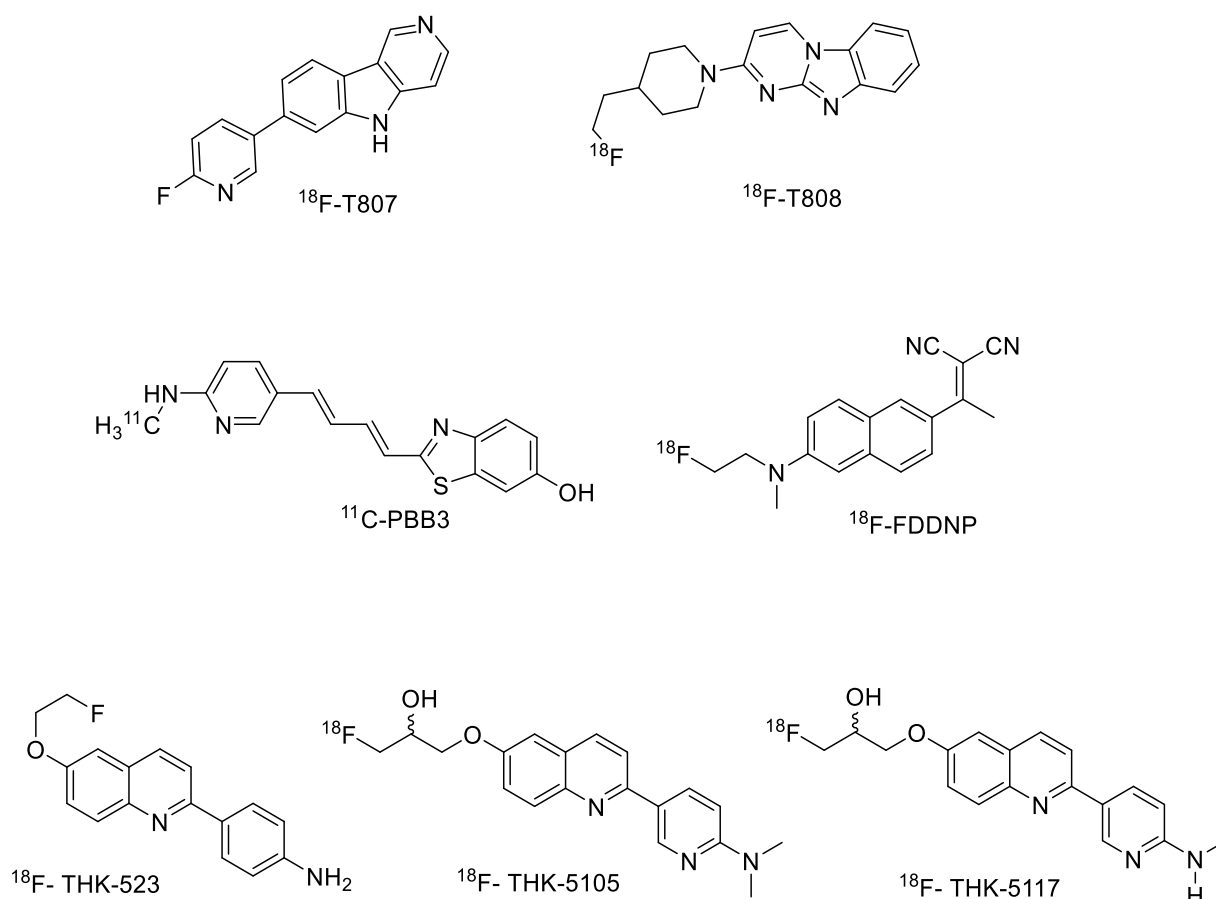


Figure 13 - Chemical structures of the first generation of tau tracers.

First-generation tau tracers:

First generation tau tracers showed higher affinity for tau than amyloid- β aggregates and have reached clinical trials in humans. However, they all present some issues that limit their use, mostly related with suboptimal pharmacokinetics and off-target binding.⁶⁵

^{18}F -FDDNP

This naphthylethylidene derivative was the first PET radiotracer to be applied in clinical PET imaging of tau pathology in patients with AD.⁷¹ ^{18}F FDDNP was initially a $\text{A}\beta$ tracer, which, in contrast to [^{11}C]PiB, also bound to neurofibrillary tangles.⁷²

^{18}F -T808 and ^{18}F -T807

^{18}F -T808, a benzo[4,5]imidazo[1,2-*a*]pyrimidine derivative, was reported as selective tau PET radiotracer.^{69,73} This radioligand showed selective labelling of tau pathology with a laminar distribution in the neocortex of AD brain sections.

Developed by Kolb et al., ^{18}F -AV1451 (formerly known as ^{18}F -T807) is a pyrido[4,3-*b*]indole which is among the most used tracers at present. However, substantial off-target binding has been found for both ^{18}F -T808 and ^{18}F -T807 in the absence of tauopathies.

¹¹C-PBB3

¹¹C-PBB3 is a pyridinyl-butadienyl-benzothiazole derivative, which was developed by Higuchi's group⁷⁴ as part of a unique chemotype class of radiotracers. As with [¹⁸F]T807, there is minimal white matter binding, but the existence of a radiolabelled metabolite constitutes a significant limitation for quantification of ¹¹C-PBB3. An important assumption underlying measurement with PET is that the radioactivity measured in brain tissue is composed exclusively of unchanged radioligand; that is without contamination by any radiometabolite that interferes and affords inaccurate measurements.⁶²

THK- radiotracers

The first generation of THK radiotracers included ^{18}F -THK-523, ^{18}F -THK-5105, and ^{18}F -THK-5117 (Figure 13), a family of quinoline analogues which show several defects, including poor disease discriminability and high white matter retention, that hindered their clinical utility.⁷⁵

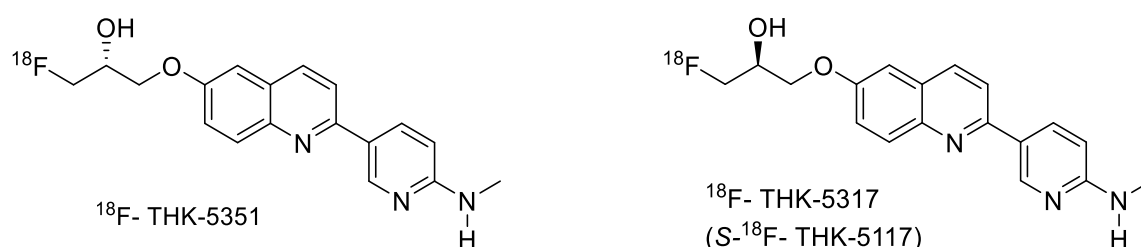


Figure 14 - Enantiomers *R* and *S* of ^{18}F -THK5117.

The use of pure enantiomers helped to reduce the limitations of this first generation. Thus, ¹⁸F-THK-5317 (*S*- enantiomer of ¹⁸F-THK-5117; Figure 14) is able to differentiate between AD and mild cognitive impairment (MCI) patients and healthy controls.

Second-generation of tau radiotracers:

The second generation of tau tracers improved some of the limitations of their predecessors, showing lower off-target binding defects and improved pharmacokinetic profiles.

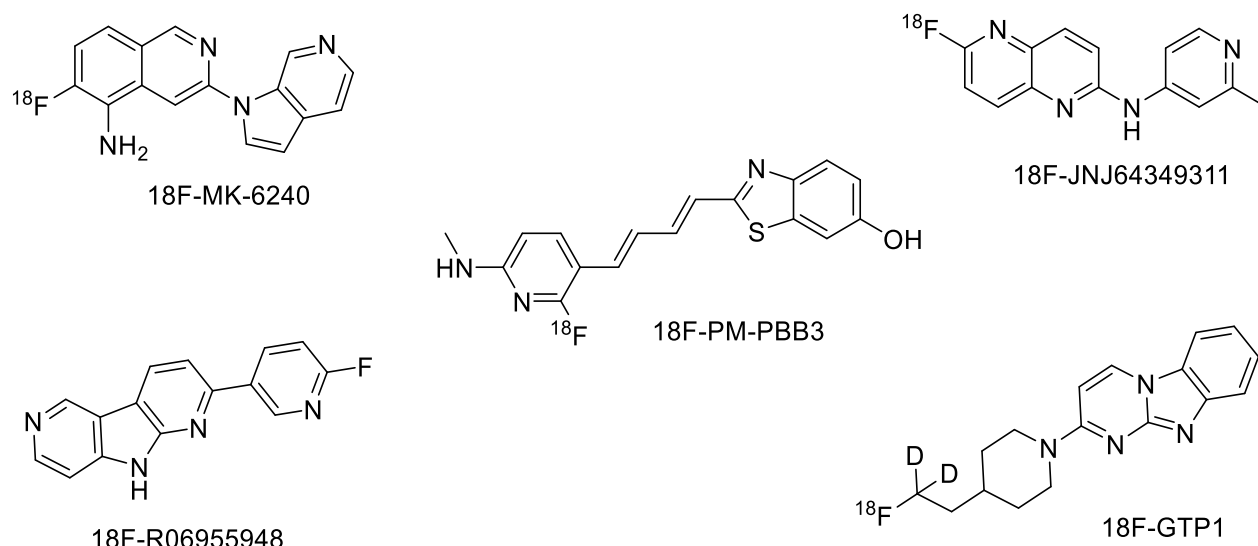


Figure 15 – Chemical structures of second generation of tau tracers.

^{18}F -MK-6240

This isoquinoline derivative showed high affinity to tau deposits specifically in the brain tissue of AD patients over those in the tissue of patients with other tauopathies, $\text{A}\beta$, and α -synuclein. However, important off-target binding has been found for ^{18}F -MK-6240.⁷⁶

^{18}F -GTP1

Genentech tau probe 1 (^{18}F -GTP1, Figure 15) possess a chemical structure that highly resembles to that of T808 (Figure 13). ^{18}F -GTP1 is one of the most recently developed tau tracers⁷⁷ with improved affinity and selectivity for tau pathology that its parent compound T808.

^{18}F -PM-PBB3

Shimada *et al.* have developed new fluorinated PBB3 derivatives, such as ^{18}F -PM-PBB3 to overcome some of the limitations present by ^{11}C -PBB3. As previously described, the chief disadvantages of the [^{11}C]PBB3 include the short half-life of carbon-11 ($t_{1/2} = 20$ min), significant off-target binding in striatal regions and limited dynamic range. Current clinical trials in AD patients show an improved profile in behalf of these new PBB3 ligands, and reduction or total absence of off-target signals in the basal ganglia and thalamus.⁷⁸

^{18}F -R06958948

Developed by Roche, ^{18}F -RO695894 is the isosteric product of ^{18}F -T807. The benzyl ring was substituted by pyridine, affording a fused pyrido-indole. As a result, ^{18}F -RO695894 improved significantly the affinity for tau tracer in AD brains, as well as the clinical profile and kinetic characteristics compared to ^{18}F -T807.⁶⁸ However, none of these tracers showed significant binding to other tau pathology such as PSP, CBD and Pick's disease.⁶

^{18}F -JNJ64349311

Rombouts and co-workers developed in 2017 the 1,5-naphthiridin-2-amines derivative ^{18}F -JNJ64349311, which showed higher affinity for aggregated tau over β -amyloid in comparison with ^{18}F -T807 in animal models

.

1.2 HYPOTHESIS, AIM AND OBJECTIVES

Neurodegenerative diseases represent a concern for the society. Tauopathies and more specifically, AD, will affect an increasing number of people in the near future. Early diagnose of these diseases is key for the success of therapies currently under development.

One of the most powerful tools for the early diagnose is PET, and development of radiotracers with high affinity for tau protein ensures not only the diagnose but also the ability to monitor the evolution of tauopathies.

We aim:

- 1.- to design, synthesise and characterize novel heterocyclic structures with potential affinity for tau protein.
- 2.- to evaluate the binding-affinity of the synthesized compounds for tau protein using different approaches (*i.e.* SPR, molecular modelling, in vitro studies) to select the most promising candidates for further development.
- 3.- to assess the stability and cytotoxicity (*i.e. ex vivo* haemolysis assay) of best candidates to tau tracers.
- 4.- to design of synthetic routes suitable for the preparation of cold ligands and radiosynthesis precursors for the selected structures.

1.3 RESULTS AND DISCUSSION

1.3.1 Introduction and background

Tau imaging and the requirements for tau PET-radiotracer design

Cognitive impairment is highly associated with tau deregulation and deposition, as it has been demonstrated in post-mortem studies and cerebrospinal fluid measurements.^{12,35,79} Tau-PET imaging is the latest addition to the array of tools for the non-invasive diagnosis of degenerative proteinopathies. It helps and improves understanding of tau aggregation and deposition in the human brain, affording insight into causes, diagnosis, and treatment of tauopathies such as Alzheimer's disease, progressive supranuclear palsy, or chronic traumatic encephalopathy among others. If administered before the event of irreversible neuronal damage, therapeutic interventions have a better chance of success. Early detection of the underlying pathological process is decisive for therapeutic strategies intended for modulation of tau aggregation and deposition.

PET imaging is advantageous because it provides quantitative information of physiological, biochemical and pharmacological processes using picomolar concentrations, without perturbing the biological system.⁸⁰

Candidates to PET probes must fulfil a series of requirements to be useful neuroimaging tracers.^{81,82} For instance, small, non-toxic lipophilic molecules that can cross the blood-brain barrier. Amyloid and tau tracers are usually polycyclic compounds, with high planarity due to the presence of aromatic residues. In addition, tracers should be rapidly cleared from blood, ideally not metabolised and possess low levels of non-specific binding. Most importantly, they should selectively, reversibly and specifically bind to the target. This is experimentally determined by biodistribution, radiometabolite, and autoradiography studies.^{62,83}

The radiolabelling is preferably done with isotopes whose radioactive half-lives are long, such as ¹⁸F (half-life~120 min) or ⁶⁴Cu (~13h), that allow centralised manufacture and regional distribution of these radiotracers.

Imidazo[1,2-*a*]pyridines

Our first objective was selecting a suitable heterocycle not previously used as tau tracer, that accomplished the requirements above mentioned and possessed a privileged scaffold with optimal pharmacological and pharmacokinetics profiles.

Imidazo[1,2-*a*]pyridine is, among the nitrogen-fused azoles, one of the most relevant moieties in the pharmaceutical and pharmacological field. Imidazo[1,2-*a*]pyridine is an advantageous scaffold: it is present in molecules with a wide range of biological activities such as antifungal, antiviral, antituberculous, anticancer, antiprotozoal, anti-inflammatory, hypnoselective, antipyretic, antibacterial, anthelmintic, analgesic, antiepileptic, anticonvulsant, antiulcer and anxiolytic.^{84–88}

They also act as β -amyloid formation inhibitors, GABA and benzodiazepine receptor agonists, H_2 receptor antagonist and cardiogenic agents.^{89–91} Marketed drugs such as olprinone (for the treatment of heart failure),⁹² zolimidine (used for the treatment of peptic ulcer),⁹³ zolpidem (for the treatment of insomnia),⁹⁴ and the optically active GSK812397, candidate for the treatment of HIV (Figure 16) are imidazo[1,2-*a*]pyridine analogues.⁹⁵ In view of this, imidazo[1,2-*a*]pyridine heterocycle arose as a strong candidate and was chosen for the development of the molecules presented in this work.

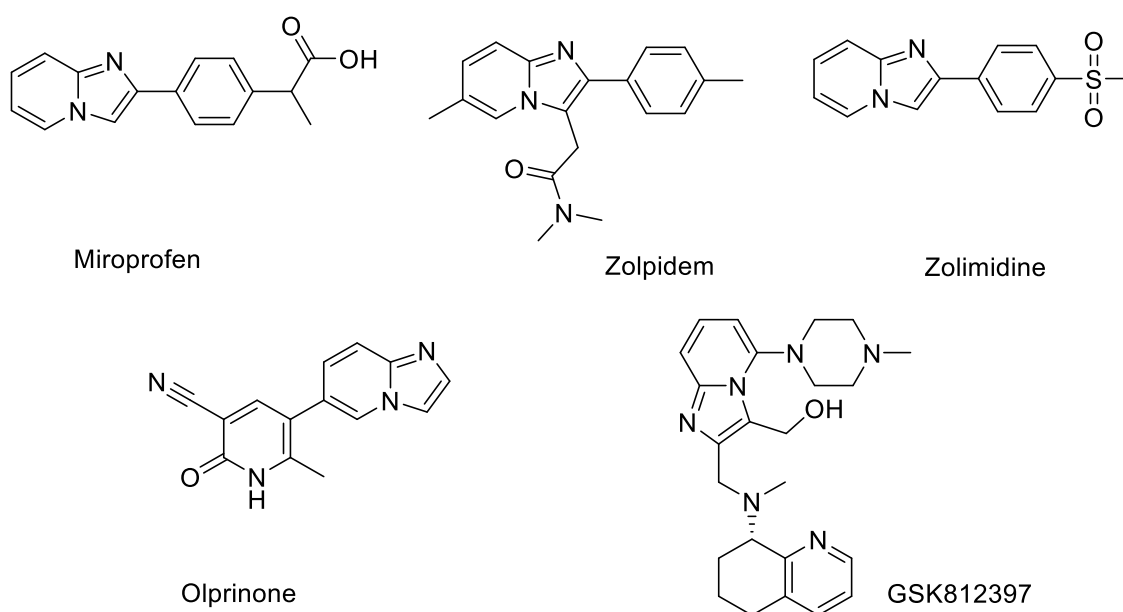


Figure 16 - Imidazo[1,2-*a*]pyridine-based marketed drugs.

Synthetic methods for the preparation of imidazo[1,2-*a*]pyridines

We would not be surprised if such a well-equipped heterocycle had a wide variety of synthetic routes for its preparation. In fact, that is the case, due to a continuous effort to develop different synthetic strategies covered in the literature^{96,97} for this privileged imidazo[1,2-*a*]pyridine scaffold.

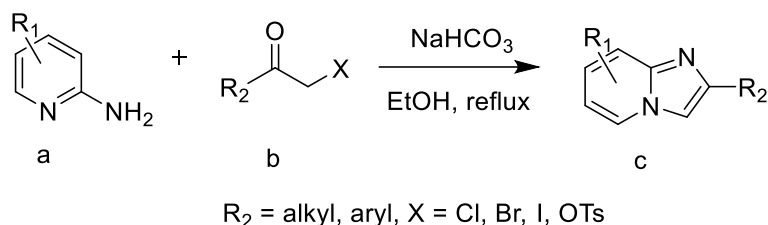
These multiple approaches can be classified into condensation, oxidative coupling and multicomponent reactions, synthesis from pyridinium salts, etc. Some of the most relevant routes are next reviewed.

Condensation reactions

From α -haloketones

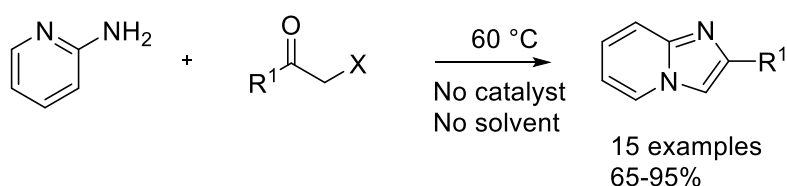
2-Aminopyridines **a** constitute key substrates for the synthesis of imidazo[1,2-*a*]pyridines **c** (scheme 1). The nucleophilic substitution of the halogen atom in the α -haloketone (**b**) by the pyridine-nitrogen

in 2-aminopyridine is the key step for these reactions. Tschitschibabin (also known as Chichibabin) pioneered the reporting of a method for the synthesis of imidazo[1,2-*a*]pyridines **3** in 1925.⁹⁷ The reaction of 2-aminopyridine with an α -haloketone at 150–200 °C in a sealed tube afforded the formation of **3**, although in low yields. This method was later adapted by using a base like sodium hydrogen carbonate under mild reaction conditions to give **3** with higher efficiency.



Scheme 1. Synthesis of imidazo[1,2-*a*]pyridines (**c**) from 2-aminopyridine (**a**) and an α -haloketone (**b**).

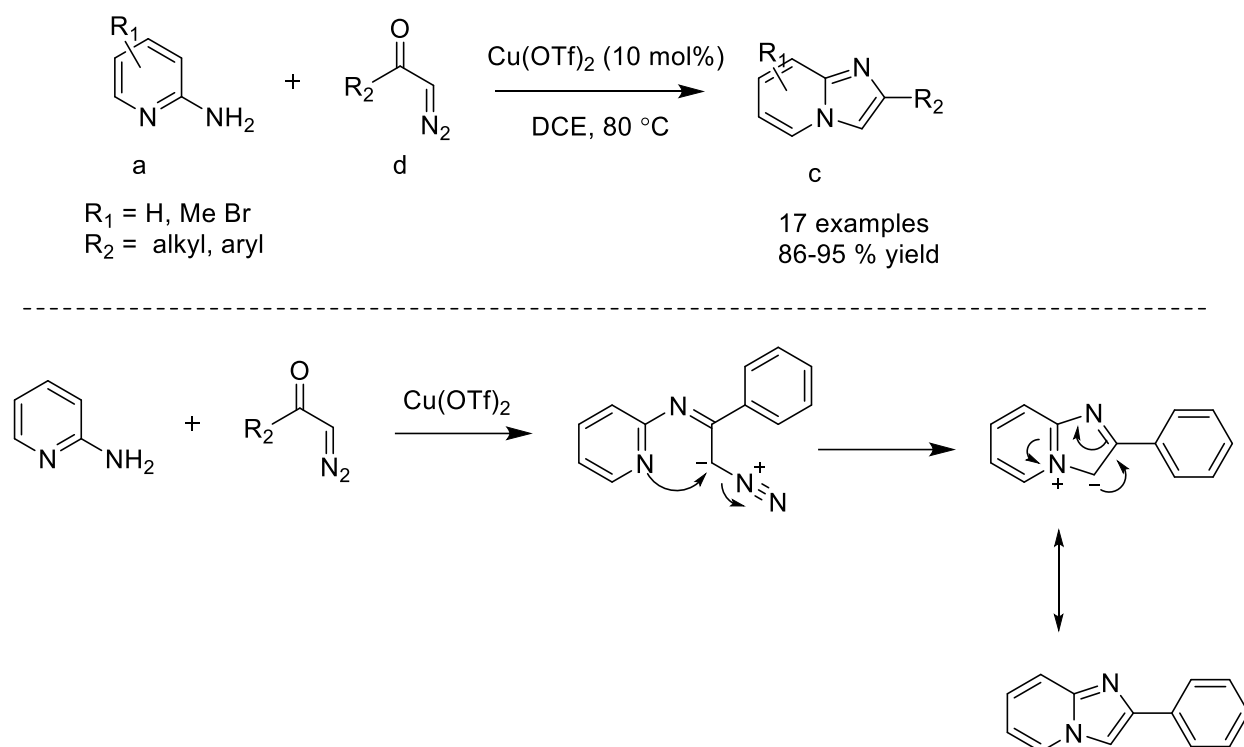
The Chen and Wu group showed that the imidazo[1,2-*a*]pyridines could be synthesized from α -bromo/chloro-ketones and 2-aminopyridines under catalyst and solvent-free conditions at 60 °C (Scheme 2).⁹⁸



Scheme 2- Solvent and catalyst-free conditions for the synthesis of imidazo[1,2-*a*]pyridines by using α -haloketones.

From diazoketones

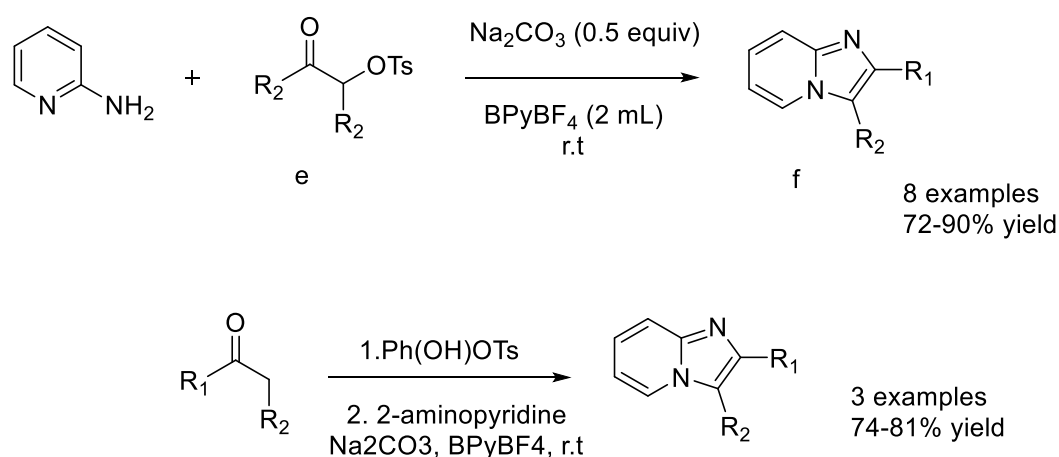
α -Diazoketones are as practical as α -haloketones for the synthesis of imidazo[1,2-*a*]pyridines. The copper-catalysed reaction between α -diazoketones (**d**) and 2-aminopyridines (**a**) afforded these heterocycles with good selectivity and good yields (Scheme 3).⁹⁹ $\text{Cu}(\text{OTf})_2$ catalysis worked efficiently for both the aromatic and aliphatic diazoketones. This reaction undergoes via the imine formation followed by nitrogen insertion. Other Lewis acids like InCl_3 , $\text{In}(\text{OTf})_3$, $\text{Sc}(\text{OTf})_3$, InBr_3 , $\text{Bi}(\text{OTf})_3$, and Brønsted acids like hetero, polyacid, PMA, Amberlyst-15 are not effective to afford the imidazopyridines by this reaction.



Scheme 3- Copper-catalysed reaction between 2-aminopyridines and α -diazoketones. Proposed mechanism of the reaction is detailed on the bottom reaction sequence.

From α -toxyloxyketones

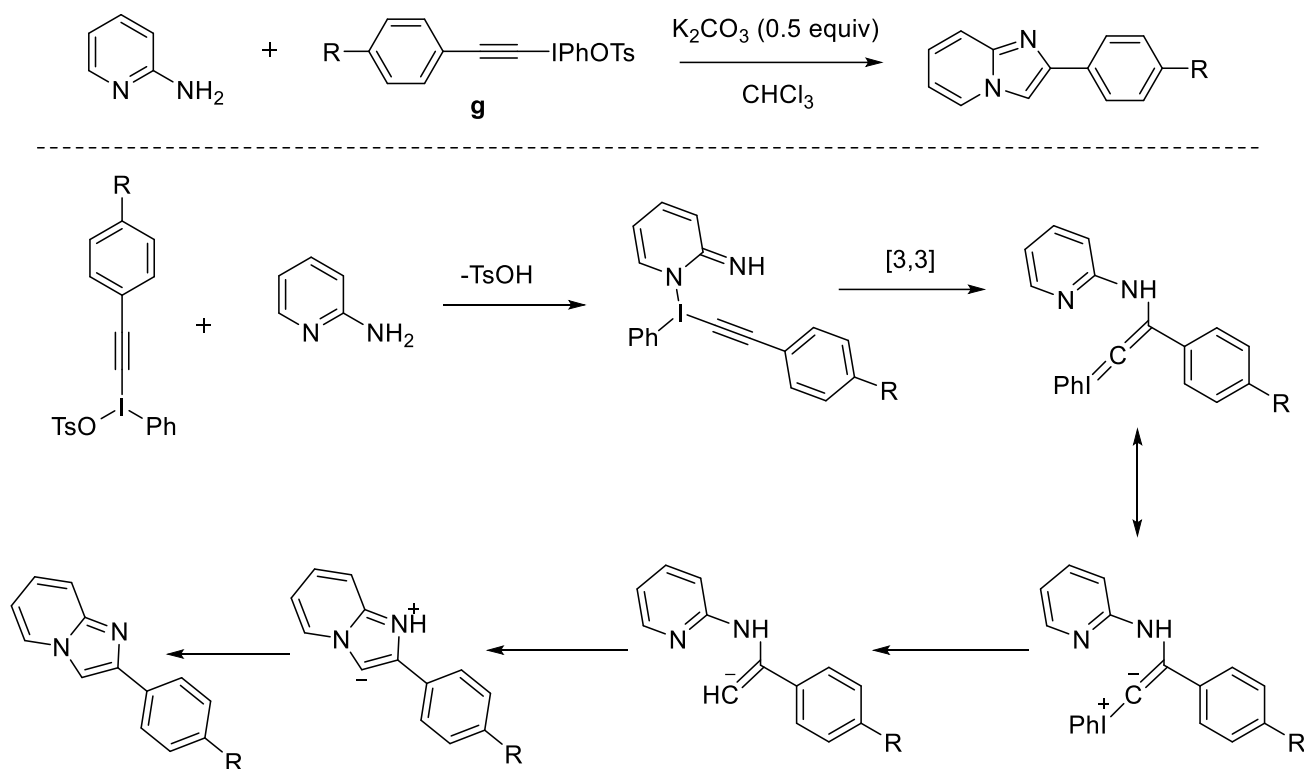
The imidazopyridines have been synthesized directly from ketones by *in situ* generation of the α -tosyloxyketones, **e** (Scheme 4).¹⁰⁰ The ionic liquid is preferable for this condensation reaction compared to common organic solvents. Ionic liquid BPyBF₄ afforded the imidazopyridine derivatives at room temperature, low temperatures and short reaction times (1h).



Scheme 4- Synthesis of imidazopyridines by using α -toxyloxyketones.

From alkynyl(phenyl)iodonium salts

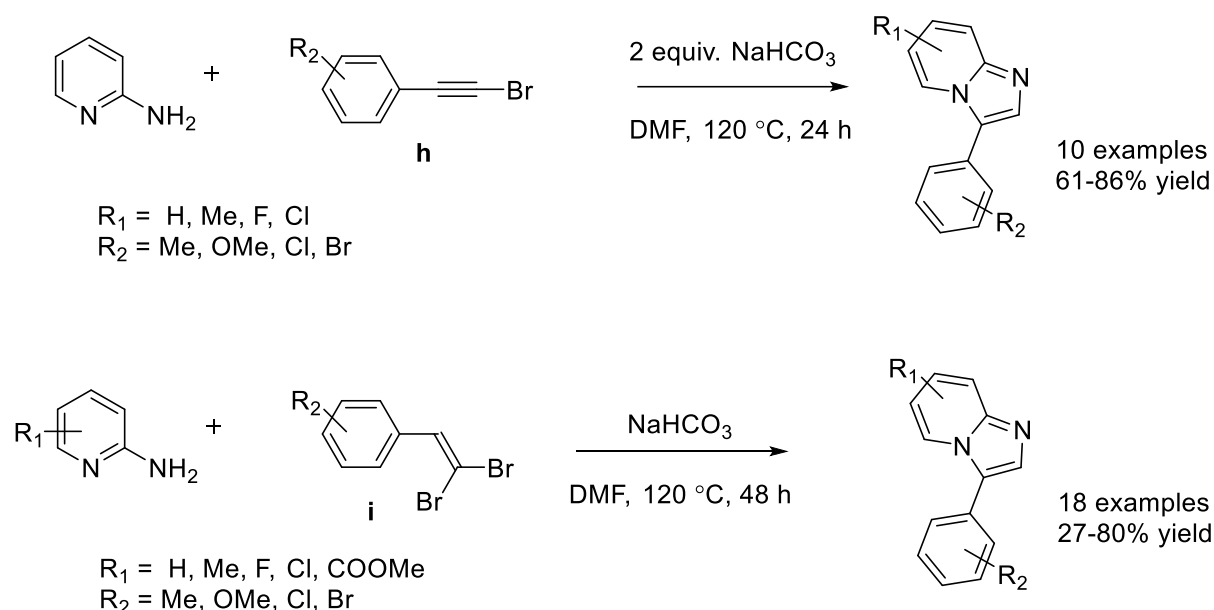
Chen group developed a simple method for the synthesis of 2-substituted imidazopyridines by the reaction of alkynyl(phenyl)iodonium salts (**g**) with 2-aminopyridine (Scheme 5).¹⁰¹ The reaction proceeds through the [3,3]-sigmatropic rearrangement followed by intramolecular cyclization. Only K_2CO_3 in chloroform is effective to carry out the reaction.



Scheme 5- Reaction of alkynyl(phenyl)iodonium salts with 2-aminopyridine.

From 1-bromo-2-phenylacetylene/1,1-dibromo-2-phenylethene

3-arylimidazo[1,2-*a*]pyridines were synthesized by the catalyst-free reaction between 2-aminopyridines and 1-bromo-2-phenylacetylenes (**h**) or 1,1-dibromo-2-phenylethenes (**i**) by Zhou group (Scheme 6). The bromoalkynes bearing electron-withdrawing groups on the aromatic ring afforded better yields in comparison to those bearing electron donating groups. More detailed reaction mechanism in reference.¹⁰²

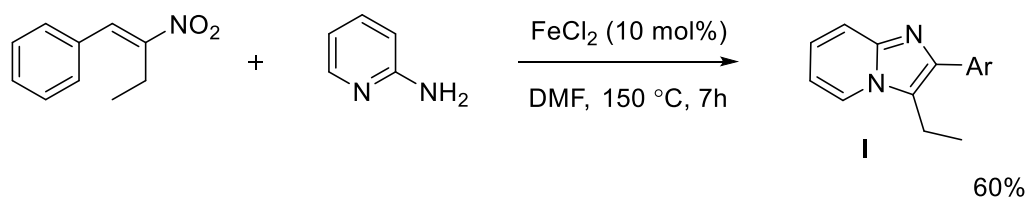


Scheme 6 Synthesis of 3-aryl-2-imidazopyridines from 1-bromo-2-phenylacetylene (**e**) or 1,1-dibromo-2-phenylethene (**i**).

Tandem reactions

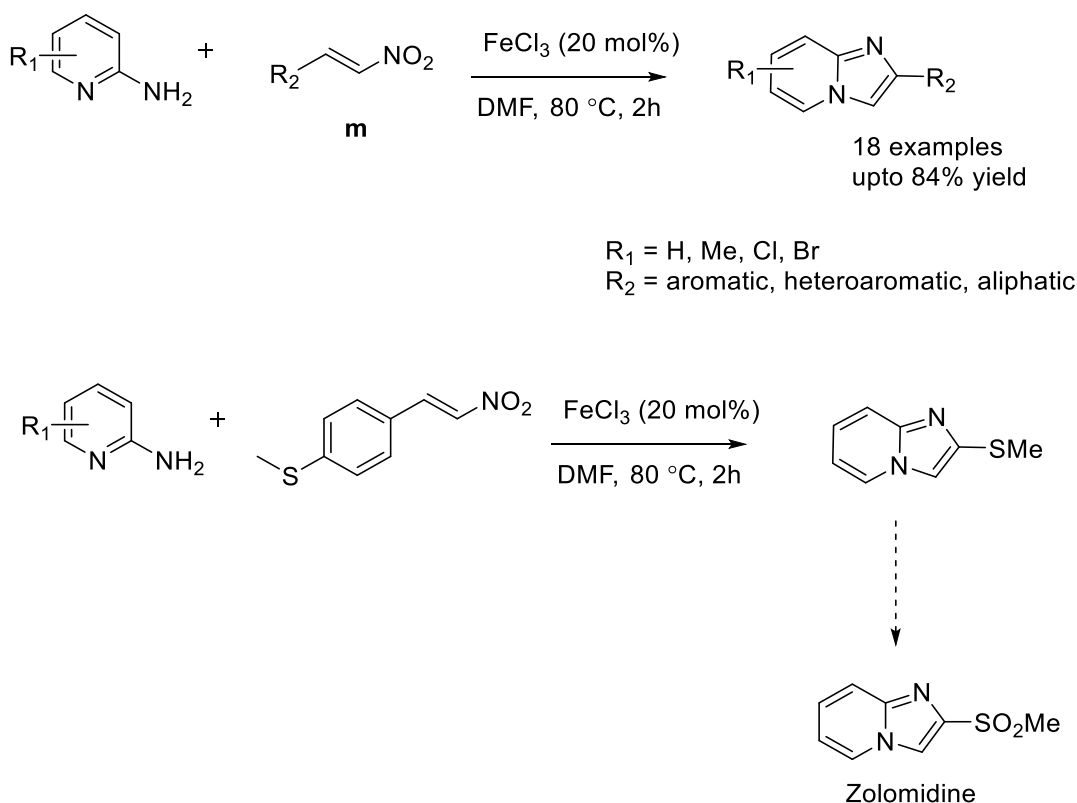
Tandem coupling between 2-aminopyridines and nitroolefins

Yan and Huang group prepared 3-methyl-2-aryl-2-imidazopyridine derivatives (**k**) by Fe(II)-catalysed tandem coupling of 2-aminopyridines and 2-methylnitroolefins (**j**) (Scheme 7).¹⁰³ FeCl₂ was more suitable compared to the other iron salts for this transformation. A library of 3-methyl-2-aryl-2-imidazopyridines were synthesized to establish the general applicability of this method. This is also applicable for the synthesis of 3-ethyl-2-phenyl-2-imidazopyridine (**l**) with good yields. The reaction proceeds through tandem Michael addition/intramolecular cyclization.



Scheme 7 - Synthesis of imidazo[1,2-*a*]pyridines through reaction of 2-aminopyridines with nitroolefins.

Synthesis of 3-unsubstituted imidazo[1,2-*a*]pyridines was possible in a cascade reaction between the bielelectrophilic nitroolefins (**m**) and binucleophilic 2-aminopyridines (Scheme 8).¹⁰⁴ Hajra *et al.* found that FeCl₃ was the most efficient catalyst among the Lewis acids such as AlCl₃, ZnCl₂, LaCl₃, BF₃·OEt₂, In(OTf)₃, Cu(OTf)₂, etc. for this reaction. This FeCl₃-catalyzed reaction is applicable for both the aromatic and aliphatic nitroolefins as well as for various substituted 2-aminopyridines. Zolimidine drug was obtained employing this strategy.

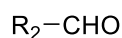
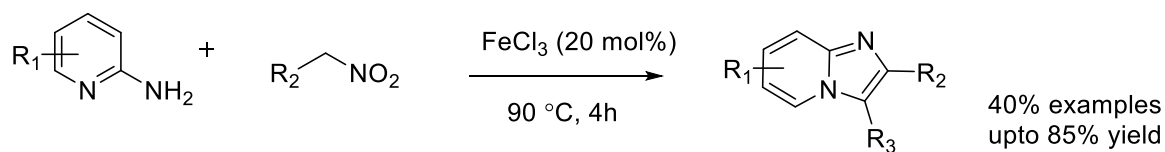


Scheme 8 - Synthesis of 3-unsubstituted imidazopyridines such as Zolimidine from nitroolefins.

Multicomponent Reactions

Multicomponent reaction of 2-aminopyridine, aldehyde and nitroalkane

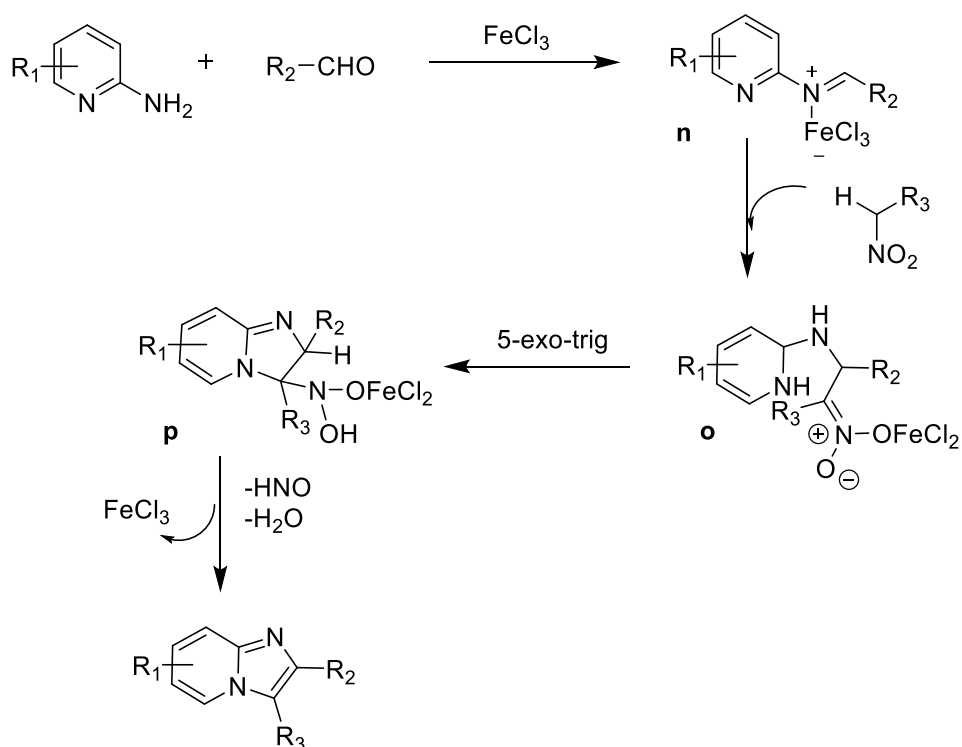
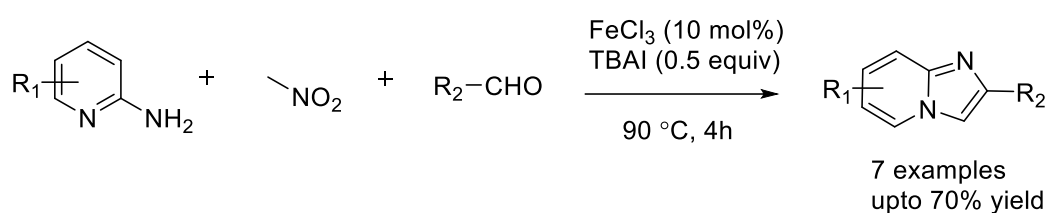
The Fe(III)-catalyzed three-component cross-coupling reaction of 2-aminopyridine, aldehyde and nitroalkane constitute a new strategy for synthesis imidazo[1,2-*a*]pyridine rings developed by Huang and co-workers¹⁰⁵ (Scheme 9). Aldehydes containing electron-withdrawing as well as electron-donating groups were well tolerated. 2-Aminopyridine with electron-withdrawing substituents gave higher yield compared to the electron-donating group containing 2-aminopyridines. Aliphatic and heteroaryl aldehydes also afforded the heterocycle.



$\text{R}_1 = \text{H, Me, F, Cl, Br, COOEt, OBn}$

$\text{R}_2 = \text{aliphatic, aromatic, heteroaromatic}$

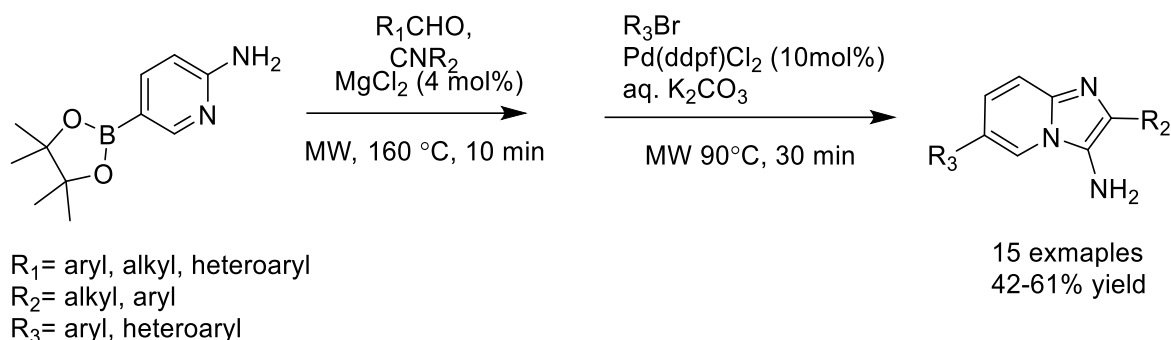
$\text{R}_3 = \text{Me, Et}$



Scheme 9 - Three component reaction to obtain imidazo[1,2-*a*]pyridines (top image). Proposed course of reaction involves the formation of an imine (**n**) between the aldehyde and the 2-aminopyridine. Next, a Michael addition followed by internal proton transfer and a second intramolecular Michael addition gave the intermediated (**p**). Loss of water and nitric acid molecules afforded the imidazo[1,2-*a*]pyridines (bottom image).

Multicomponent reaction of 2-aminopyridine, aldehyde and isonitrile

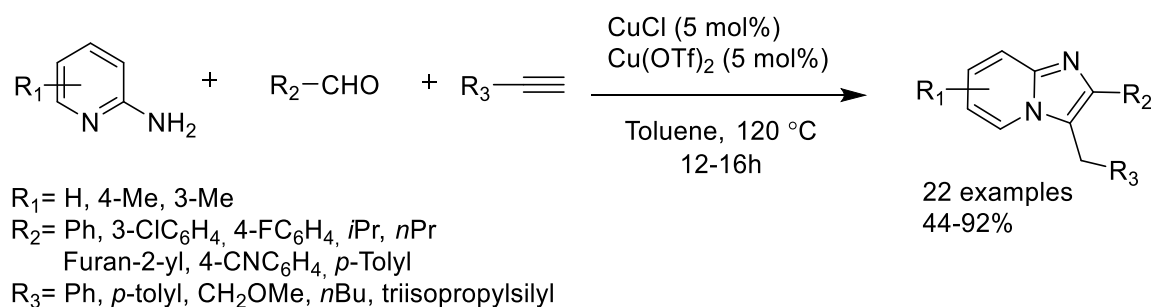
DiMauro and co-workers prepared several 2,6-disubstituted- 3-amino-imidazopyridines using a microwave-assisted one-pot cyclization/Suzuki coupling approach (Scheme 10).¹⁰⁶ The reaction works for various aldehydes, isonitriles and bromo derivatives. 2-aminopyridine-5-boronic acid pinacol ester constitute a robust and versatile building block for the synthesis of diverse compound libraries. The boronate functional group was notably tolerant to the Lewis acid catalysed cyclisation.



Scheme 10 - One-pot reaction for the synthesis of 3-amino-imidazopyridines using Ugi-type reagents. The reaction involves cyclization followed by Suzuki cross coupling reaction.

Multicomponent reaction of 2-aminopyridine, aldehyde and alkynes

Gevorgyan et al. reported a smart method for the synthesis of imidazo[1,2-a]pyridine derivatives by the copper-catalyzed three-component coupling reaction of aldehydes, 2-aminopyridines and terminal alkynes (Scheme 11).¹⁰⁷ This transformation led to imidazoquinoline and imidazoisquinoline frameworks prepared in good yields from 2-aminoquinoline and 2-aminoisquinoline coupling partners. This one-pot route permitted the obtention of the marketed drugs alpidem and zolpidem.

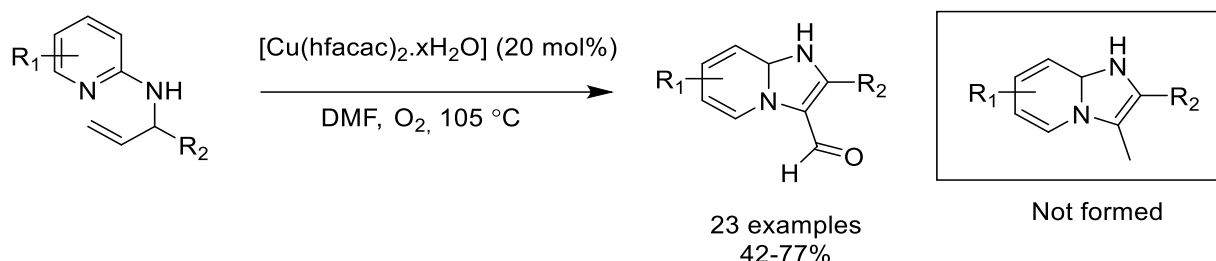


Scheme 11 - Cooper catalysed three component reaction to obtain imidazopyridines.

Aminooxygenation and hydroamination

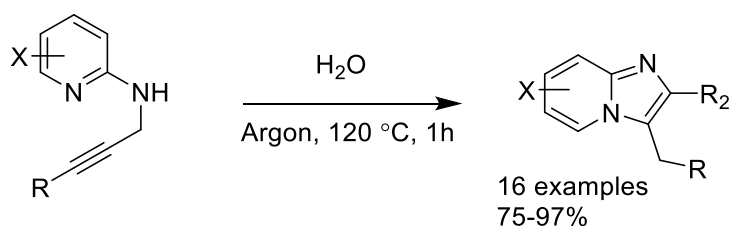
An unprecedented and novel intramolecular dehydrogenative aminooxygenation reaction for the preparation of imidazopyridines containing a formyl group was achieved by Zhu and co-workers

(Scheme 12).¹⁰⁸ This copper-catalyzed methodology was carried out under an oxygen atmosphere employing simple acyclic precursors. DMF or DMA were the most effective solvents, whereas copper salt was essential for this transformation. A library of imidazo[1,2-*a*]pyridine-3-carbaldehydes with a wide substrate scope was synthesized in moderate to good yields.



Scheme 12 - Copper-catalysed synthesis of imidazo[1,2-*a*]pyridines-3-carbaldehyde.

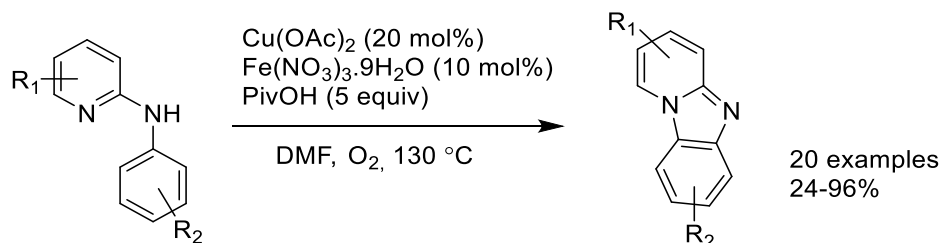
The intramolecular hydroamination of *N*-(prop-2-yn-1-yl)pyridin-2-amines (Scheme 13) was also possible in aqueous medium without any catalyst, affording the methylimidazo[1,2-*a*]pyridines.¹⁰⁹ Water apparently plays a dual role as a solvent and catalyst since polar and non-polar organic solvents (except ethanol) were not able to afford the product in the absence of any transition metal catalyst.



Scheme 13 - Intramolecular hydroamination of *N*-(prop-2-yn-1-yl)pyridin-2-amines.

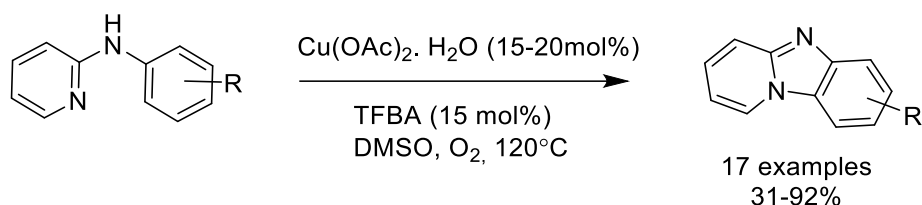
Intramolecular C-H amination

Copper-catalysed synthesis of fused imidazo[1,2-*a*]pyridines, in particular pyrido[1,2-*a*]benzimidazole, have been reported independently by Zhu *et al.* and Maes *et al.* Zhu group employed iron salts that did not promote the reaction itself but improved the yield significantly. Pivalic acid was used as an additive which also increased the yield (Scheme 14).¹¹⁰



Scheme 14 – Copper-catalysed synthesis of pyrido[1,2-*a*]benzimidazole through aromatic C-H amination.

On the other hand, Maes group evaluated the influence of the acid additive by employing different carboxylic acids. Acetic acid, pivalic, butyric and benzoic acid produced the product with good efficiency. 3,4,5-trifluorobenzoic (TFBA) acid was clearly a superior additive since it provided a faster reaction and complete conversion of the starting material. Non-carboxylic acids were also useful when used in catalytic amount. (Scheme 15).¹¹¹

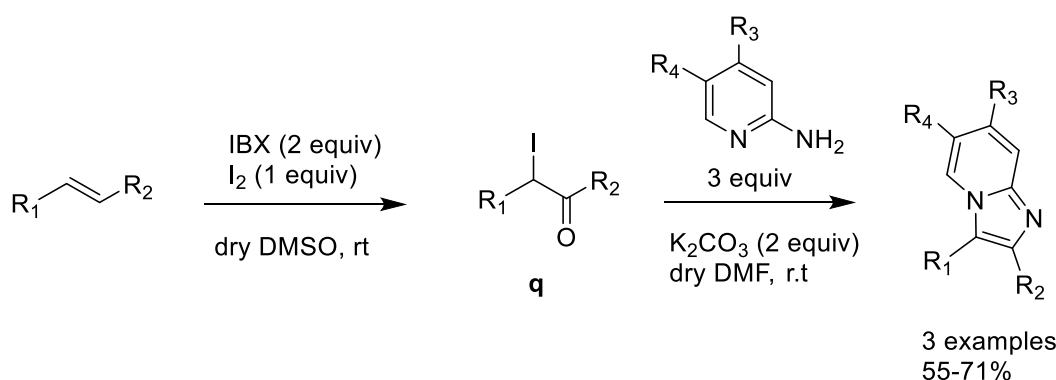


Scheme 15- Direct copper-catalysed synthesis of pyrido[1,2-*a*]benzimidazole with TFBA as acid additive.

Oxidative coupling

Oxidative coupling between 2-aminopyridine and alkene

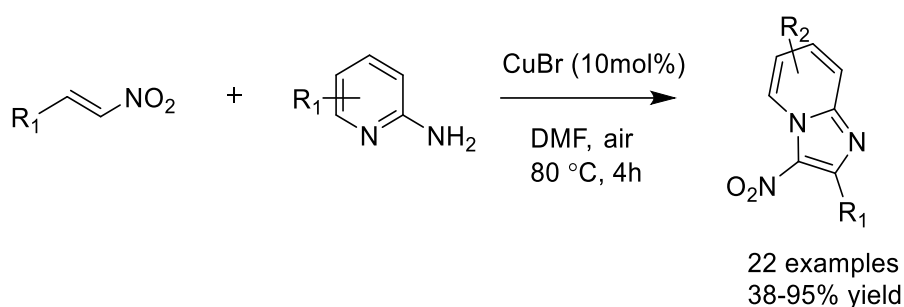
In 2012, Donohoe and co-workers developed an effective method to generate α -halo carbonyl compounds by the reaction of alkenes with 2-iodoxy-benzoic acid/ iodine/ dimethyl sulfoxide (Scheme 16). The *in situ* generated reactive α -iodoketones (**q**) were further used for the synthesis of diverse heterocycles such as imidazo[1,2-*a*]pyridines.¹¹²



Scheme 16 In situ generation of α -haloketone from alkenes as intermediate reagent for the synthesis of imidazopyridines.

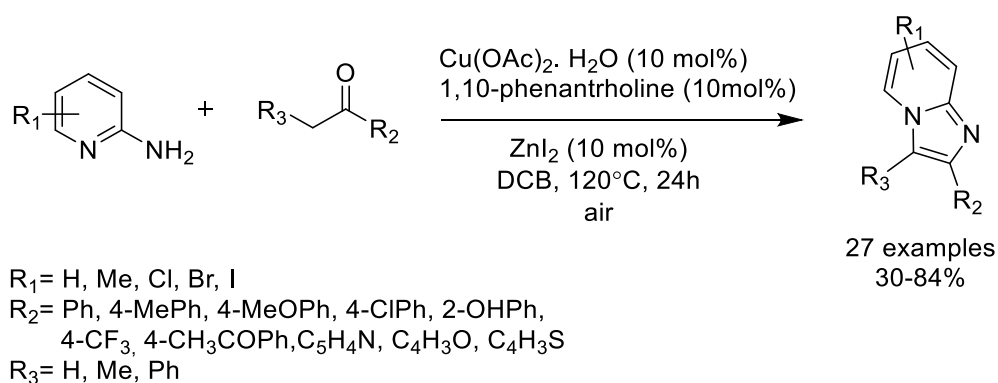
Cu-catalysed oxidative coupling from 2-aminopyridine

The next copper-catalysed oxidative coupling of nitroolefins with 2-aminopyridines is another tandem reaction as those described in Schemes 7 and 8. The reaction produced 3-nitro imidazo[1,2-*a*]pyridines in moderated to excellent yields (Scheme 17).¹¹³ Aerial oxygen was enough to act as the terminal oxidant. Authors found copper bromide was the most efficient catalyst.



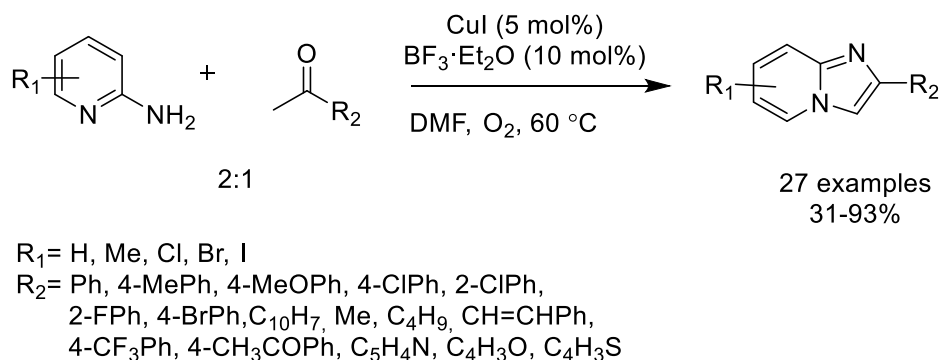
Scheme 17- Nitroolefins afford imidazopyridines under cooper-catalysis.

Although being classified here as an oxidative coupling reaction, the obtention of next imidazo[1,2-*a*]pyridine derivatives involved the C–H amination of the aryl ketones with zinc-salt as the additive under ambient air (Scheme 18).¹¹⁴ Among the various copper salts evaluated in the study, best results were obtained with $Cu(OAc)_2 \cdot H_2O$. This process was employed for the one-step synthesis of Zolimidine on a gram-scale.

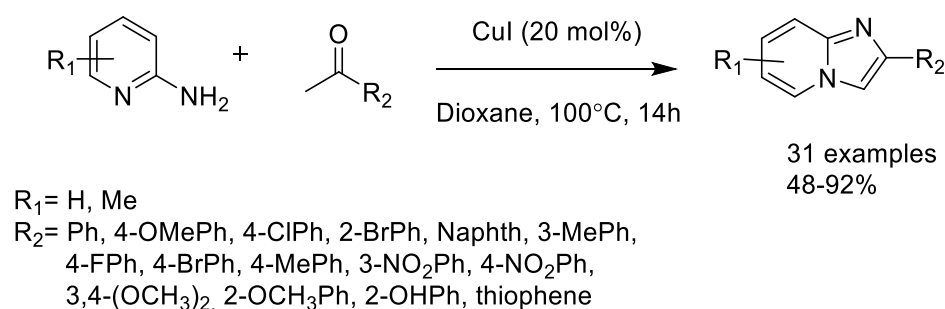


Scheme 18 - Direct copper-catalysed oxidative coupling between 2-aminopyridines and ketones.

Adimurthy *et al.* reported a similar methodology employing CuI as the catalyst in DMF (Scheme 19).¹¹⁵ Kumar group reported a ligand-free approach for the synthesis of imidazopyridines using CuI in dioxane medium (Scheme 20).⁹⁷

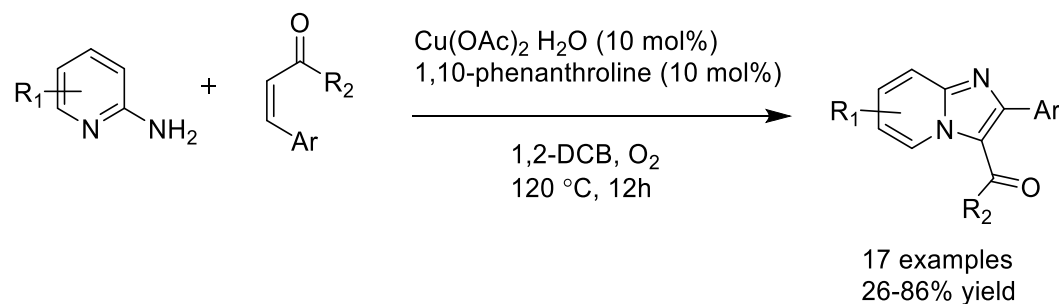


Scheme 19 - CuI catalyses the coupling reaction of 2-aminopyridines and ketones.



Scheme 20 - Additive-free synthesis of 3-unsubstituted imidazopyridines.

Chalcones have also been used for the synthesis of imidazo[1,2-*a*]pyridine derivatives. Hajra and co-workers used copper-catalysed oxidative coupling under oxygen atmosphere, which is applicable for a wide range of chalcones in gram-scale (Scheme 21).¹¹⁶

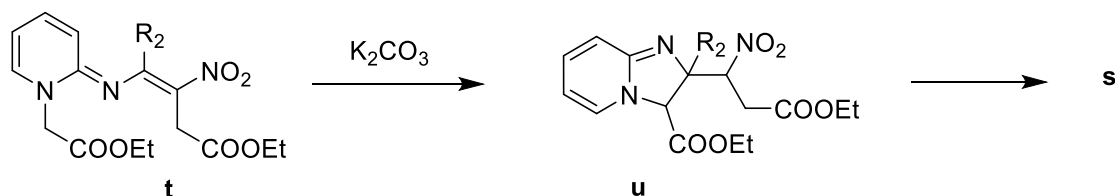
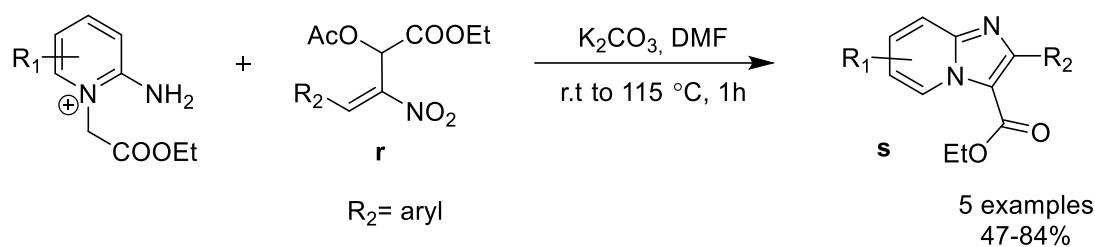


Scheme 21 - Synthesis 3-aryl-imidazo[1,2-*a*]pyridines from chalcones and copper-catalysis.

From pyridinium salts

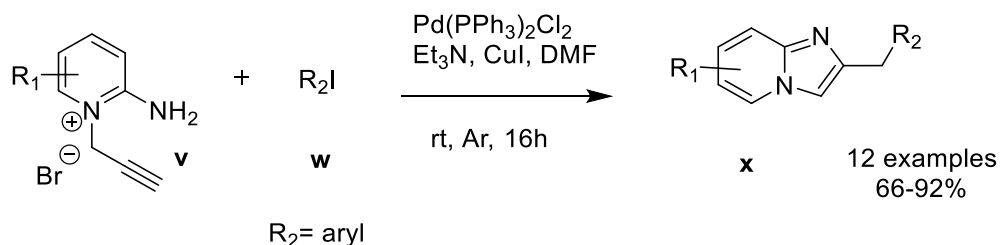
Interesting synthetic routes for imidazo[1,2-*a*]pyridine use pyridinium salts as a key precursor. Zhu *et al.* demonstrated the potential utility of Morita–Baylis–Hillman (MBH) acetates for the synthesis of structurally diversified heterocyclic motifs by varying the nucleophilic partners (Scheme 22). The method was highly efficient and allowed the reactivity of all the electrophilic sites (α , β , γ , and δ) of Morita–Baylis–Hillman acetates, affording imidazo[1,2-*a*]pyridines, indolizines, pyrroles, pyrazoles, and benzo[*b*][1,6]oxazocin-2-ones with their respective coupling partners.

It was found that MBH acetates bearing electron-withdrawing substituents gave good yields of substituted imidazo[1,2-*a*]pyridines **j** when compared to Morita–Baylis–Hillman acetates containing electron-rich groups. The mechanism involved Michael elimination and rearrangement (intermediate **t**) followed by intramolecular Michael addition at the α -position of **t**, affording **u** which transformed into the desired imidazo[1,2-*a*]pyridine **s** through elimination of the ester counterpart.¹¹⁷



Scheme 22- MBH acetate provided diverse nitrogen-based heterocycles when combined with pyridinium salts.

The quaternization of 2-aminopyridines with propargyl bromide gave 2-amino-1-(prop-2-ynyl)pyridinium bromides **m**.¹¹⁸ These salts afforded 2-benzylimidazo[1,2-a]pyridines **o** on subsequent Sonogashira coupling and intramolecular cyclization. Electron-withdrawing groups on the aryl iodide **n** were essential because in the case of iodobenzene, 2-methylimidazo[1,2-*a*]pyridine was the major product which is the heterocyclization product without Sonogashira coupling (Scheme 23).



Scheme 23- Sonogashira coupling followed by intramolecular cyclization for the synthesis of 3-substituted imidazopyridines from pyridinium salts.

1.3.2 Results and discussion

Synthesis of imidazo[1,2-*a*]pyridine derivatives

Imidazo[1,2-*a*]pyridine derivatives (**1**, Figure 17) were prepared via two-step procedure consisting on Suzuki-Miyaura cross-coupling and heterocyclization reactions.

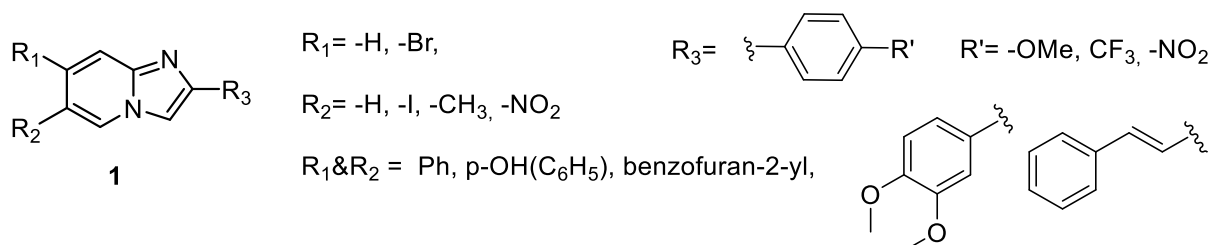
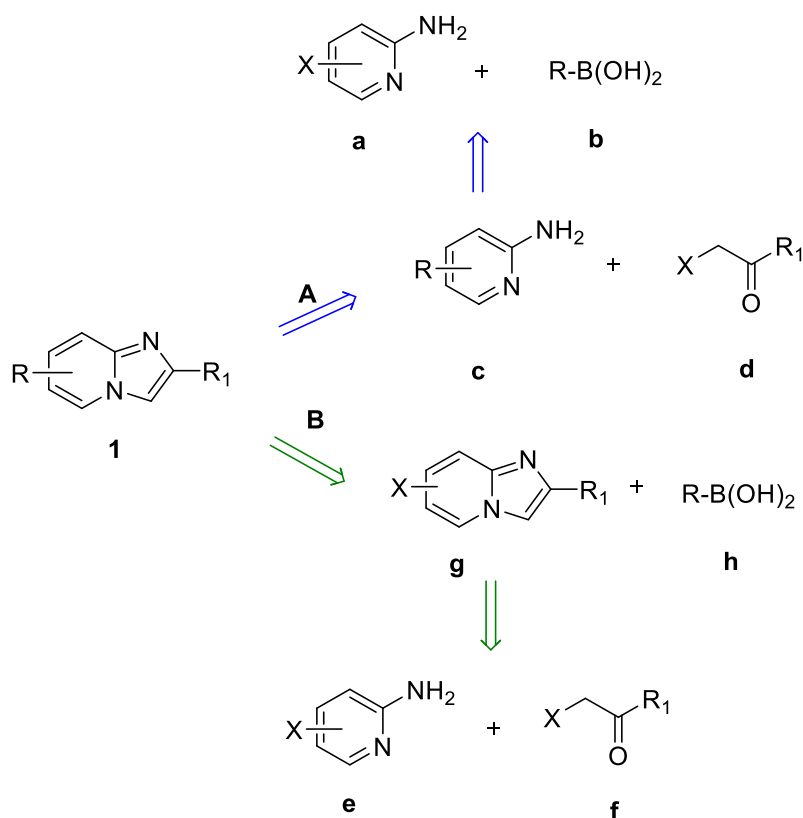


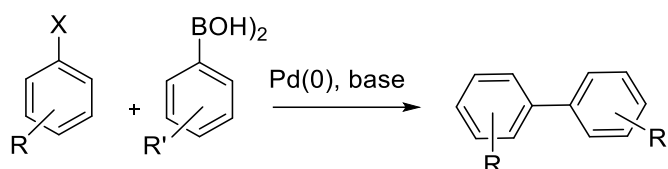
Figure 17 – General chemical structure of imidazo[1,2-*a*]pyridine derivatives presented in this work.

The sequence of reactions could be interchanged, cross-coupling followed by heterocyclization or *vice versa*, so we employed two different methods (A or B) depending on the order of the reactions performed (Scheme 24).



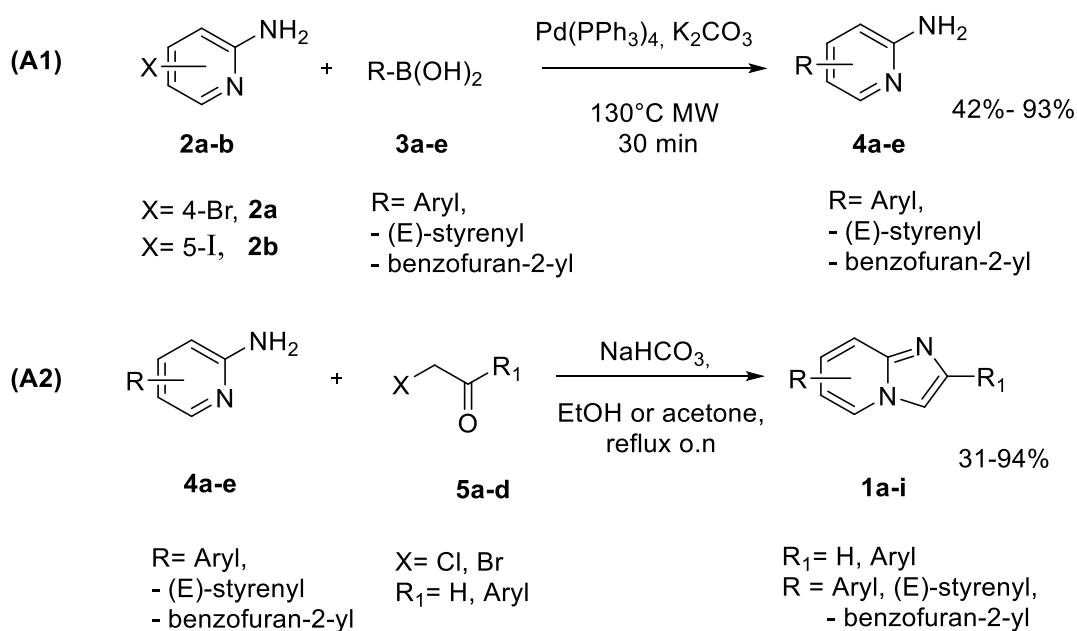
Scheme 24 – Retrosynthetic analysis for the preparation of imidazo[1,2-*a*]pyridine analogues. Two synthetic routes were followed: **A** (blue arrows) and **B** (green arrows).

The reaction of organometallic and organohalides compounds can be achieved by using a catalytic source of Pd(0), with additional ligands, base and solvent. The Suzuki reaction (palladium-catalysed cross-coupling of aryl halides with boronic acids) is one of the most versatile and used reactions for the formation of carbon-carbon bonds, in particular biaryls (Scheme 25).



Scheme 25- Schematic representation of the Suzuki cross-coupling between aryl halides and boronic acids with palladium (0) catalysis.

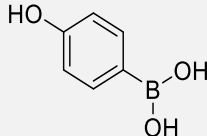
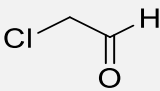
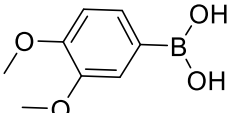
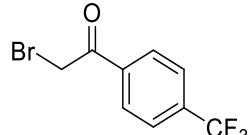
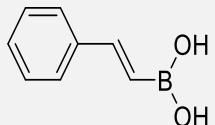
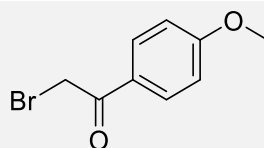
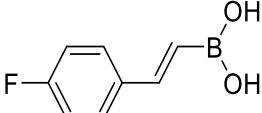
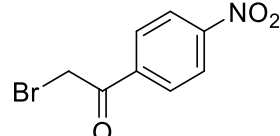
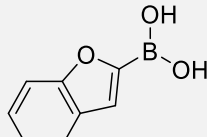
Method A:



Scheme 26 - Method A. Synthetic route for the obtention of imidazo[1,2-*a*]pyridine derivatives comprising two subsequent steps: Suzuki's cross coupling (A1) and heterocyclization (A2).

4-bromo and 5-iodo 2-aminopyridine are commercially available, as well as the aryl boronic acids (R-B(OH)₂) and α-haloketones (Table 1, entries 1-9).

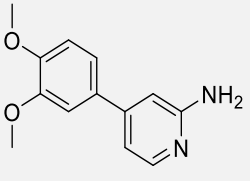
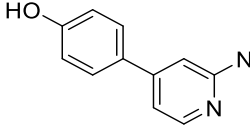
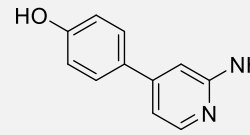
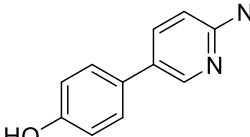
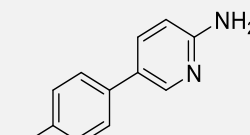
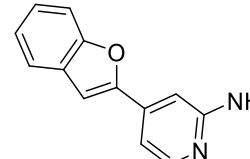
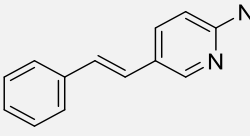
Table 1 - Chemical structures of boronic acids (entries 1-5) and α -haloketones (entries 6-9) utilised in the present study.

Entry	R-B(OH) ₂ (3a-e)	Entry	α-haloketone (5a-d)		
1		3a	6		5a
2		3b	7		5b
3		3c	8		5c
4		3d	9		5d
5		3e			

Enguehard *et al.* observed that differently substituted 6-haloimidazo[1,2-*a*]pyridines at the C2 influenced the Suzuki reaction,¹¹⁹ when using well-known conditions for Suzuki cross-coupling, that is, Pd(PPh₃)₄ as catalyst, a base and DME/H₂O as solvent. In that work, 2-alkyl and 2-arylimidazo[1,2-*a*]pyridines appeared to be much more reactive towards the Suzuki-coupling than those without substitution on C2. Based on this knowledge, and with the scope of avoiding the potential effect of the substitution on carbon 2, the Suzuki reaction was carried out prior to forming the imidazopyridine core *via* condensation with α -haloketones. With this method, intermediates (**4a-e**) were obtained with mild to excellent yields (Table 2).

The metal catalyst of choice was the tetrakis(triphenylphosphine)palladium(0) ([Pd(PPh₃)₄]), and K₂CO₃ the chosen base. Solvents for Suzuki's reaction were either DME/H₂O, H₂O/EtOH/Toluene or 1,4-dioxane/H₂O.¹²⁰⁻¹²²

Table 2 - Reagents and conditions explored for the preparation of intermediates **4a-d** and **12a**. Key: DME, dimethoxyethane. Reactions were carried out in a microwave reactor.

Entry	Comp. 2 (a-b)	Boronic acid 3a-e	Base	Solvent	Time (min)	Temp (°C)	Compound 4a-e	Yields (%)
1	2a	3a	K ₂ CO ₃	H ₂ O/MEOH/ Toluene (1:2:8)	60	150	 4a	93
2	2a	3b	K ₂ CO ₃	H ₂ O/ MEOH/ Toluene (1:2:8)	60	150	 4b	30
3	2a	3a	K ₂ CO ₃	DME: H ₂ O (4:1)	30	110	 4b	84
4	2b	3a	K ₂ CO ₃	DME: H ₂ O (4:1)	30	110	 4c	25
5	2b	3a	NaHCO ₃	1,4- Dioxane: H ₂ O (3:1)	45	110	 4c	42
6	2a	3a	K ₂ CO ₃	DME: H ₂ O (4:1)	30	110	 4d	82
7	2a	3c	K ₂ CO ₃	DME: H ₂ O (4:1)	30	110	 12a	58

Compound **4a** was successfully synthesised applying the conditions in Table 2 (entry 1)¹²¹ using the boronic acid **3b** (Table 1). However, when those conditions were used with 4-hydroxyphenylboronic acid **3a**, the yield dropped drastically (Table 2, entry 2). In addition, more side-products were observed by NMR, that could not be isolated. The substitution of the solvent by the mixture DME:

H₂O afforded the desired p-hydroxy-biaryl compound **4b** (Table 2, entry 3) in a cleaner reaction.¹²⁰ The solvent in cross-coupling reactions plays an important role because of its significant influence on the rate and selectivity of the reaction, as well as equilibria. By choosing the correct solvent, the life-time of the catalyst and activity of acids and bases can be enhanced.¹²³ Time and temperature were also reduced under these new conditions.

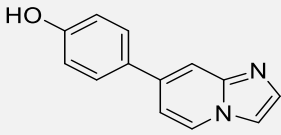
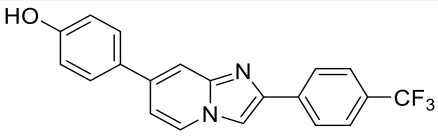
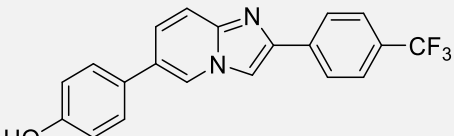
Interestingly, when the conditions to synthesize **4b** were applied for the synthesis of **4c** (structural isomer of **4b**), the yield decreased notably (Table 2, entry 4). In this case, the 5-halo-2-aminopyridine reagent had an iodine atom instead of a bromine, which it is known to have a different reactivity on cross-coupling reactions.¹²⁴ Therefore, the solvent and the base were changed¹²² (Table 2, entry 5), which improved the overall yield, although still far away from the values obtained with its isomer **4b**.

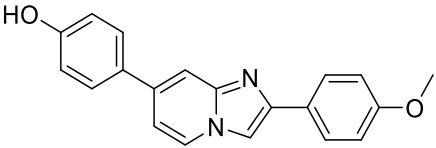
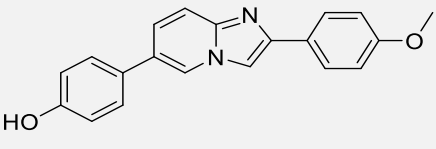
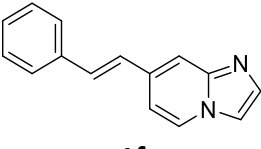
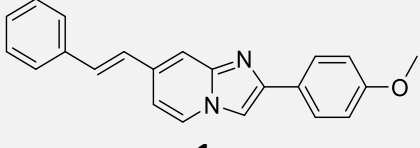
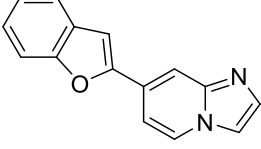
Intermediates **4d** and **12a** were satisfactorily prepared with the corresponding boronic acids under the conditions applied to **4b**, with mild to good yields (Table 2, entries 6 and 7).

In general, the use of the microwave reduced reaction times drastically: from 8-24h as described in the literature,^{120,125} to 10-60 minutes.

The subsequent heterocyclization reaction (**A2**) used NaHCO₃ as base and one of the α -haloketones shown in Table 1 (entries 6-9).¹²⁶

Table 3 – Method A, step A2. Reagents and conditions for the preparation of imidazo[1,2-*a*]pyridine analogues **1a-i**.

Entry	Comp. 4 (a-e)	Comp. 5 (a-d)	Exp. Conditions* / Solvent	Comp. 1 (a-i)	Yields (%)
1	4a	5b	NaHCO ₃ , reflux overnight / EtOH	 1a	74
2	4c	5b	NaHCO ₃ , reflux overnight / EtOH	 1b	54
3	4a	5a	NaHCO ₃ , reflux overnight / EtOH	 1c	31

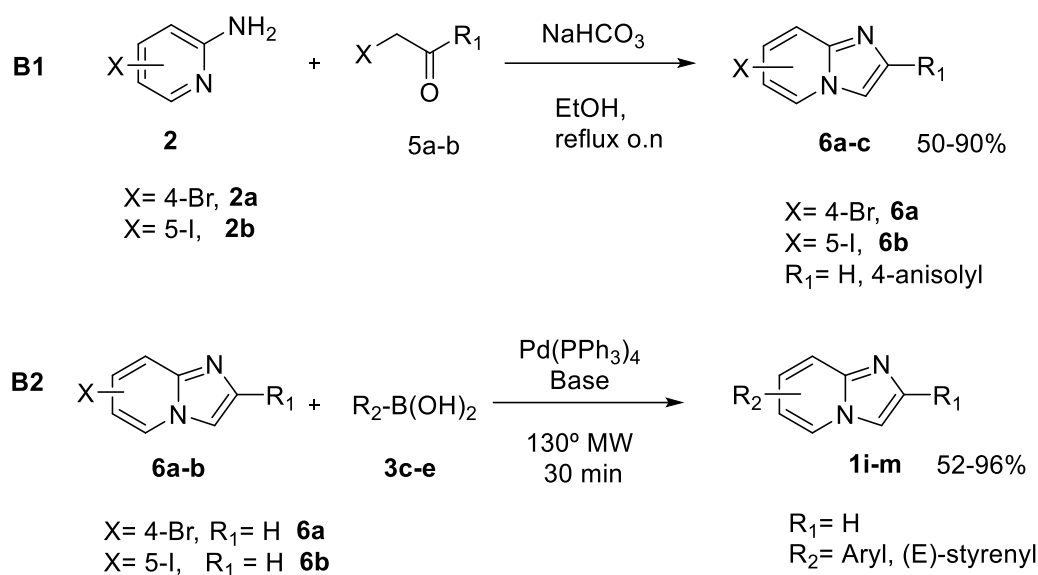
4	4c	5c	NaHCO ₃ , reflux overnight / Acetone	 1d	67
5	4a	5b	NaHCO ₃ , reflux overnight / Acetone	 1e	60
6	4e	5a	NaHCO ₃ , reflux overnight / Acetone	 1f	94
7	4e	5c	NaHCO ₃ , reflux overnight / Acetone	 1g	47
9	4d	5a	NaHCO ₃ , reflux overnight / EtOH	 1i	45

The condensation of intermediates **4a-d** and **12a** with the α -haloketones shown in Table 1 (entries 5-9), allowed us to easily obtain the desired imidazo[1,2-*a*]pyridines **1a-i** from mild to excellent yields (Table 3).

Only in the case of **1c**, which showed lower solubility, presented some issues during the purification process that led to lower yields. This problem was solved by replacing ethanol by a less polar solvent, for instance, acetone. Hence, chemically related compounds such as **1d** and **1e**, precipitated in acetone once the reaction cooled down and were readily isolated in higher yields.

Method B:

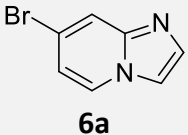
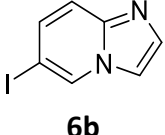
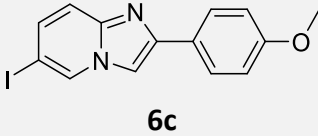
The alternative route for the synthesis of imidazo[1,2-*a*]pyridine analogues **1i-m** is shown in the next scheme:



Scheme 27- Synthetic route for the obtention of imidazo[1,2-*a*]pyridine comprising heterocyclization (**B1**) followed by Suzuki's cross coupling reaction (**B2**).

Intermediates **6a-c** were prepared from commercially available 5-iodo-2-aminopyridine and the corresponding α -halo carbonyl compound (Table 1, entries 6 and 8) in refluxing EtOH.

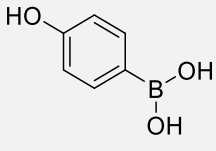
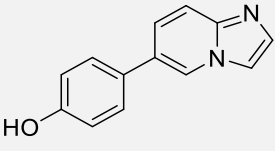
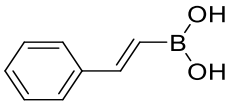
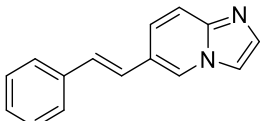
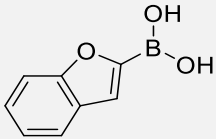
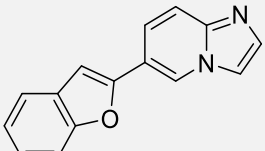
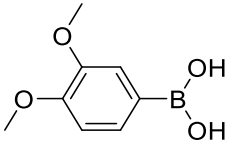
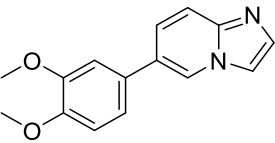
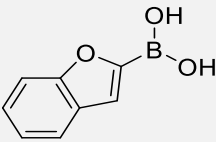
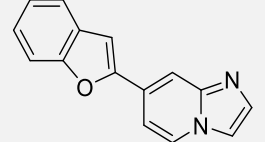
Table 4 - Method B, step B1. Reagents and conditions for the synthesis of intermediates **6a-c** via condensation of 4- and 5-halo-2-aminopyridines and α -haloketones.

Entry	Comp. 2 (a-b)	α -haloketone 5a	Exp. Conditions /Solvent	Compound 6 a-c	Yields (%)
1	2a	5a	NaHCO ₃ , reflux overnight / EtOH	 6a	86
2	2b	5a	NaHCO ₃ , reflux overnight / EtOH	 6b	90
3	2b	5b	NaHCO ₃ , reflux overnight / EtOH	 6c	47

Intermediate **6c** was obtained in less yield because of the lower number of equivalents of 2-chloroacetaldehyde (**5a**) employed, 1.5 instead of 2 equivalents, in the attempt of adjusting the quantity of this reagent. However, optimal results were obtained with 2 equivalents of halo-ketone **5a**, as it was the case of intermediate **6b** (90% yield, 1 g scale).

Compound **6b** was used in the Suzuki's reaction with a boronic acid to obtain imidazo[1,2-*a*]pyridine derivatives **1j-m** in good to excellent yields (Table 5). Different solvent mixtures were used in the cross-coupling reaction, such as DME/H₂O (4:1) or 1-4-dioxane/H₂O (3:1).

Table 5 – Method B, step B2. Reagents and experimental conditions for the synthesis of imidazo[1,2-*a*]pyridines **1i-l**.

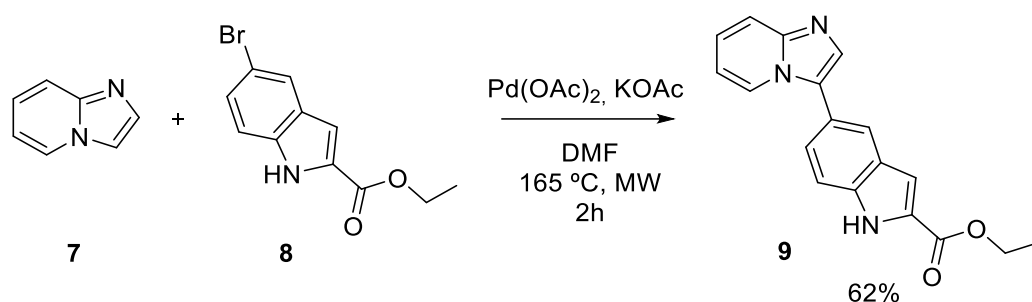
Entry	Comp. 6 (a-b)	Boronic acid 3a-3c, 3e	Exp. Conditions*/ Solvent	Compound 1i, 1j-m	Yields (%)
1	6b	 3a	Pd(PPh ₃) ₄ K ₂ CO ₃ / DME:H ₂ O 4:1	 1j	70
2	6b	 3c	Pd(PPh ₃) ₄ K ₂ CO ₃ / 1-4-dioxane: H ₂ O (3:1).	 1k	91
3	6b	 3e	Pd(PPh ₃) ₄ , NaHCO ₃ / DME:H ₂ O (4:1)	 1l	52
4	6b	 3b	Pd(PPh ₃) ₄ K ₂ CO ₃ / H ₂ O:MeOH:Tol (1:2:8)	 1m	96
5	6a	 3e	Pd(PPh ₃) ₄ K ₂ CO ₃ / DME:H ₂ O (4:1)	 1i	70

*110 °C in a microwave reactor, 30 min.

These compounds were alternatively synthesised, so the heterocyclization step was carried out before the Suzuki cross-coupling. Thus, compounds **1k** and **1i** were obtained firstly via heterocyclization and secondly cross-coupling reaction. Compound **1i** had been previously

synthesised by Suzuki reaction followed by heterocyclization with 2-chloroacetaldehyde (**5a**) to afford **1i** in 45% (Table 3, entry 9). By contrast, the same compound significantly improved the yield to 70 % when the 5-haloimidazo[1,2-*a*]pyridine was firstly synthesised and then reacted further with the corresponding boronic acid (**3e**) (Table 5, entry 5). Therefore, the lack of substitution in carbon 2 is not always related with poorer yields, as it has been suggested by Enguehard *et al.*¹¹⁹ One more example to probe this fact was the preparation of compound **1k** and **1m**, which were also obtained from the cross-coupling reaction between 5-iodo-imidazo[1,2-*a*]pyridine in excellent yields (Table 5, entries 2 and 4). This value was very similar to the 94% obtained in the synthesis of **1f** (structural isomer of **1k**) which was obtained via Suzuki's-heterocyclization route.

Additionally, an imidazo[1,2-*a*]pyridine analogue substituted in C3 was prepared *via* Heck reaction.¹²⁷ The commercially available imidazo[1,2-*a*]pyridine and the unsaturated halide **8** reacted via palladium (II) catalysis with potassium acetate base dissolved in DMF for 2 h, affording the desired imidazo[1,2-*a*]pyridin-3-yl-indole **9** (Scheme 28).



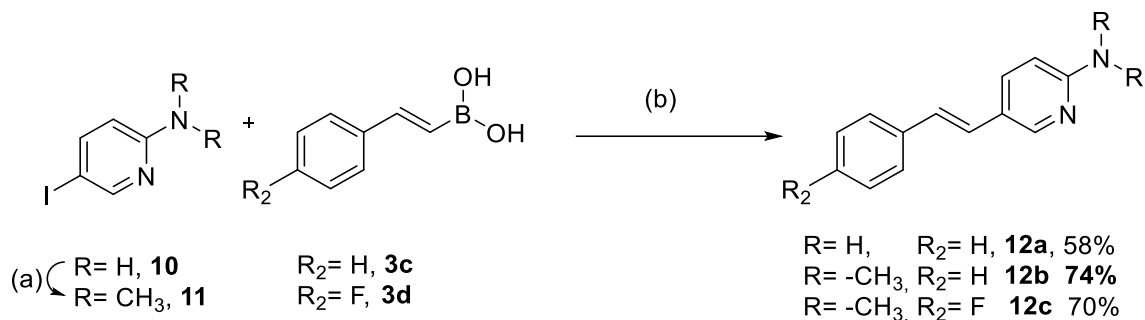
Scheme 28- Palladium-catalysed C-C coupling between an aryl halide (**8**) and imidazo[1,2-*a*]pyridine (**7**) afforded the substituted indole-2-carboxylate analogue (**9**). Key: MW, microwave reactor.

The Heck reaction is one of the most widely used catalytic carbon-carbon bond forming tools in organic synthesis.¹²⁸ The reaction allows the arylation or alkenylation of olefins via palladium (0) catalysis by substitution of the olefinic proton. The reaction can occur with Pd (II) precursor, which is reduced to form Pd (0) *in situ*. The C2-C3 double bond present in the imidazo[1,2-*a*]pyridine scaffold behaves as an isolated double bond. Although lacking the presence of the reductive phosphine, the C-H arylation of C3 carbon was possible by palladium (II) catalysis (Scheme 28).

Synthesis of 2-aminopyridine derivatives

Whilst synthesizing the imidazo[1,2-*a*]pyridine derivatives, we realised that intermediates **4a-d** highly resembled to some PET radiotracers described in the literature. In fact, the *N*-methyl-aminopyridine moiety is a common feature of several radiotracers of amyloid fibrils, (see article review⁶⁵). Thus, we wanted to explore the effect of double methylation of the amine group in the pyridine as well as leaving the ring “opened”, instead of forming the imidazo[1,2-*a*]pyridine core. Intermediate **12a** was selected as starting point for the synthesis of new 2-aminopyridine derivatives as potential tau radiotracers. Compounds **12c** and **12b** were prepared in a two-step procedure (Scheme 32). First, methylation of 5-iodo-2-aminopyridine with MeI afforded *N,N*-dimethyl-pyridine-2-amine. Then, the

desired products were prepared via Suzuki cross-coupling reaction with palladium (0) catalysis and K_2CO_3 as a base.



Conditions: (a) NaH, MeI, DMF (anh) from $0^\circ C$ to r.t., 1.5 hours (62%). (b) $Pd(PPh_3)_4$, K_2CO_3 in 1,4-dioxane/ H_2O (3:1), $110^\circ C$, 30 min, MW.

Scheme 29- Synthetic route for the synthesis of 2-aminopyridine analogues **12a-c**.

Three analogues were prepared, **12a-c**, to evaluate the effect of the free amine in **12a** compared to the dimethylated analogue (**12b**) as well as the cold ligand **12c**, which already possesses a F atom in its structure. All compounds were obtained in good yields, being **12a** the analogue with lower yield, may be due to the free amine group, which can bind palladium, thus reducing the effective amount of catalyst in the reaction.

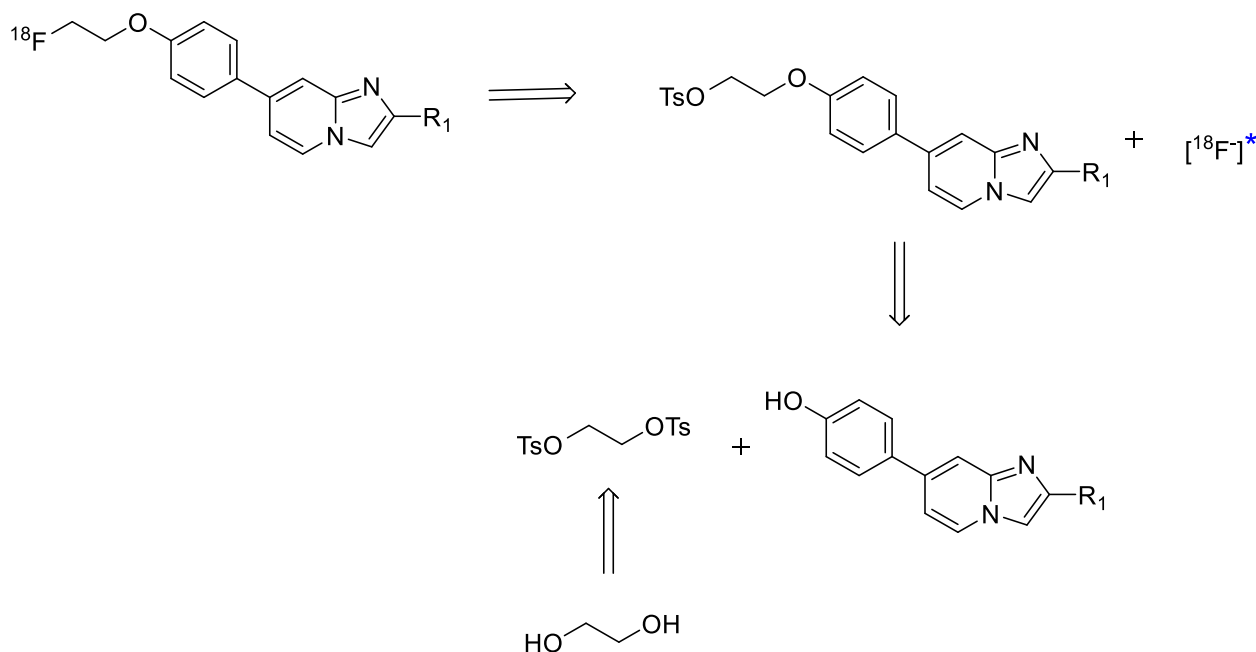
Cold ligands and radiosynthesis precursors

Positron emitting nuclides possess relatively short half-lives (recall ^{18}F fluorine, whose $t_{1/2}$ is 110 min). Thus, the synthesis time for a PET imaging probe should be as short as possible. As Ametamey and colleagues suggest in their work, the synthesis and purification period should not exceed 2 to 3 times the physical half-life of the radionuclide, and in consequence, the radioactive label is introduced in the synthetic route as late as possible.⁶⁰ Therefore, the radiosynthesis precursor is the molecule obtained immediately before the introduction of the radioactive label. In nucleophilic radiolabelling strategies, these precursors typically possess a good leaving group in their structure, such as mesylate, tosylate, bromo, iodine and sulfonate groups, which are substituted by the radioligand *via* S_N2 mechanism.^{60,129} ^{18}F -fluoride ion is accomplished by applying cryptands in combination with tetra-*n*-butylammonium cation or alkali salts. A cryptand serves to capture the metal cation (usually K^+) and to separate it from the ^{18}F -fluoride ion, thereby enhancing its nucleophilicity. A large cation without cryptand (Cs^+ , Et_4N^+ , tBu_4N^+) serves the same purpose of charge separation.¹³⁰ Quaternary ammonium or the alkali (K, Cs, Rb) salts are readily obtained from their respective hydroxides or carbonates and bicarbonates, respectively. Practical reaction times for this last step ranges from 1 to 30 minutes, and can be assisted by microwave heating.¹³¹

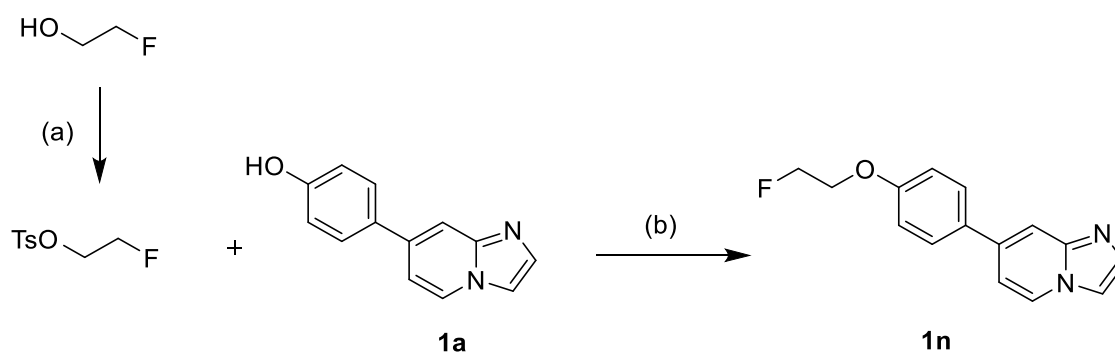
For practical reasons, studies with radiotracers which do not need the presence of the radionuclide are carried out using the so-called cold ligands, which are the unlabelled counterpart of PET probes.

Cold ligands are useful for studying pharmacodynamics and pharmacokinetics characteristics of new radiotracers, since isotopes of the same atom do not alter their biological activity.⁸⁰

In the next scheme, a retrosynthetic route is proposed for the synthesis of ^{18}F -PET radiotracers with the imidazo[1,2-*a*]pyridine core (Scheme 30, top route). Additionally, and as a probe of concept, compound **1a** was transformed into its cold ligand counterpart, by means of adding the fluoroethanol side-chain (Scheme 30, bottom route). Compound **1n** was obtained in good yields for its assessment as a model of potential radiotracer.



* Conditions for nucleophilic radiofluorination: $[\text{K/K222}]^+ [^{18}\text{F}-]$, K_2CO_3 , CH_3CN



Conditions: (a) TsCl , pyridine, atm Ar, 24h (35%). (b) KI , $n\text{Bu}_4\text{NI}$, $\text{DMF}(\text{anh})$, 70°C , 3h. (72%).

Scheme 30 – Proposed retrosynthetic route for the synthesis of imidazo[1,2-*a*]pyridine-based ^{18}F -PET radiotracers (top). Synthetic route for the obtention of cold ligand **1n** (bottom).

1.3.3 Tau Aggregation and Morphological Studies

In Alzheimer's Disease (AD), the assembly of tau proteins into paired helical filaments (PHFs) is linked to neurodegeneration.¹³² Ultrastructurally, tau inclusions are made of paired helical filaments (PHFs) and straight filaments (SFs). Tau protein degenerates from soluble tau to tau fibrils which eventually will form the neurofibrillary tangles (NFTs).^{133,134}

Unlike other amyloidogenic proteins, such as prion protein, α -synuclein and amyloid beta, tau does not spontaneously aggregate *in vitro* under physiological conditions, and even high temperatures or extreme pHs do not lead to aggregation.¹³⁵ *In vitro* aggregation of Tau⁴⁴¹ requires the use of heparin or other polyanions (such as arachidonic acid, an abundant fatty acid in human brain) to accelerate tau nucleation^{136,137} by acting through one or more mechanisms, including 1) neutralizing positive charges on tau molecules and thereby reducing electrostatic repulsion that might retard aggregation; 2) binding to tau, thereby unfolding the molecule and exposing hydrophobic regions that are needed for nucleation to proceed or by bringing multiple tau molecules into close proximity and 3), promoting hydrophobic interactions by stabilizing H-bonds of surrounding water molecules.¹³⁴

Tau aggregation has been described under a vast variety of conditions varying length of incubation period, temperature, pH, ionic strength, protein concentration and protein to heparin molar ratios. We started an optimization process for tau aggregation where protein concentration, buffer composition (and consequently the ionic strength), incubation period, tau to heparin ratio and pH were modified and optimised. Transmission electron microscopy and atomic force microscopy techniques were used to confirm the structure of tau assemblies upon tau incubation with heparin in increasing lengths of time.

The ionic strength of a buffer

Ionic strength, I , is a measure of the concentration of electrically charged species in solution. It is calculated by the equation:

$$I = \frac{1}{2} \sum_i C_i Z_i^2$$

Equation 1.1

in which C_i is the concentration of ion i of charge Z_i .¹³⁸

In our first attempt to form tau fibrils, we were more focused on protein concentration than any other factor, knowing that higher concentrations facilitate the aggregation process.¹³⁹ Lyophilised protein was resuspended in the aggregation buffer for a final concentration of 11 μ M. Aggregation buffer composition included 500 mM MES, 1000 mM NaCl and 100 mM ammonium acetate. Heparin was added at 2.75 μ M molar ratio tau to heparin 4:1. As additive, the preparation contained the chelating agent EDTA, final concentration 5 mM. The final pH of the preparation was 7 ± 0.03 , whereas the calculated ionic strength of this buffer was $I = 1.6$. Tau protein was aggregated at 37 °C for 8 days. TEM images were obtained to verify the formation of tau fibrils (Figure 18).

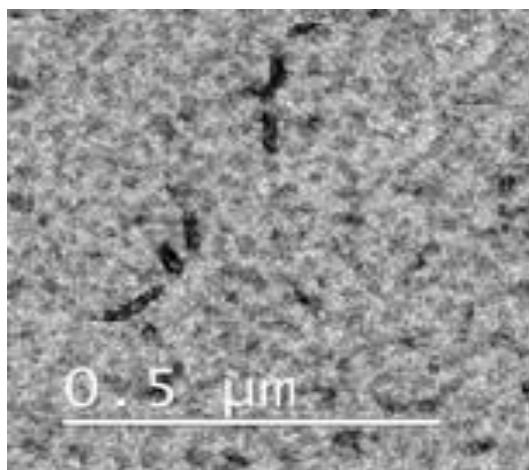


Figure 18 - TEM images of tau assembly into spherical nucleation units (SNUs) and their linear assembly. Tau was incubated for 8 days at 37 °C. A drop with tau aggregates was placed in a copper coated grid and negatively stained with uranyl acetate aqueous solution (2% w/v) for 30 seconds. Presence of SNUs -highlighted by a white circle-, and short fibres formed by multiple SNUs linked together is detected in the 8th day of incubation.

The first tau assemblies observed in our samples were the spherical nucleation units (SNUs) (Figure 18). These spherical assemblies, that resemble a string of beads formed by linearly aligned spheres, are thought to be the initial building blocks of nascent tau fibrils. It has been reported that these oligomers adopt a conformation that allows thioflavin T binding, indicative of β -sheet secondary structure.¹⁴⁰ The appearance and size of individual SNUs and those arrayed into assemblies were similar in size and diameters of 19.3 ± 2.4 nm ($n=10$), in close agreement to those reported by Xu *et al.* (21.1 ± 2.9 nm).¹³⁴ The protein concentration was optimal for its self-assembly; however, the high ionic strength of the buffer was detrimental for tau aggregation. It has been observed that the formation of PHFs is highly sensitive to elevated ionic strength.⁴⁴ In fact, previous works involving tau *aggregation in vitro* used aggregation buffers whose ionic strength ranged from 0.07 to 0.24 (6 to 22-fold less concentrated than our buffer).^{141–143}

In view of these results, we set up a new test for tau aggregation. In this occasion, incubation period and pH and were increased. By contrast, the final concentration of tau was reduced, as well as the ionic strength. Tau protein was incubated at 2.18 μ M (4-fold less concentrated), in aggregation buffer composed by 20 mM Tris-HCl, 50 mM NaCl and 1 mM DTT additive, pH 7.4. The ionic strength of this preparation was $I=0.07$. Heparin was added, so the molar ratio heparin to tau protein was 1: 1.87. Tau fibrils were formed at 37 °C for 13 days. Two different structures were prevalent in the images as indicated by AFM imaging; short and long fibrils.

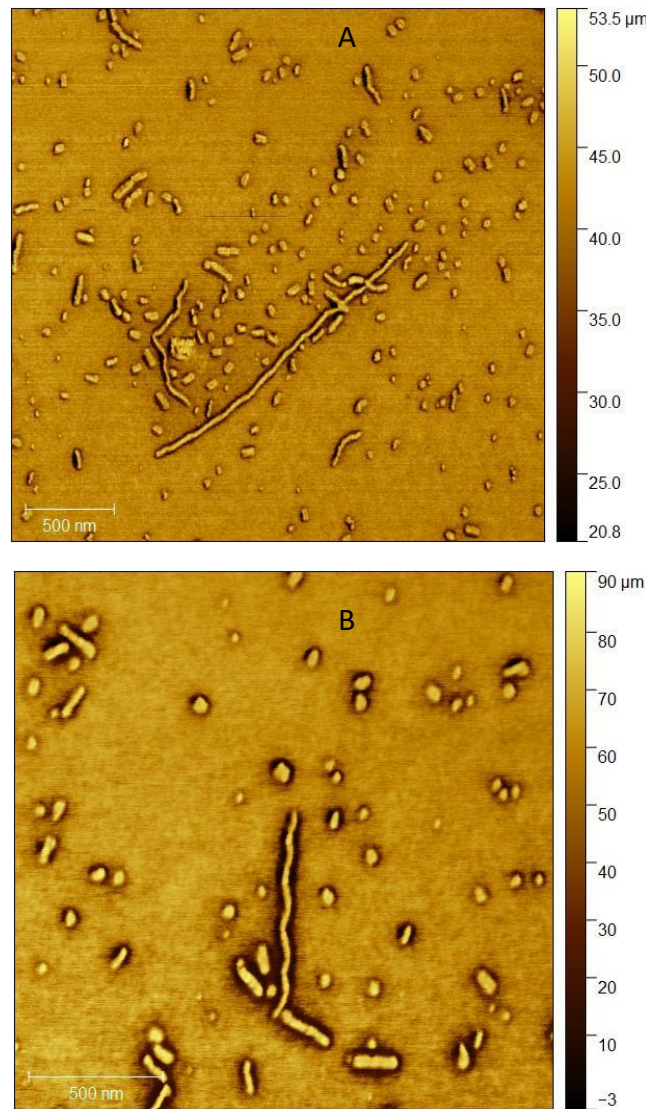


Figure 19 - AFM imaging of Tau 441 assemblies into granular oligomers and mature tau fibrils. 5 μ L of aggregation buffer containing tau aggregates was placed in the surface of a mica disk and analysed with tapping mode AFM (**A** – zoom n. 1, and **B** – zoom n. 2).

SNUs evolved to form spherically or elliptically shaped granules and long fibrils (Figure 19.A and 19.B). As the incubation period increases, SNUs seem to coalesce to form straight and twisted ribbon-like filaments as well as paired-helical filaments, similar to those found in human tauopathies.¹⁴⁴

Maeda and co-workers¹⁴⁰ used the *major-to-minor* axis ratio to define granules ($1 \leq \text{major-to-minor axis ratio} \leq 2$) and fibrils and PHFs ($2 \leq \text{major-to-minor axis ratio}$). Ratio X/Y values between 1 and 2 are descriptors of granules, whereas values above 2 indicate mature fibrils such as PHFs. According to this formula, after 13 days of aggregation, PHFs could be appreciated, reaching lengths of above 700 nm (Figure 19), although granules were also abundant at this stage.

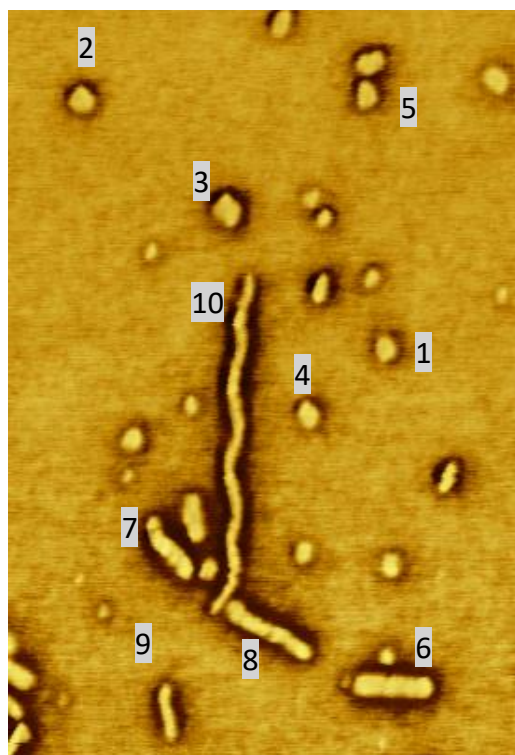


Table 6 - Major (X) and minor (Y) axis length (μm) of tau assemblies (Figure 20) measured after 13 days of incubation.

	X (μM)	Y (μM)	RATIO (X/Y)
1	0.047	0.063	1.3
2	0.066	0.047	1.40
3	0.102	0.063	1.61
4	0.066	0.039	1.69
5	0.063	0.035	1.8
6	0.152	0.043	3.53
7	0.129	0.035	3.68
8	0.170	0.045	3.77
9	0.137	0.035	3.91
10	0.776	0.025	31

Figure 20 - AFM imaging of Tau 441 assemblies into granular oligomers and mature tau fibrils - Enlarged section from Figure 19.B. Different tau aggregates were selected for elucidating their aggregation state (granular tau oligomers or fibrils) according to their major-to-minor axis ratio.

Fibril n.10 showed an x/y ratio above 2 (Table 5), and 24 ± 1.7 nm-wide, values that are similar to those previously reported in the literature (15-25 nm).^{134,140} Additionally, shorter fibrils (Figure 20, tau structures labelled as 4 and 5) presented a x/y ratio above 2 and higher diameter (>40 nm), indicative of an intermediate state in the formation of mature fibrils.

Our samples with tau aggregates presented filamentous species with periodical twists. The topography along the backbone of the fibre (blue line, Figure 21.B) showed a ~ 130 nm twist periodicity (white arrows, Figure 21.A and 21.C), which is in accordance to what is found in the literature for PHFs.¹⁴⁵

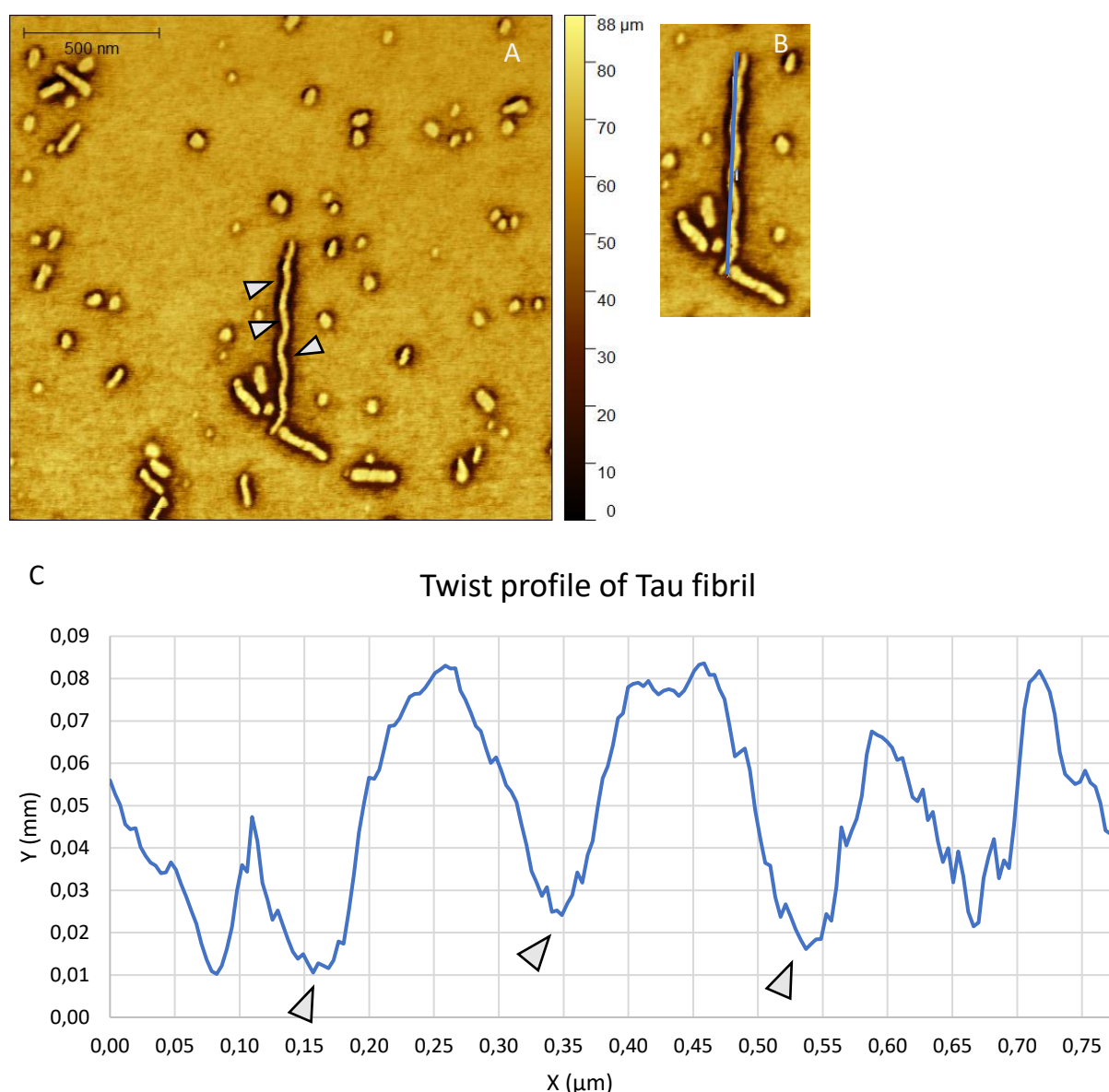


Figure 21 - Tau fibrils twist profile. The backbone of the fibre (B) was plotted (C) as the registered height (Y) versus the traversed distance by the cantilever. Data was processed with Gwyddion software and plotted on Excel.

The reduction of the ionic strength and the increased length of the incubation period clearly improved the formation of tau aggregates, as more mature fibrils were observed. This highlights the ionic character of the interaction between tau and heparin, which would be shielded by salts.⁴⁴

Our third preparation of tau fibrils was however carried out in reduced pH (6.0). Lyophilised tau protein was resuspended in water (final tau concentration was 4.36 μM), and the final composition of the aggregation buffer was 100 mM MES, 200 mM NaCl. Additives were 0.5 mM EGTA and 1 mM DTT. Heparin was added to prone tau aggregation at molar ratio was 1: 1.87 (heparin to tau protein). The ionic strength of this preparation was $I = 0.3$.

Fibrils were visualized by TEM, confirming the presence of granulates and PHFs (Figure 22).

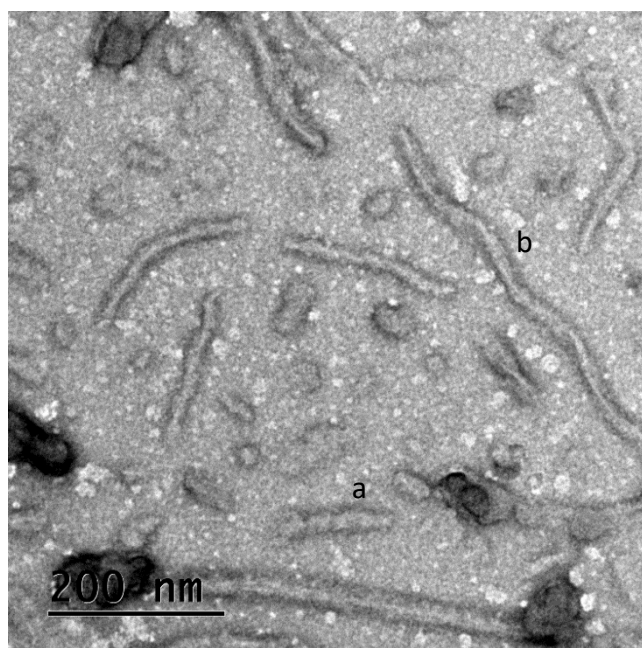


Figure 22 - TEM imaging of tau fibrils. Granular tau oligomers (a) and PHFs (b) were visible after 13 days of incubation at 37 °C.

Compared to the previous experiment, the ratio heparin to tau was kept constant, whereas the ionic strength increased, and the pH was reduced. This third conditions also generated mature tau fibrils; however, in our experience, the second conditions were the best as far as fluorescence-based assays with Thioflavin T are concerned, since higher fluorescence values were obtained when working with these fibrils.

In summary, the present morphological study allowed us to find the optimal conditions for the obtention of PHFs and a better understanding of the aggregation process followed by Tau⁴⁴¹ protein. SNUs were more abundant at early time points (8 days) and decreased progressively during longer periods of incubation with an increased abundance of non-filamentous granules and mature tau fibrils. SNUs combined and ultimately coalesced together in granular tau oligomers.¹⁴⁰ These non-filamentous granules are considered an intermediate species of tau fibril which will evolve to form mature tau fibrils as the incubation period increases. Tau filaments undergo a series of conformational rearrangements before fibrillization is fully complete. Different states of tau fibrils can coexist in the same reaction mixture, and it has been hypothesised that fibrils of different morphologies can be interconvertible, depending on conditions in the microenvironment.¹³⁴ Hence, tau fibrils were obtained after 13 days incubating under two suitable conditions to form granulates of tau oligomers as well as the characteristic PHFs and SFs in AD. These aggregates were used further in different studies to identify new candidates for tau radiotracers.

1.3.4 Binding studies with Surface Plasmon Resonance

Surface plasmon resonance (SPR) is a technique that allows label-free, real-time investigation of detailed and quantitative studies of protein-protein interactions, as well as determination of their equilibrium and kinetic parameters.

The SPR-based binding method entails immobilization of a ligand (in our specific case, tau protein) on the surface of a sensor chip which has a monolayer of carboxymethylated dextran covalently attached to a gold surface. Immobilization is possible due to the formation of amide bonds between free amine groups in the ligand and the activated carbonyl groups in the dextran matrix. (Figure 23 A). The ligand is immobilized on the surface of the sensor chip by means of well-defined chemistry, allowing solutions with different concentrations of an analyte (for us, the compounds under study) to flow over it and to characterize its interactions to the immobilized ligand. The SPR signal is obtained from changes in the refractive index at the surface of the gold sensor chip. A binding event implies an increase in mass that causes a proportional increase in the refractive index, which is detected as a change in response.¹⁴⁶ The response signal is quantified in resonance units (RU) and represents a shift in the resonance angle (θ), where 1 RU is equal to a critical angle shift of 10^{-4} deg or 10-12 gmm⁻².

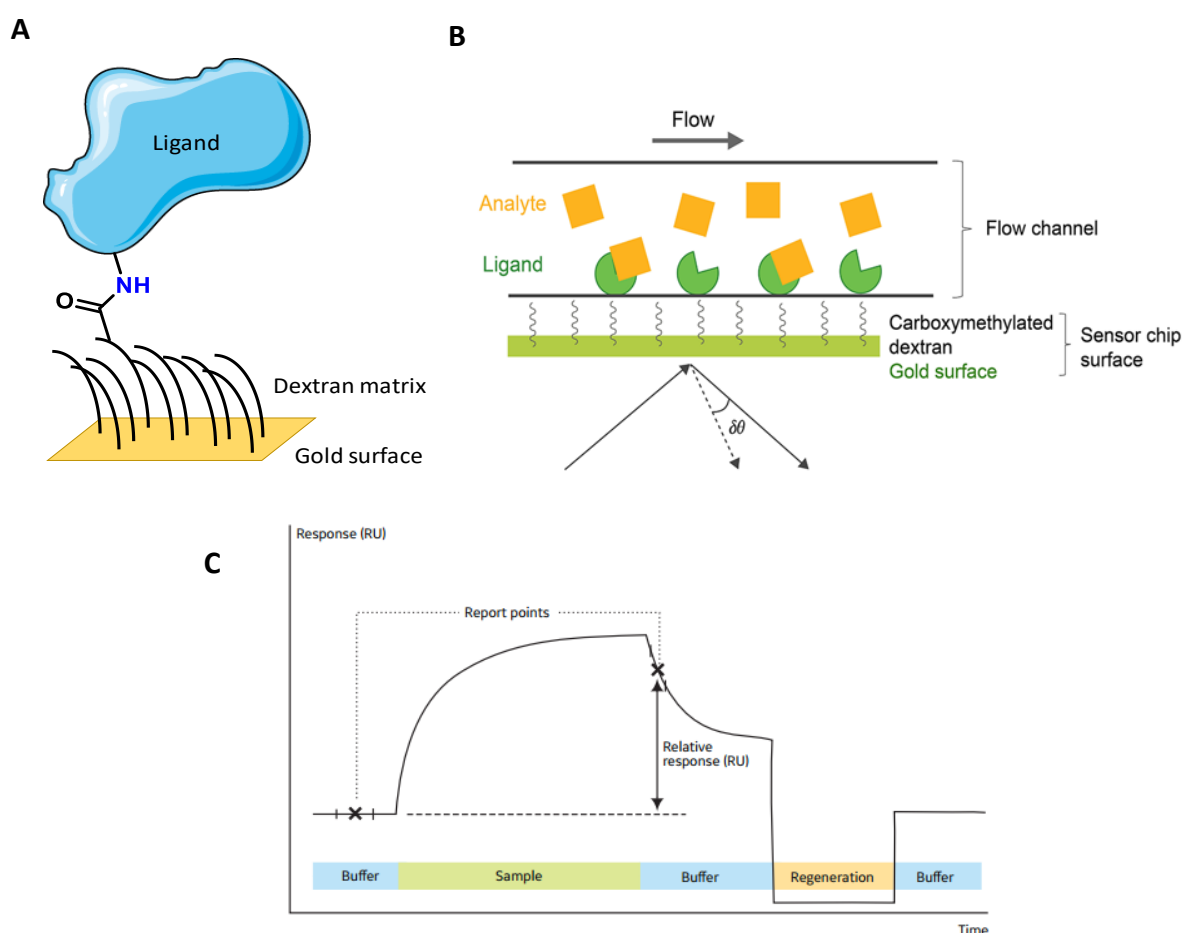


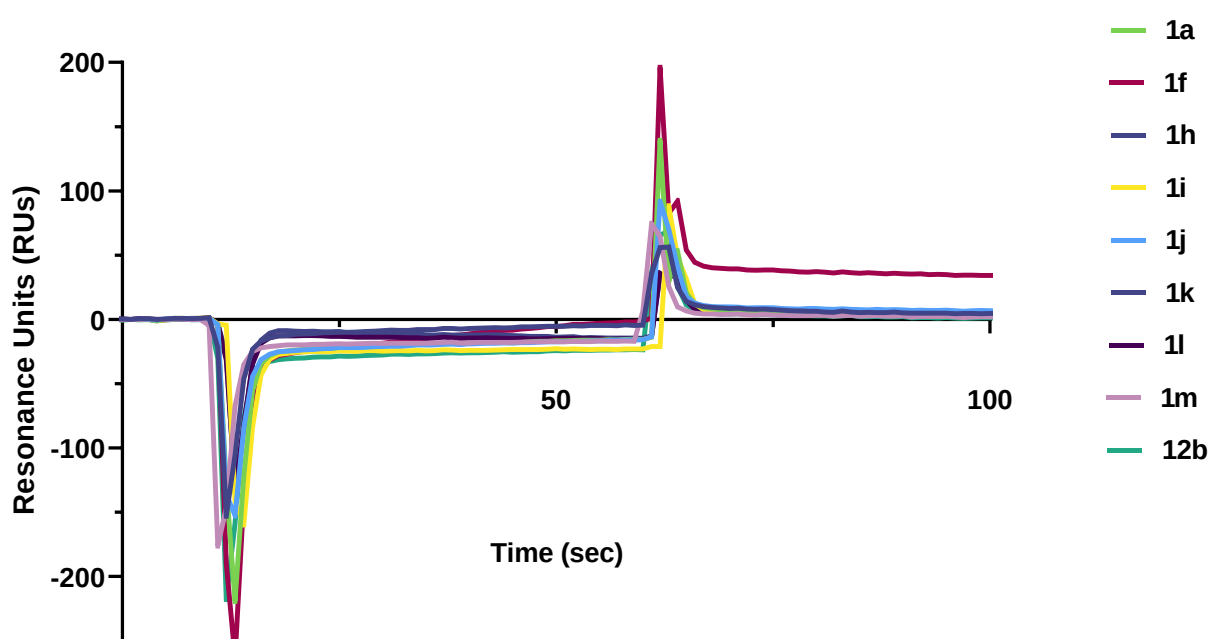
Figure 23 - Schematic representation of amine coupling (A) in SPR. (B) Experimental set up of an SPR experiment. Binding events provoke changes in the resonance angle ($\delta\theta$) of refracted light when the

analyte, flowing through the channel, binds to the immobilized ligand and increases in density at the sensor chip. (C) Typical shape of a sensorgram after one run or analysis cycle. Resonance Units (Y axis) are represented as function of time (X axis). Bars below the sensorgram curve represent type of solutions that pass over the sensor surface. Picture B has been taken from reference ¹⁴⁶ and picture C has been taken from the Sensor Surface Handbook (Biacore™).

Monitoring the responses over the time on the SPR signal it is possible to observe different stages of a binding event (Figure 23.C). Once the sample is injected, we can observe a binding response increase due to the formation of analyte–ligand complexes at the surface of the sensor-chip. This is the association phase. After a certain time of injection, binding and dissociation molecules are in equilibrium: a steady state is reached, and the binding response tends to *plateau*. In the next phase, the dissociation phase, a decrease in response is observed indicating the separation of the complexes. Regeneration phase removes bound analyte from the ligand surface and allows to reach the baseline again. This step is not always necessary, as it depends on the dissociation rate of the tested ligand. Binding affinities, association (k_a) and dissociation (k_d) rate can be obtained by fitting the sensorgram data to an appropriate kinetic binding model.

Surface Plasmon Resonance (SPR) analysis was carried out to evaluate the interaction between imidazopyridines derivatives and tau protein. Tau aggregates were immobilized in the surface by amine coupling at 7800 RUs. The second channel was blocked as reference. Compounds were subjected to a systematic screening, where each compound was injected at 100 μ M and exposed to tau aggregates for a whole cycle, comprising association, binding and dissociation steps (Figure 24).

A



B

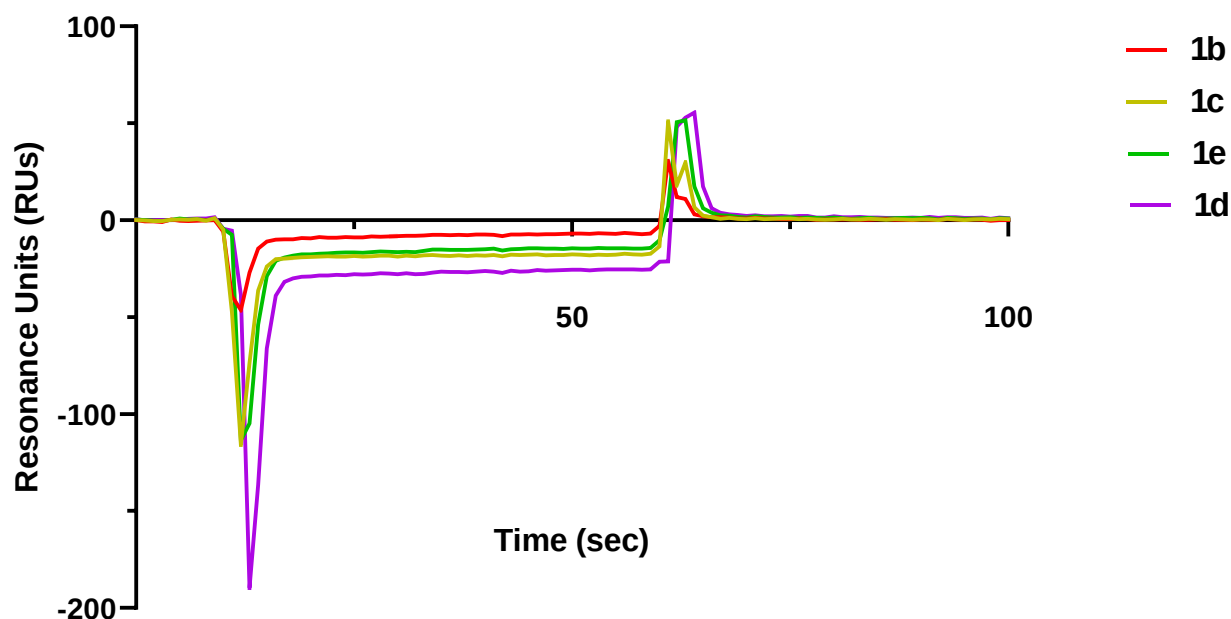
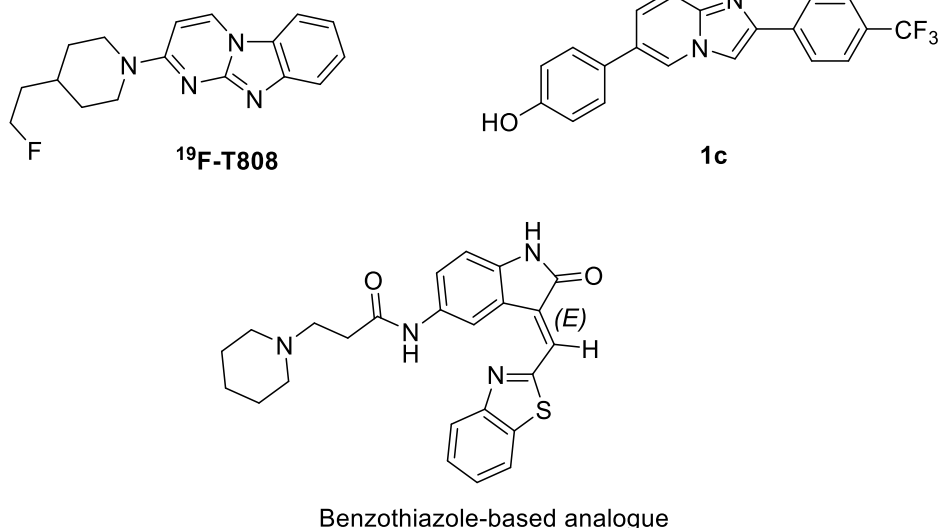


Figure 24 – Binding data obtained by SPR with immobilized tau aggregates on sensor chip CM5. Sensorgrams were obtained after exposing imidazo[1,2-*a*]pyridines (Sensorgrams A and B) to immobilized tau aggregates. Compounds were injected at 100 μ M.

None of the tested compounds presented affinity for tau aggregates, as it can be appreciated by the plain shape of the sensorgrams in Figure 24. In all experiments, compounds were flown through the reference channel (channel 1), where no ligand is immobilised, and this channel was used as blank. Response generated by compounds is reported as signal observed in the main channel minus the signal observed in the reference channel. In many cases, compounds had a certain level of unspecific interactions with the surface of the reference channel, which explains why the signal was negative.

Two positive controls were also injected (Figure 25A). The first one was ^{18}F -T808 (T808 for short), a known radiotracer used in clinics to imaging PHF-tau.⁶⁹ The second was a compound property of our group that had previously shown very good affinity for tau aggregates on SPR experiments. This last compound was not structurally related to the imidazopyridines or T808, since it has a benzothiazole-based core, but served as a good indicator of the correct performance of the analysis.

A



B

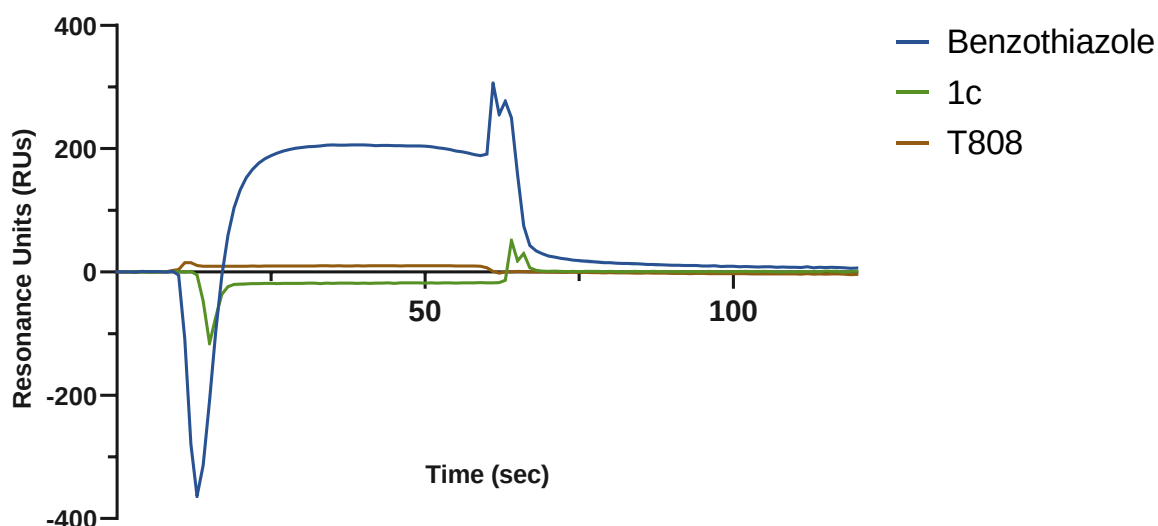


Figure 25 – (A) Chemical structures of radiotracer T808, compound 1c and benzothiazole-based compound (internal control) and their sensorgrams with binding affinities for tau aggregates **(B)**. Compounds were injected at 100 μ M.

To our surprise, T808 displayed a very low affinity for tau aggregates, even though it is known as a selective radioligand for Tau imaging.⁷³ On the other hand, the benzothiazole analogue showed noticeable affinity for tau assemblies with a good profile of association and dissociation.

At this point of the investigation, two new radiotracers were published. Their structures highly resembled those presented in this work. The first one was ^{11}C -RO6924963, developed by Gobbi and co-workers at Hoffmann-La Roche (Figure 26, molecule A). Most interestingly, the new imidazo[2,1-*a*]pyridine analogue presented high affinity for tau deposits over A β plaques *in vitro* autoradiography tests. In addition, this compound showed the absence of significant off-target binding to any other CNS targets.⁶⁸

The second compound was designed and developed by Zhang et al. as imaging probe for amyloid- β plaques for single photon emission computed tomography (SPECT/CT). This radioligand presents a benzene and pyridine rings interconnected by a conjugated double bond¹⁴⁷ (Figure 26, molecule B). Authors did not evaluate the affinity of A β plaques over tau aggregates.

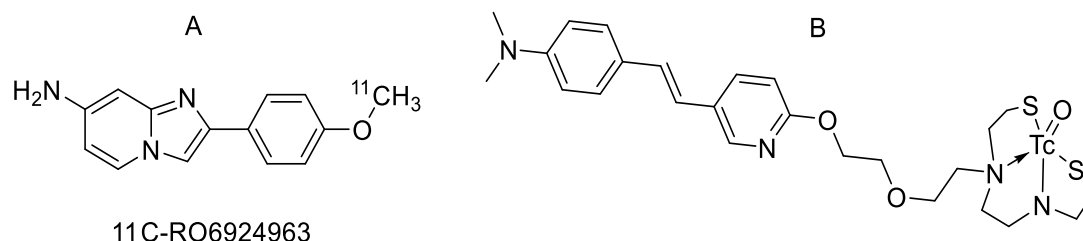


Figure 26 - Chemical structures of one tau radiotracer (**A**) and a radioligand for amyloid- β plaques (**B**).^{68,147}

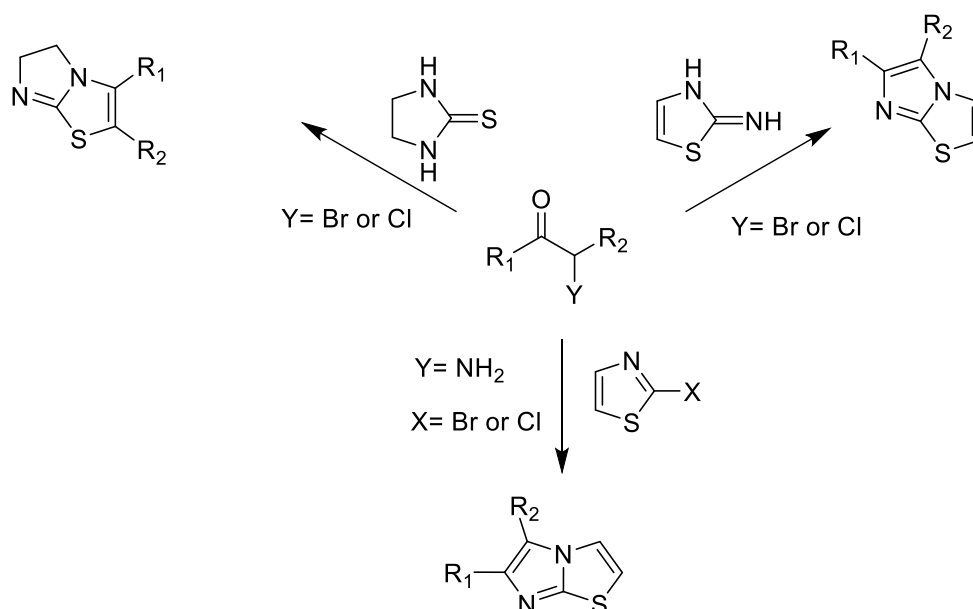
Encouraged by these findings and intrigued by the low response observed with T808 on SPR, we re-adjusted our investigation. In addition to the imidazopyridines analogues, a new family of compounds was synthesised. The new structural motif was based on an imidazo[2,1-*b*]thiazole scaffold, and the affinity for tau aggregates for the new synthesized molecules was also evaluated by SPR. Moreover, in order to understand the results obtained by SPR, and to compare them with a different technique, further fluorescence-based binding affinity studies with Thioflavin T were also carried out.

1.3.5 Synthesis and SPR studies on imidazo[2,1-*b*]thiazole derivatives

Previous work

Imidazo[2,1-*b*]thiazole scaffold is a privileged and well-considered heterocycle in the field of medicinal chemistry due to its wide spectrum of biological activities.¹⁴⁸ It is commonly found as core unit in anthelmintic and immunomodulatory agents such as Levamisol,¹⁴⁹ modulators of 5-HT receptor or inhibitors of p53 MAK kinase.^{150,151} To date, the use of imidazo[2,1-*b*]thiazole analogues as imaging probes for amyloid aggregates remains unreported.

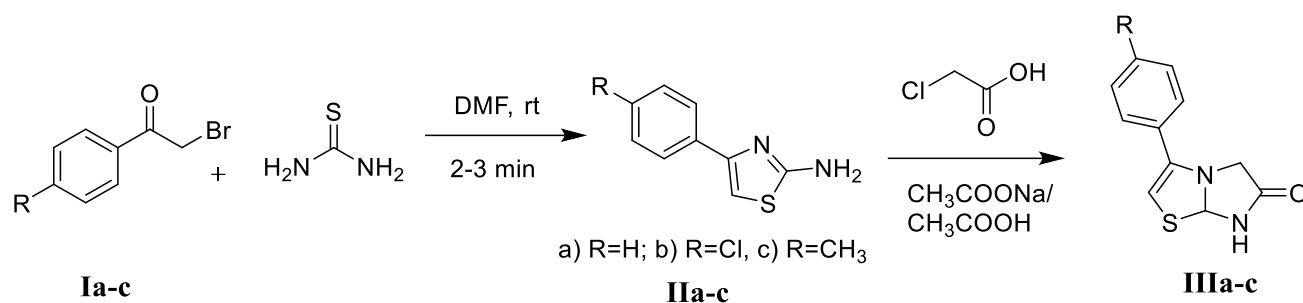
Traditional methods for the preparation of imidazothiazole involve the reaction of α -substituted ketones with sulphur heterocycles.¹⁵²



Scheme 31- Some traditional synthetic routes for preparing imidazothiazoles. Taken from reference ¹⁴⁸.

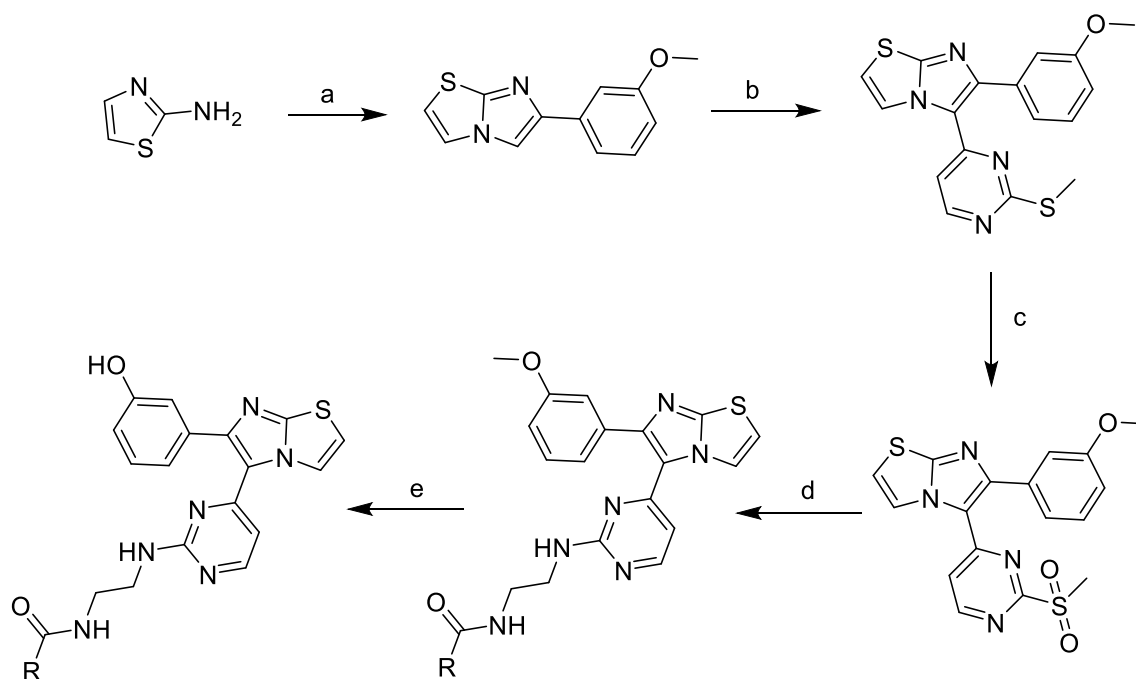
Esters, acyl chlorides or even carboxylic acids substituted on α -position with a halogen atom can react with sulphur heterocyclic systems to afford the desired imidazo[1,2-*b*]thiazole.^{153,154}

For example, 2-amino-4-arylthiazoles (**IIa-c**) were prepared from phenacyl bromide and thiourea, and further reacted with chloroacetic acid for the obtention of various 3-arylimidazo[2,1-*b*]thiazole-6(5*H*)-one analogues (**IIIa-c**) (Scheme 32).¹⁵⁴



Scheme 32- Synthesis of 3-arylimidazo[2,1-*b*]thiazol-6(5*H*)-one.

Park *et al.* synthesized an imidazo[2,1-*b*]thiazole-based compound for the treatment of melanoma from α -bromo-3-methoxyacetophenone and 2-aminothiazol as starting materials. The obtained cyclic 6-(3-methoxyphenyl)imidazo[2,1-*b*]thiazole reacted further in a C-C arylation, via Heck reaction, in presence of palladium acetate and triphenylphosphine giving the corresponding methylthiopyrimidinyl intermediate. The side chain was introduced as the corresponding amine derivative after the oxidation of the sulphide moiety with oxone. Finally, demethylation afforded the target antitumoral compound (Scheme 33).¹⁵⁵

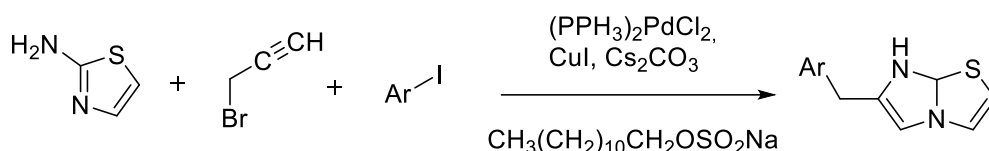


Reagents and conditions: a) α -bromo-3-methoxyacetophenone, ethanol, reflux-16 h; b) 4-iodo-2-(methylthio)pyrimidine, Pd(OAc)₂, CsCO₃, PPh₃, DMF, 80 °C, 12 h; c) oxone, methanol, water, rt, 16 h; d) H₂NCH₂CH₂NHCOR, DIPEA, DMSO, 80 °C, 8 h; e) BBr₃, CH₂Cl₂, -78 °C, 30 min then rt, 1 h.

Scheme 33- Synthetic route followed by Park and co-workers for the synthesis of an antitumoral agent with an imidazothiazole core.

Metal-catalysis has also been employed for the synthesis of this imidazothiazole scaffold.

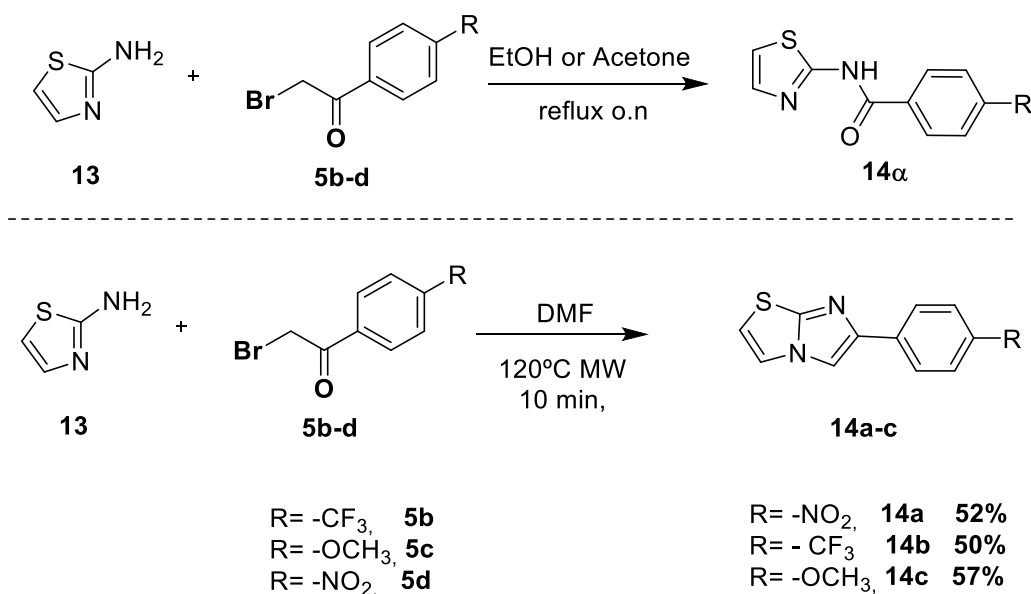
Kamali *et al.* developed a successful coupling of a 2-amino thiazole with an alkyne using palladium/copper as catalyst (Sonogashira coupling) in the presence of sodium lauryl sulphate as the surfactant in aqueous medium.¹⁵⁶



Scheme 34 - Sonogashira coupling for the synthesis of 6-arylmethyl imidazo[2,1-*b*]thiazole.

Synthesis of imidazo[2,1-*b*]thiazole derivatives

Compounds **14a-c** were prepared via heterocyclization between 2-aminothiazole (**13**) and several α -bromo-phenylethanones **5b-d** (Table 1, entries 6-8)¹⁵⁷ with moderate yields (Scheme 35).



Scheme 35 – Initial (top) and final conditions (bottom) explored synthesis of imidazo[2,1-*b*]thiazole derivatives.

The synthesis of compounds **14a-c** was not possible under the conditions applied previously with imidazopyridine derivatives, this is, EtOH or acetone refluxing overnight. The 2-aminothiazole ring is less reactive, due to the presence of the S atom which withdraws electron density from the N atom

in the ring. Thus, only the reaction intermediate **14a** was isolated. The use of microwave irradiation allowed the obtention of imidazo[2,1-*b*]thiazole scaffold as previously described elsewhere.¹⁵⁷ The described conditions included K₂CO₃ as base, however, higher number of side-products was observed on the TLC, in addition to a more difficult extraction process. By this procedure, compound **14c** was obtained in 52% yield.¹⁵⁸ Derivatives **14b-c** were prepared without the base and were obtained in similar yields with easier purification processes and less side-products.

Binding affinity studies on SPR with imidazothiazole derivatives

The affinity of imidazo[2,1-*b*]thiazole analogues for tau aggregates was also assessed by SPR. Similar to imidazo[1,2-*a*]pyridines, none of the imidazothiazoles tested displayed a positive sensorgram, indicating the absence of binding to tau protein aggregates (Figure 27).

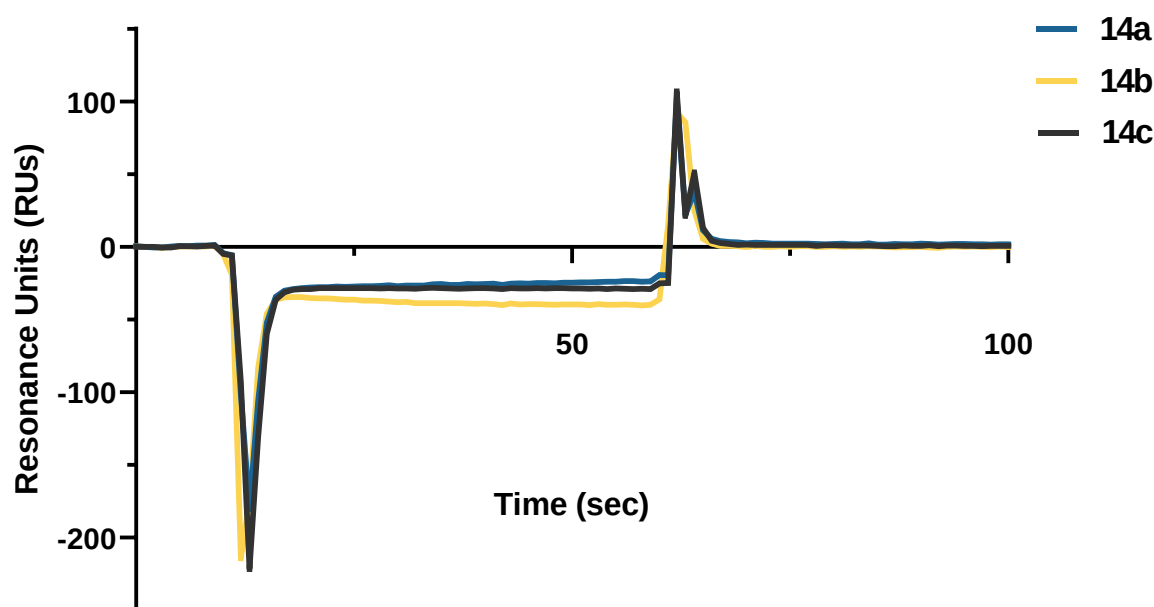


Figure 27 - Binding data obtained by SPR with immobilized tau aggregates on sensor chip CM5. Sensorgrams were obtained after exposing imidazo[2,1-*b*]thiazole analogues to immobilized tau aggregates. Compounds were injected at 100 μ M.

In view of these results and bearing in mind the demonstrated potential of imidazo[1,2-*a*]pyridine derivatives, alternative *in vitro* binding affinity studies were designed and carried out.

1.3.6 Fluorescence-based affinity studies - Thioflavin T competition assay

Thioflavin T and S dyes (ThT and ThS) are known as molecular rotors: when their rotational freedom is restricted, they show a dramatic increase in fluorescence (Figure 28).¹⁵⁹ This restriction manifests in confined spaces within proteins as well as in high viscosity solvents. These dyes possess a flat structure and therefore, are particularly well suited to bind β -sheet rich proteins. Thioflavin staining is the gold standard in the post-mortem diagnosis of AD.

The principle

Taking advantage of this ability to bind β -sheets, we carried out fluorescence-based competition assays with ThT to assess the binding affinity of imidazopyridines and imidazothiazole derivatives to amyloid aggregates. In ThT competition assays, changes in the ThT fluorescence are monitored in the mixture comprising protein aggregates, ThT and the competitor (our compounds under study). If the affinity for the fibrils is high enough, the competitor may displace Th T, so the emitted fluorescence of ThT would drop. The bigger the reduction of ThT fluorescence, the higher the affinity of the competitor for the aggregates.

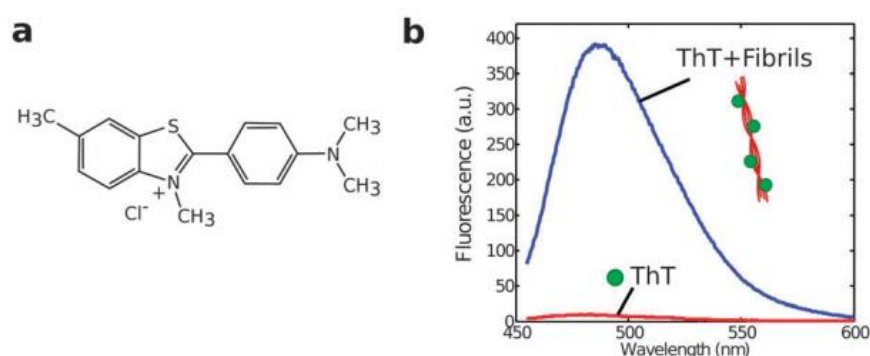


Figure 28 - (a) Chemical structure of Thioflavin T dye. (b) Changes in ThT emission fluorescence spectrum upon binding to amyloid fibrils. Picture taken from reference 160.

ThT competition assay with insulin aggregates

Insulin is a peptide that readily assembles into amyloid fibrils under low pH and elevated temperature. Many proteins in humans undergo misfolding processes that lead to the formation of amyloid fibrils. Insulin has been widely employed to study amyloid formation.^{161–163} *In vitro*, human and porcine insulin can be converted to amyloid fibrils at low pH and high temperatures (60 °C) in few hours.¹⁶⁴ With our first approach with insulin, we wanted to evaluate the general binding affinity of the synthesised compounds for amyloid fibrils as well as find suitable conditions to perform the ThT competition assay.

The thioflavin T competition assay required optimization regarding molar ratios of ThT to protein molar ratio and protein to compound as well as determination of potential intrinsic fluorescence of

compounds. Insulin aggregates were prepared by incubating 2 mg/mL in glycine-HCl buffer, pH = 2 for 8 hours and then located in 96-well plates with a fixed concentration of ThT and a range of molar ratio of the aggregated protein to the competitor between 1:20 to 5:1 (protein to competitor). After an incubation period of two hours, the fluorescence of ThT was measured and compared with the control (no competitor added).

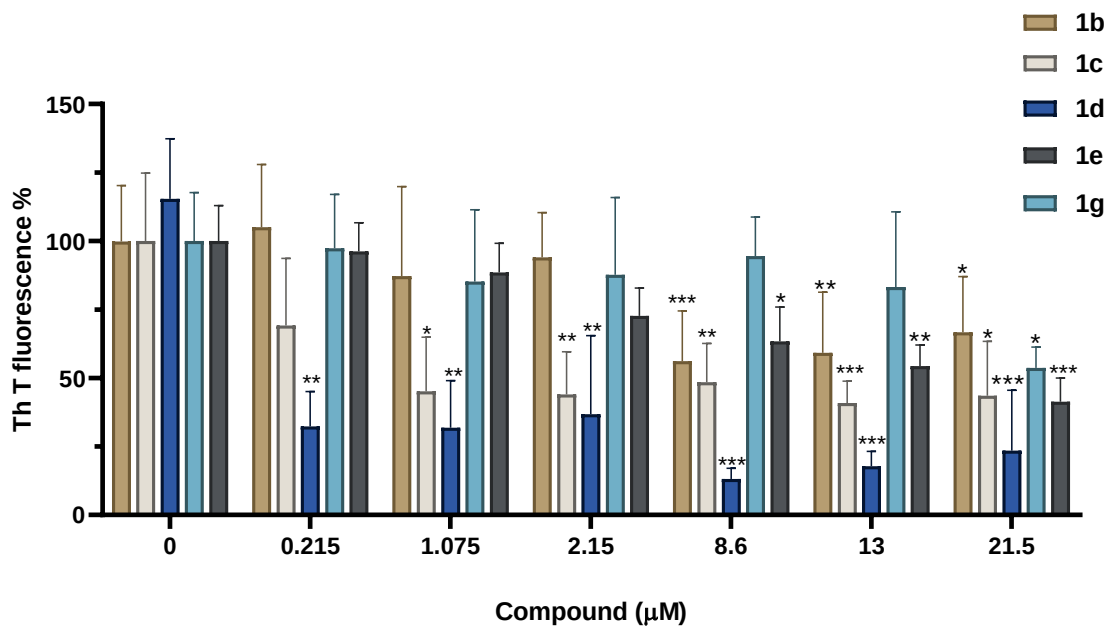
We also determined if our fused imidazole presented intrinsic fluorescence. The intrinsic fluorescence of compounds was in all cases $\leq 25\%$ of the background at the highest concentration tested, although most concentrations were found below 5%. We corrected these values by baseline subtraction in every test performed.¹⁶⁵

We also assessed whether any of the imidazopyridines and imidazothiazole could behave as Th T, eliciting an increase in fluorescence in the presence of insulin or tau aggregates that could interfere in the assay. Thus, for every concentration of compound tested in the assay, the fluorescence in the presence of protein aggregates was obtained. The conditions mimicked those employed in the competition assay (*i.e.* length of exposition to amyloid fibrils, temperature and concentration of aggregates), except for the absence of Th T, which was substituted by the appropriate buffer used in the assay. None of the evaluated compounds did show an increase of fluorescence when mixed with amyloid aggregates.

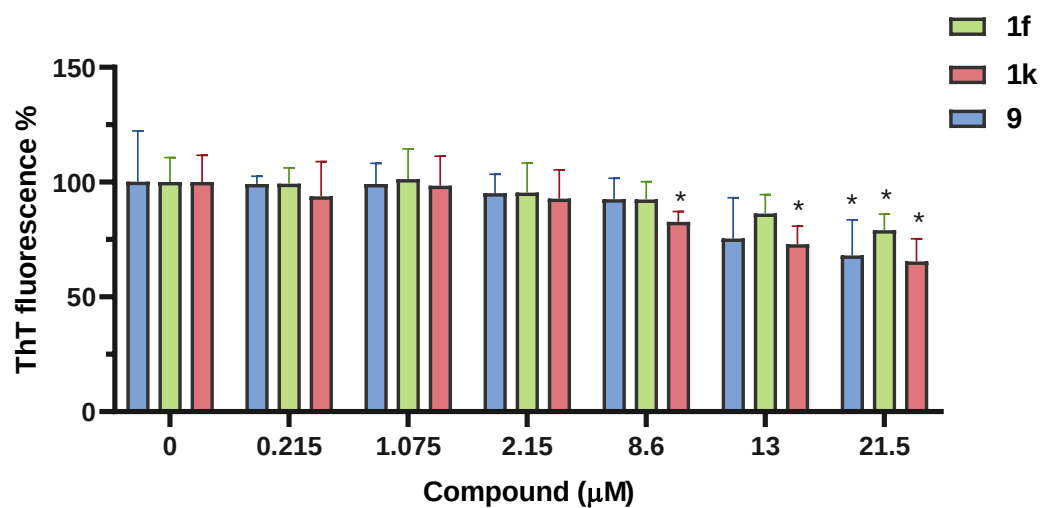
The ratio of ThT to protein was selected based on previous work,¹⁶⁶ where the final concentration of the dye was half the concentration of the aggregates. Insulin was added at 4.3 μM and thus ThT was tested at 2.15 μM .

Since the assay is based on a direct competition between the ligands and Th T, and there is little known about the number of possible binding sites present in insulin, the concentrations of compound tested were chosen using the concentration of ThT as reference. Hence, compounds were tested at molar ratios [1:1], [1:4] [1:6] [1:10] (ThT: compound), as Lockhart and co-workers reported previously.¹⁶⁶ Additionally, molar ratios [1:0.1] and [1:0.5] were also tested. Results after the competition assay on insulin aggregates are shown in Figure 29 below. The chemical structure of studied compounds is shown in Figures 30-32.

A



B



C

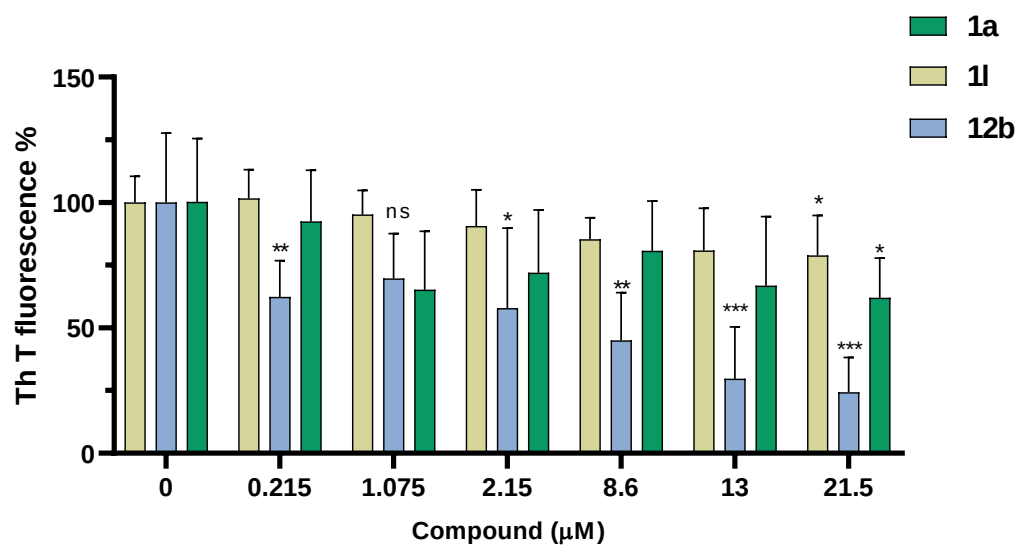


Figure 29 - Binding affinity of imidazo[1,2-*a*]pyridines-based compounds expressed as remaining ThT fluorescence (%). The lower the percentage value, the higher the affinity of competitor for tau aggregates. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing control samples (no competitor added) with test samples. One-Way ANOVA with Dunnett's post-hoc test. Mean $\pm$ SD (n=6).

Among all the compounds tested, those which had a disubstituted imidazopyridine core carrying phenol rings showed the maximum ThT displacement (Figure 29). Compound **1d** was the most potent compound, which was able to displace ThT when tested at 1.075 μ M, a concentration $\sim$ 4-fold times below the insulin concentration (4.36 μ M). The ability of **1d** to displace ThT increased in a concentration-dependent manner. However, the highest concentration tested in these assays (21.5 μ M) gave similar results to lower concentrations. This might be due to lower solubility of **1d** at high concentrations under the conditions of this assay. If the competitor precipitated, and therefore moved out of the solution, it could not bind the aggregates.

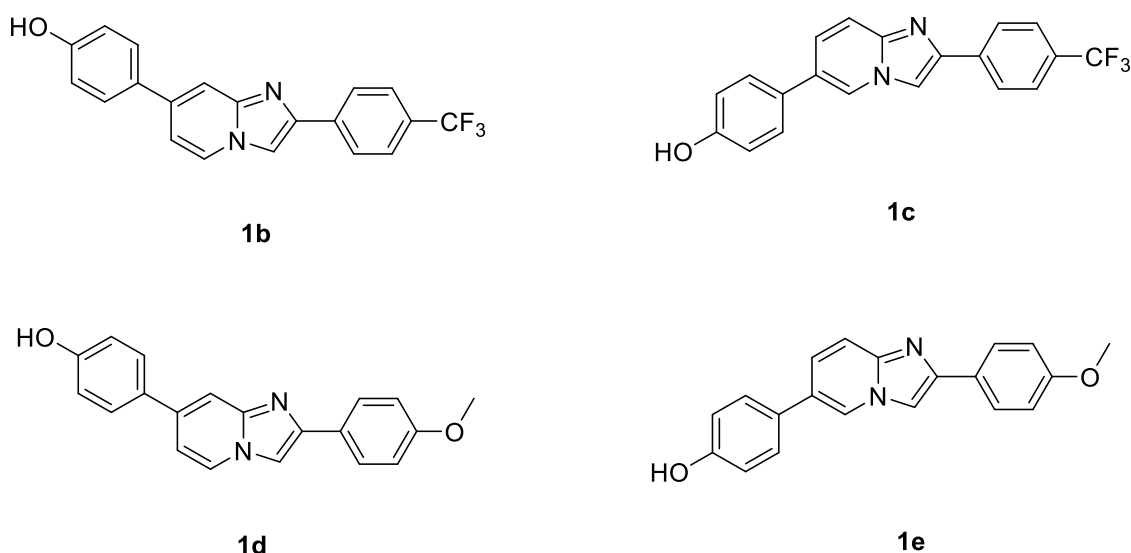


Figure 30 - Chemical structure of imidazo[1,2-*a*]pyridine derivatives **1b-1e.**

Inside the same subfamily of compounds, imidazopyridine **1e** and **1c** gave also good results, with a ThT remaining fluorescence of $41 \pm 8\%$ and $36 \pm 9\%$, respectively. Interestingly, compound **1g**, which possess a styryl moiety instead of the phenol present in **1d** (Figure 30), showed much lower affinity for insulin amyloid fibrils. Only 21.5 μ M of **1g** effectively displaced ThT (remaining fluorescence $\sim 55\%$)

Similar behaviour was shown by **1f** and **1k**, both imidazopyridines with the styryl moiety in position -7 and -6, respectively (Figure 31). **1k** displaced ThT slightly better than **1f**. All together, these observations could imply that the styryl moiety in position -7 is detrimental for the affinity for the aggregates. The reason could be the inability of forming H bonds with the protein (as it is possible

with the phenol moiety of **1d**). Another explanation could be the bulkiness of the styryl moiety, whose hindering effect was more prevalent if present in the position -7.

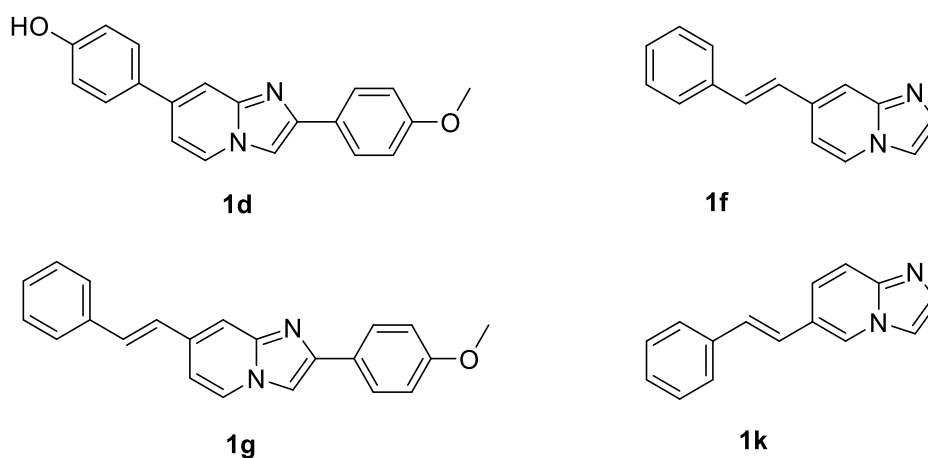


Figure 31 - Chemical structures of imidazo[1,2-*a*]pyridine derivatives **1d**, **1f**, **1g** and **1k**.

Other imidazopyridines with low affinity for insulin aggregates were **1l** and **1a**. **9** showed modest affinity for the aggregated protein, reducing the ThT fluorescence up to 25% (Figure 32).

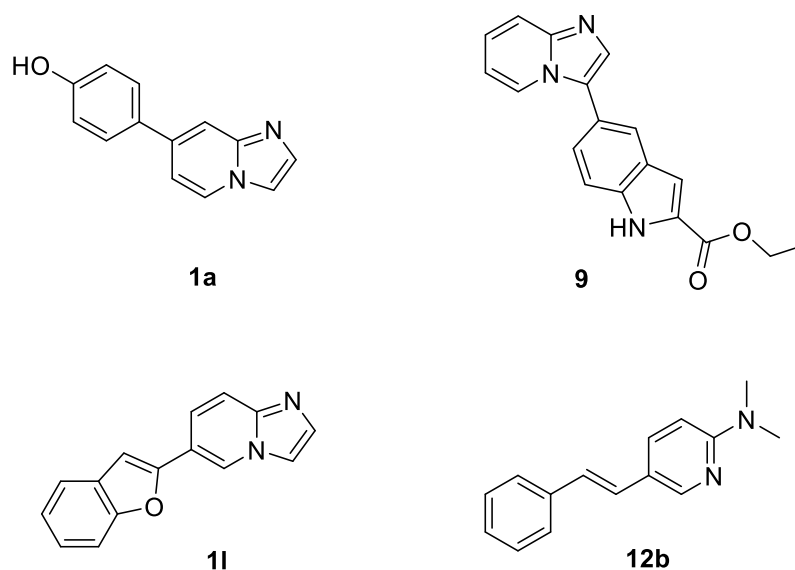


Figure 32 – Chemical structures of compounds imidazopyridine derivatives **1a**, **9** and **1l** and the N,N-dimethyl-2-aminopyridine analogue **12b**.

The substitution on the C2 of the imidazopyridine also played a role in amyloid affinity: compounds **1a** and **1d** differed only on the substitution on that carbon. **1d** showed higher affinity for insulin aggregates than **1a**, revealing the importance of the substitution on the C2. This effect has been observed previously in the literature.⁶⁸

12b, whose chemical structure contains a pyridine ring instead of the imidazopyridine core, showed a good level of affinity for insulin aggregates (Figure 29C). **12b** did displace ThT at almost every

concentration tested, with remaining fluorescence values below 30% at the highest concentrations tested (13 and 21.5 μM). However, no statistical difference was found between these two groups, which means that 21.5 μM of **12b** were as good as 13 μM when binding insulin aggregates.

The family of imidazo[2,1-*b*]thiazole derivatives showed mild to high affinity for insulin aggregates (Figure 33). The best analogue was **14c**, which reduced the ThT fluorescence below 50 % at 21.5 μM . **14a** and **14b** behaved similarly towards ThT, and both displaced the dye ~45%.

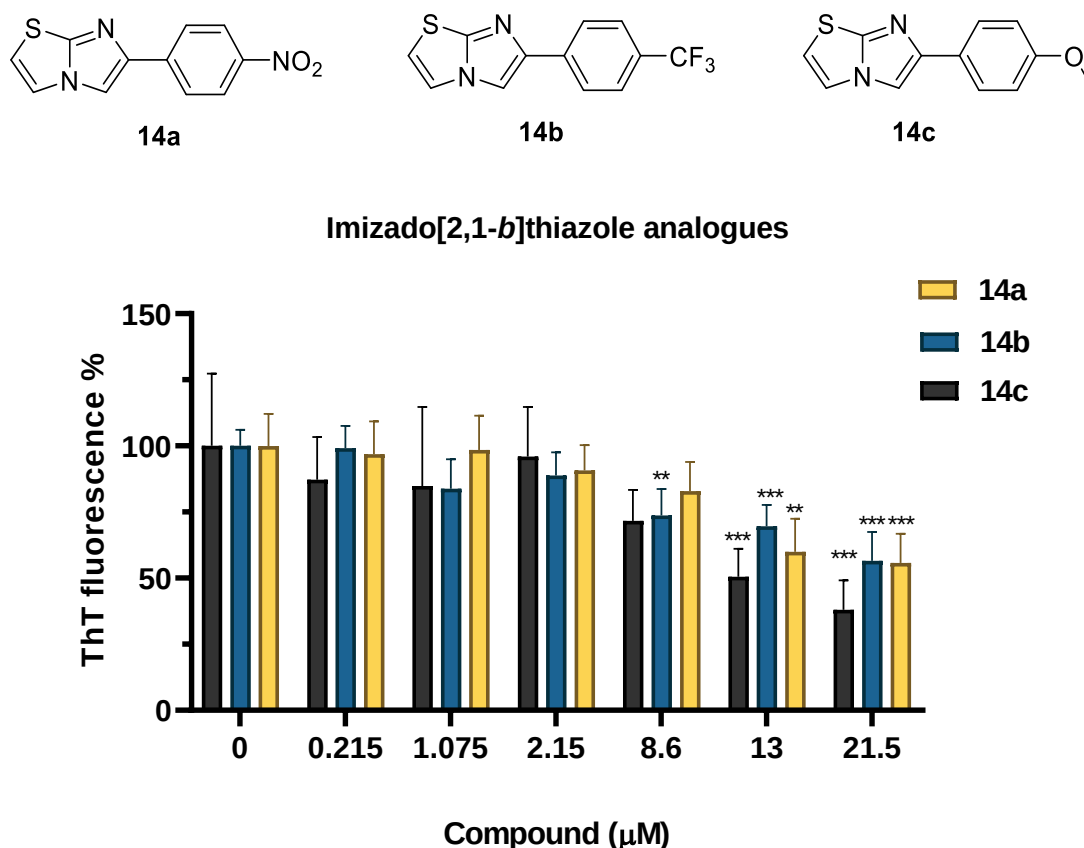


Figure 33 – Chemical structures (top) and binding affinity (bottom) of imidazo[2,1-*b*]thiazole-based analogues for insulin aggregates, expressed as remaining ThT fluorescence (%). Binding affinity is expressed as remaining ThT fluorescence (%), the lower the percentage value, the higher the affinity of competitor for tau aggregates. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing control samples (no competitor added) with test samples. One-Way ANOVA with Dunnett's post-hoc test. Mean $\pm$ SD (n=6).

When comparing the affinity of imidazo[1,2-*a*]pyridines analogues with imidazo[2,1-*b*]thiazole compounds for insulin aggregates at equal concentration employed, it is possible to observe that imidazo[1,2-*a*]pyridines derivatives presented in general higher affinity for insulin aggregates than imidazo[2,1-*b*]thiazole analogues.

Overall, these results showed that the synthesised imidazo[1,2-*a*]pyridine and imidazo[2,1-*b*]thiazole analogues did bind amyloid fibrils by means of ThT displacement and showed mild to good affinity for insulin aggregates.

Our next step was the assessment of the affinity of these compounds for aggregated human Tau protein.

Th T competition assay with Tau protein

Optimization of the assay

Recombinant or normal brain tau protein can polymerize spontaneously but slowly into a PHF-like structure *in vitro*, as denoted by the binding of several β -sheet preferred fluorescent dyes, light scattering spectroscopy and electron microscopy. Molecules such as heparin or arachidonic acid, an abundant fatty acid in human brain, dramatically accelerates PHF formation in tau isoform.¹⁴²

Tau protein aggregates were obtained after 13 days incubating at 37°C in the presence of heparin to trigger the aggregation. Our first goal was performing a screening with the most relevant compounds selected from the assay with insulin aggregates.

The available amount of tau protein forced us to use lower protein concentrations in each assay, (below 1.5 μ M). According to our previous tests performed with insulin, ThT concentration would be below 1 μ M (ratio protein to ThT 2 to 1). We wondered if this ratio would be still optimal for tau protein or a different ThT:protein ratio would be needed.

Thus, a test with insulin aggregates was set, where this protein was tested at 0.55 or 1.09 μ M (mimicking the concentrations planned for tau aggregates), with ThT added at different molar ratios [1:1], [1:2] and [1:5] to protein. The objective was to find the minimum molar ratio at which a significant difference in fluorescence emission, ΔF , between negative control (ThT alone) and test samples (ThT + insulin aggregates), considering that ThT does not emit fluorescence in the absence of amyloid aggregates. With this scope, equation 1.2 was applied.

$$\text{Fluorescence variation } (\Delta F) = \text{Fluorescence [ThT + insulin]} - \text{fluorescence [ThT alone]}$$

Equation 1.2

The fluorescence emitted by ThT alone was subtracted from the fluorescence emitted by ThT in the presence of insulin aggregates. The resulting fluorescence variation (ΔF) is expressed in arbitrary units (a.u).

We set two orders of magnitude as the minimum fluorescence variation to ensure the good performance of future assays. The fluorescence of ThT alone was measured at 0.1 μ M, 0.5 and 0.25 μ M when corresponding.

Table 7 – Optimization test of ThT: protein ratio. Tests were done in triplicates. Data is shown as mean $\pm$ SD.

Entry	Pair ThT – Insulin / ratio	Control (ThT alone)	Test (ThT + Insulin)	Fluorescence variation (ΔF)
1	Th T 0.5 μ M + Insulin 0.5 μ M [1:1]	5.28E+04	1.28E+06	1.22E+06
2	Th T 0.5 μ M + Insulin 1.09 μ M [1:2]	5.28E+04	4.04E+06	3.99E+06
3	Th T 0.25 μ M + Insulin 0.5 μ M [1:2]	5.27E+04	1.64E+06	1.12E+0.6
4	Th T 0.1 μ M + Insulin 0.5 μ M [1:5]	5.26E+04	3.28E+05	2.76E+05

Ratios 1 to 1 and 1 to 2 (ThT to protein) did satisfied our criteria, and all the combinations tested at that ratios showed a fluorescence increment of two orders of magnitude. Comparing entries 1 and 2, we could observe that the increment of fluorescence was higher when insulin aggregates were added at 1.09 μ M, indicating that ThT fluorescence increases linearly in a concentration dependent manner/trend, in accordance with the literature.⁴⁴

By contrast, the ratio 1:5 showed a smaller increment of fluorescence, one order or magnitude lower than the ratio 1:2 (Table 7, entry 4 and 3). This could mean that that 0.1 μ M of ThT were not enough for binding all the available binding sites of insulin aggregates, so this ratio was discarded.

The pair 0.5 -0.25 μ M generated a similar ΔF to that one obtained in the combination 0.5-1.09 μ M and 0.5-.05 μ M. However, 0.5-0.9 μ M was chosen as the most appropriate concentration because it kept the same ratio as previously used in ThT competition assays with insulin and afforded higher fluorescence emission.

In order to determine optimal molar ratios tau protein fibrils to compound, in the next ThT competition assay, different molar ratios tau to **12b** were tested (Figure 34). A positive control (with active competitor benzothiazole analogue) were also added. As positive control, the benzothiazole derivative active in SPR was used. **12b** was selected to find the suitable conditions for the programmed screening since it was one of the best candidates as well as possess higher solubility at low DMSO percentage contained in the assay buffer.

While incubating, changes in ThT fluorescence were monitored every 15 minutes in the fluorimeter. After 2 hours of total acquisition, the highest peak of fluorescence in the control wells was found 30 minutes after starting the assay.

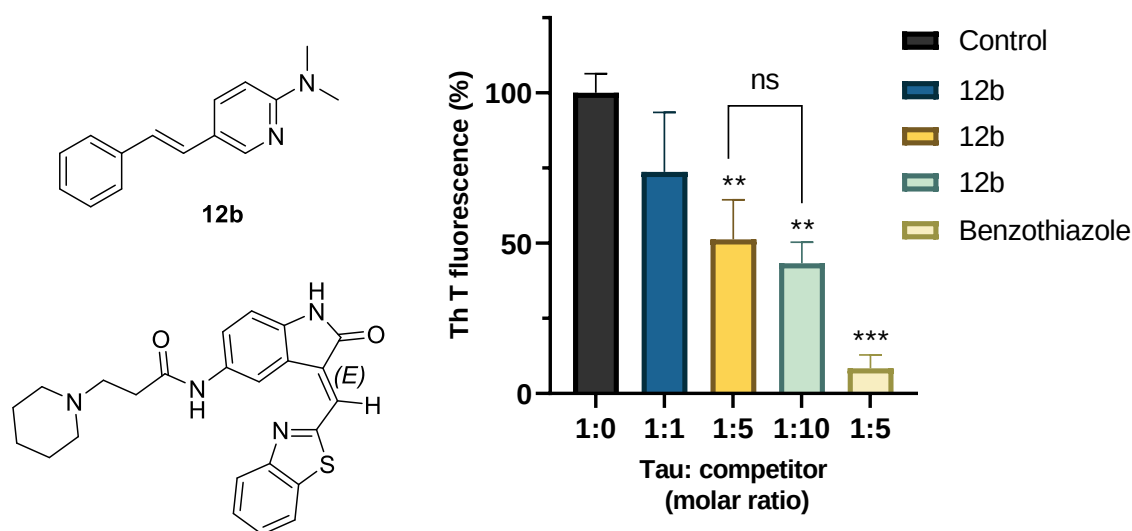


Figure 34 - ThT competition assay results. Binding affinity of **12b** compound and benzothiazole (positive control) expressed as remaining ThT fluorescence (%). Chemical structures of **12b** (top) and benzothiazole (bottom) are shown on the left. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are relative to control values. P values *no significant* $ns > 0.05$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, indicate multiple comparisons between groups. One-Way ANOVA with Dunnett's post-hoc and Tukey's Post-hoc test. Mean $\pm$ SD ($n=3$).

Compound **12b** showed affinity for Tau fibrils above 1: 5 molar ratio. This pyridine-based compound reduced the ThT fluorescence emission 50%. The positive control, benzothiazole analogue, showed a remarkable ability to displace Th T, whose fluorescence dropped below 15%.

These results validated the assay as well as provided the minimum molar ratio needed for the experimental design of the screening assay for the selected imidazopyridine and imidazothiazole analogues.

Screening of imidazopyridine and imidazothiazole analogues with tau aggregates

Tau protein was aggregated for 13 days at 37 °C prior to performing the screening assay. Compounds were tested at 1:5 molar ratio, with tau aggregates concentration of 1.09 μ M (Figure 35).

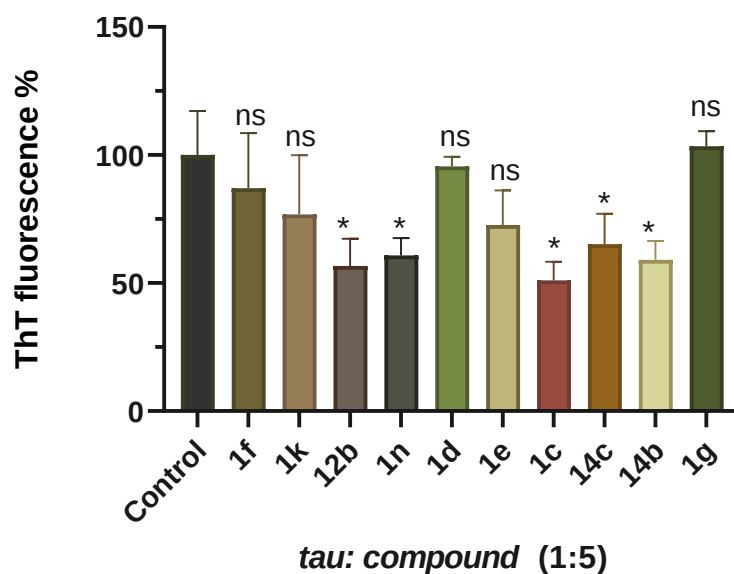


Figure 35 - Binding affinity of compounds for tau aggregates expressed as remaining ThT fluorescence (%). Student's t-test. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are shown for competitors relative to control. P values > 0.05 were statistically *no significant* (ns). Comparisons between compounds were analysed by One-way ANOVA and Tukey's post hoc test. Mean $\pm$ SD (n=3).

Some interesting insights were obtained after this first screening. First, compound **1d**, which positioned itself as the best candidate after ThT competition assay with insulin aggregates (Figure 29.A), appeared now as the compound with less affinity in the tested set. With a similar behaviour, compound **1e**, the structural isomer of **1d**, displaced slightly ThT ($73 \pm 13\%$ remaining fluorescence), although this value did not have statistical significance. On the other hand, other chemically related compounds such as compound **1c** kept its affinity for tau aggregates (Th T fluorescence reduction $49 \pm 7\%$). Chemical structures of isomers **1b-1c** and **1d-1e** were shown on Figure 30.

We aimed to observe differences in affinity values between compounds that only differed in one substituent, as it happens with **1c** and **1e**. **1c** possesses a trifluoromethyl group whereas **1e** has a methoxy group (Figure 36). Apparently, **1c** showed higher affinity than **1e** for tau aggregates (Figure 35); however, that difference was not statistically significant ($p > 0.05$, ANOVA and Tukey's post hoc test), and therefore we could not assume it as real.

Compounds **1f**, **1g** and **1k** showed low affinity for tau amyloid fibrils (Figure 35). By contrast, the cold ligand **1n** reduced the ThT fluorescence $40 \pm 7\%$. These values were very similar to those obtained by **12b** in this test.

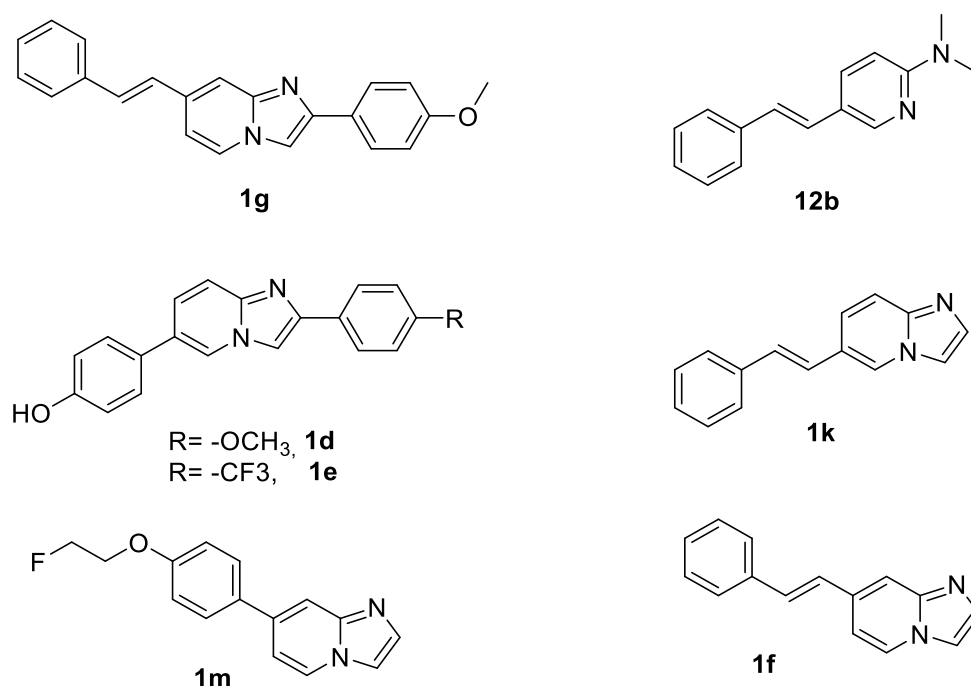


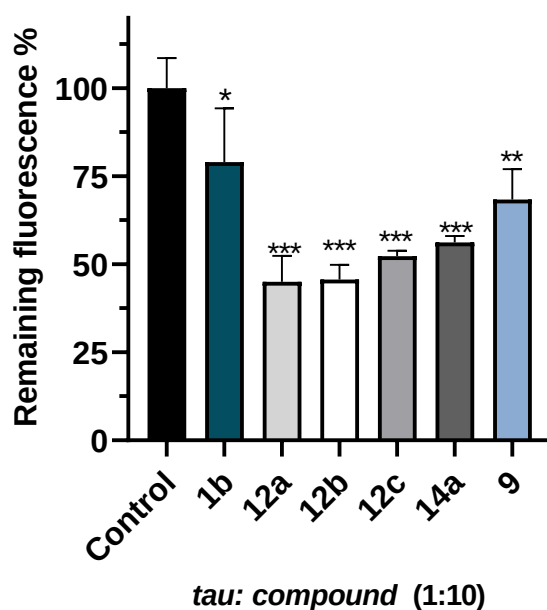
Figure 36 – Chemical structures of compounds **1g**, **1m**, **1f**, **1k** and **12b**.

Another interesting observation is related with the ring-opening in compound **12b**. Used as control in this assay, compound **12b**, repeated score and displaced ThT so the fluorescence reduction was $44 \pm 11\%$. By contrast, when the 2-aminopyridine ring was closed to form the imidazopyridine analogues **1f** and **1k**, the affinity dropped strikingly, especially in the case of **1f**, which reduced the ThT fluorescence 13% (no statistically different compared to control). **1k** was slightly better (24% reduction), although still above the values obtained with **12b**.

Regarding imidazothiazoles, both **14c** and **14b** displaced ThT, the figures being $35 \pm 12\%$ and $41 \pm 7\%$, respectively. However, the affinity of **14c** for insulin aggregates was clearly higher than its affinity for tau aggregates, the figures being 62% versus 35 % fluorescence reduction, respectively (molar ratio protein to compound was 1:5 in both assays) (Figures 33 and 35).

Next, a second screening of compounds was carried out to evaluate the affinity for tau aggregates. This time, the ratio competitor to protein was increased, as we wanted to evaluate the effect of doubling the proportion of competitor. Thus, compounds were tested at 1:10 molar ratio under the same conditions as the previous set (Figure 37):

A



B

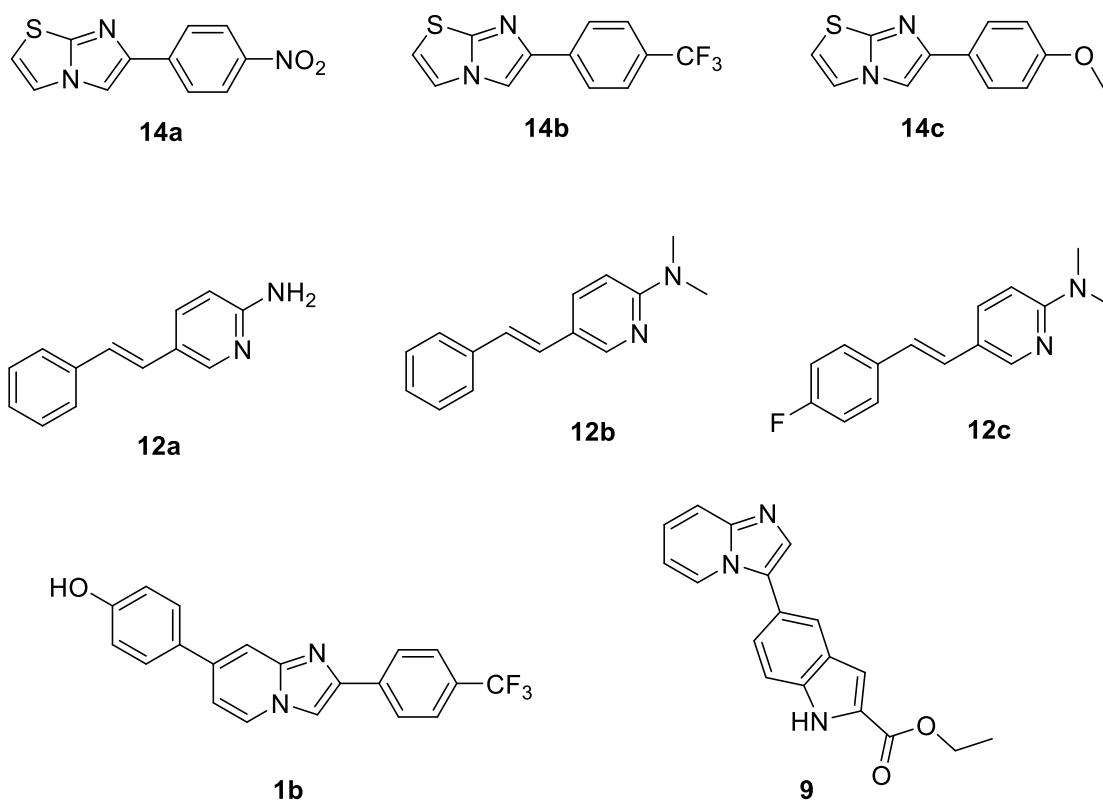


Figure 37 – (A) Binding affinity of compounds for tau aggregates expressed as remaining ThT fluorescence (%). Molar ratio tau aggregates: compound was 1:10. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are shown for competitors relative to control (Student's t test). P values > 0.05 were statistically *no significant* (ns). Student's t-test Mean $\pm$ SD ($n=3$). **(B) Chemical structures of tested compounds.**

The third imidazothiazole analogue **14a** reduced the ThT fluorescence $44 \pm 1\%$, in a similar trend to its analogues **14b** and **14c**.

Compound **12b** is the *N,N*-dimethylated analogue of **12a**. Both compounds showed the same ability to displace the ThT dye, indicating that the presence or absence of methyl groups did not affect the affinity for tau aggregates. The fluorinated analogue of **12b**, **12c**, was also equally effective displacing Th T. Therefore, **12c** could be a potential radiotracer as its structure allows the use of ^{18}F as radioligand.

In the imidazo[2,1-*b*]thiazole family, **14b** showed the highest affinity for tau aggregates, with similar results to those obtained by the imidazopyridine **1c** and 2-aminopyridine analogue **12b**. Therefore, the imidazothiazole scaffold could be a good alternative to imidazo[1,2-*a*]pyridine heterocycle.

Additionally, this second screening was used to evaluate the affinity of **1b** for tau aggregates. Interestingly, even if tested at higher concentration than its structural isomer **1c** (Figure 30), **1b** showed less affinity for tau aggregates (Th T fluorescence reduction was $21 \pm 15\%$, Figure 37.A). The ThT fluorescence reduction observed for compounds **1b**, **1c**, **1d**, **1e** against tau aggregates gave us valuable information (Figure 35). For example, the phenolic group in position -6 is preferred over the position -7. Compound **1e** showed higher affinity for tau aggregates than **1d**, as it happens with the couple **1b** and **1c**, the former showing more affinity than the latter.

In summary, whereas compounds **1b**, **1d** and **1e** present higher affinity for insulin than for tau aggregates, compound **1c** showed good affinity for both aggregates. These observations suggest that compound **1c** could be considered as a good starting point for the development of molecules with higher selectivity for tau aggregates over other amyloids, such as insulin or β -amyloid aggregates. In fact, more studies with β -amyloid aggregates are planned to confirm this potential selectivity for tau aggregates over other amyloids.

1.3.7 Computer-assisted modelling studies

Computational modelling is a powerful tool that can provide the knowledge necessary for rational design of drugs and radiotracers, as well as help to explain the interactions taking place between protein and its ligand.

Amyloid fibrils, including tau filaments, represent non-canonical binding targets for small molecules, because of the absence of deep pockets normally related to high-affinity, stereo-specific binding. Instead, they possess narrow, solvent exposed channels that extend along their surfaces.¹⁶⁷ Although tau fibrils do not fit the “lock and key” model for ligand binding, some molecules do bind tau protein aggregates with higher affinity than others.

Lemoine et al. investigated the binding profile of several radiotracers, such as ^{18}F -T807, ^{18}F -THK5351, and PBB3 in brain tissue (Figure 13). They reported the existence of multiple high-affinity binding sites for all the tracers.¹⁶⁸ Overall, from *in vivo* competitive binding assay studies using PBB3, THK5117 and T807 tracers, it has been gleaned that there are at least three different high affinity binding sites available in tau fibrils.¹⁶⁹

Until recently, computational binding studies on tau filaments remained untested due to the lack of 3D structures for the tau protofibrils. Very recently, Fitzpatrick and co-workers have elucidated the atomic structure of tau filaments PHFs and SPs.⁴⁶ Based on this findings, Murugan *et al.* have performed modelling studies (comprising docking, molecular dynamics and binding free energy calculations) with known PET tracers to declare the presence of 4 different high-affinity binding sites in tau protofibril, namely S1, S2, S3 and S4.¹⁷⁰ Sites 1, 3 and 4 are termed core sites as they are buried inside the fibril, whereas site 2 is named surface site as it is located in the outer face of the fibril (Figure 38).

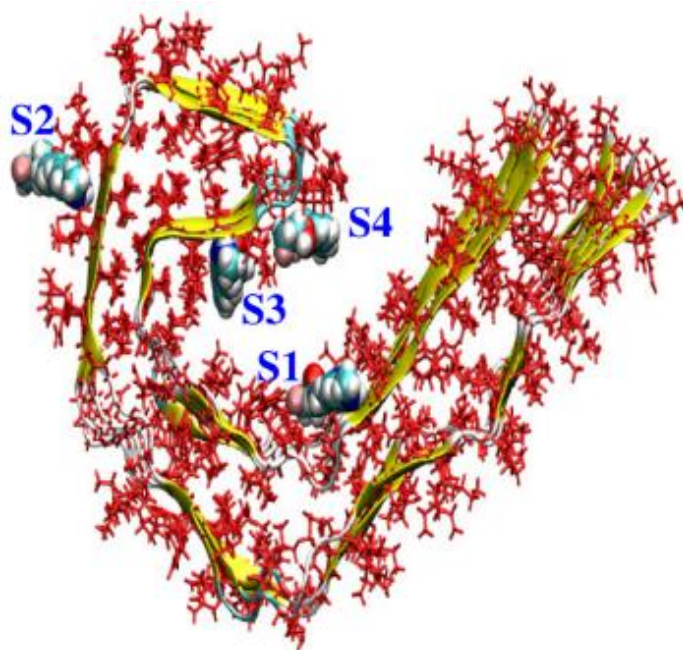


Figure 38 - Tau protofibril structure showing the various high-affinity binding sites S1-S4.

Our results from ThT-based assays revealed clear differences of binding affinity when different isomeric forms were tested on tau aggregates (i.e. **1b** versus **1c**). In view of this, computational studies were carried out for a better understanding of the interactions occurring between tau protofilaments and the selected molecules (Figure 39). In particular, molecular docking was employed as a prediction tool for gaining insights about the preferential binding site for the most promising compounds. The candidate **12c** was also studied, as well as RO6924963, the latter used as benchmark for computing the relative activity of radiotracers, and herein used as positive control.⁶⁸

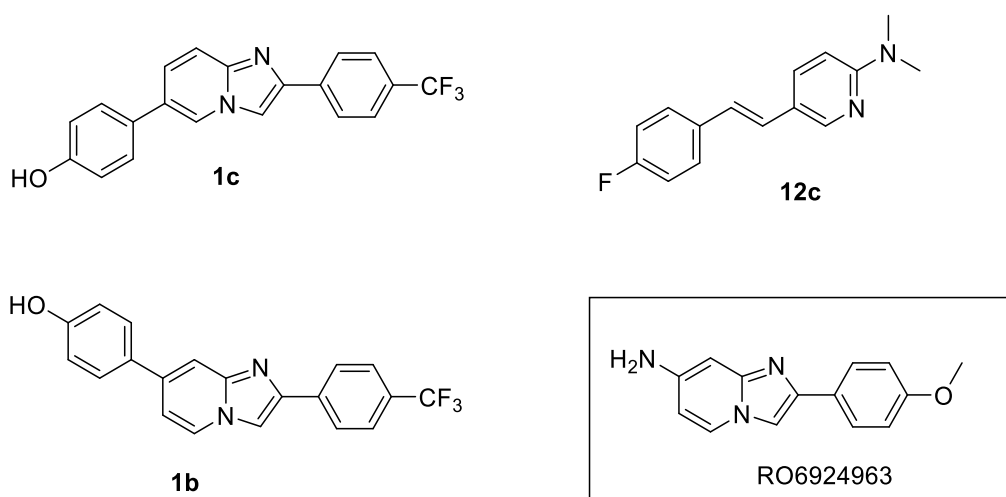


Figure 39 - Structure of selected compounds (1b, 1c, 12b and RO6924963) for molecular docking simulations. RO6924963 is a known tau selective radiotracer used as reference in this study.⁶⁸

The results of docking simulation on tau protofilament are reported in Table 8. Docking poses were re-evaluated using the HINT scoring function, which provides a HINT score (HS) based on the empirical Log P-based assessment of interatomic interactions at the ligand-protein contact point and it may correlate with the free energy of binding. In general terms, the higher the score, the stronger the interaction, as demonstrated by several works.^{171–173} Therefore, the HS could be used to propose the preferential binding site of ligands under analysis.

Table 8 – HINT scores obtained after rescoring docking results of tracer candidates compared with a known tau radiotracer, RO6924963.

Compound	Binding site			
	S1	S2	S3	S4
RO6924963	631	-355797	409	403
12c	707	-12652	345	422
1c	978	-136917	684	603
1b	716	-416252	598	556

Compounds were ranked for their relative theoretical binding potency compared to the reference radiotracer. Of note, a precise system validation could not be possible due to the shortage of experimental data in background preventing an optimal quantitative comparison of ligands and sites

of interaction. However, on the basis of the HS recorded, all four compounds under analysis showed a theoretical preferential binding for S1. Interactions with the other sites (S3 and S4) were not completely excluded, but they were considered hypothetically less favoured compared to S1 on the basis of the lower score recorded. In particular, each compound showed similar scores at S3 and S4, which might indicate comparable and low-specificity binding events, while the scores at S1 were much higher compared to the others for all four compounds. By contrast, none of the assessed compounds showed an appreciable interaction for the external binding site S2, as suggested by the negative scores recorded. Moreover, to our delight, the synthesized compounds showed higher HS than the positive control RO6924963 (Table 8), which might indicate a higher binding affinity (especially for **1c** that recorded the highest score). However, a more precise quantitative comparison was not possible due to the lack of an appropriate validation, as mentioned above.

Compound **1c** presented higher theoretical binding activity than its isomer **1a** for all sites. These results are in highly agreement with our previous findings observed in ThT competition assays and confirm the suitability of the substitution in position -6 instead of -7 in the imidazopyridine ring. A hydrogen bond is established between the phenolic -OH in **1c** and the His³⁶² of the protofibril, whilst it is absent in the case of **1b**. The lack of this interaction provided the chemical rationale of the differences found in HSs between these two isomers. The following preferred binding site for both isomers was S3, with HS values of 684 and 603 for **1c** and **1b**, respectively. These values were slightly higher than those found for S4.

Compound **12c** occupied the third position in the ranking, which also showed higher affinity for S1, although it bound with less computed affinity if compared with the values shown by **1c**. The styryl-pyridine analogue bound strongly S1, higher than RO6924963, however, in the case of S3, the tau tracer showed higher affinity than **12c**.

1.3.8 Comparison of ThT competitive assay and SPR interaction study: a theory supported by molecular modelling

The most intriguing aspect in the present study has been the consistent lack of signal in SPR when testing the imidazopyridine and imidazothiazole derivatives. Those results did not correlate with the results obtained with the ThT competition assay, where some compounds clearly bound tau fibrils. There are some factors that could explain this contradictory occurrence.

The aggregation technique of tau protein has been optimized in the first stage of this study, thus the last sets of aggregated tau (used in the ThT assays) were richer in fibrils compared to those used in preliminary screening studies with SPR. The optimization of the buffer (*i.e.* improved control of the ionic strength and antioxidant components) and the optimization of the incubation period led to the formation of a higher number of matured fibrils.

Our compounds show preferential selectivity for the S1 binding sites of tau protofibrils which is the most shielded of the available binding sites (Figure 38). In the study of Murugan and co-workers,¹⁷⁰ a variety of known tau tracers was selected to evaluate the affinity for each binding site. The study revealed that each radiotracer had a preferential binding site, which explains why different molecules non-structurally related to each other presented high affinity for tau fibrils.

Our molecular docking studies predicted S1 as the preferable site for the imidazopyridine derivatives **1b**, **1c** and **12c**. S1 was also preferred by T807, as predicted by Murugan *et al.* Structurally speaking, T807 is highly related with T808 (Figure 13), which was used as reference in our studies on SPR. However, SPR could not detect binding of T808 to tau aggregates.

The immobilization of tau aggregates on the sensor-chip surface could also be another factor. The amine coupling takes place between the carboxyl groups present in the dextran matrix and the amine groups of the protein (Figure 23). This implies that the bond formation between tau aggregates and SPR sensor-chip is random and it occurs in different positions alongside the aggregates. Therefore, it is possible that during the immobilization, the tau protofibril is bound facing downwards, this is, with the S1 facing the dextran matrix. In consequence, S1 binding-site would remain completely buried and unreachable for the compounds flowing over the matrix. This occurrence can be true and extended to the whole population of tau fibrils present in the solution, to a greater or lesser degree. Therefore, the actual number of available S1 binding sites would be fewer than what is reflected on the sensor-chip by the number of immobilized RUs of protein. In other words, the ratio *bound fibril: available binding site* will not be 1:1 but potentially lower. This theory would also explain the results observed with T808.

Given that SPR is a surface technique, where one component has limited freedom of movement and possibly a constrained orientation, mass transport effects, etc, caution is required when comparing SPR with solution-based assays such as the ThT competition assay, where aggregates are suspended in the solution and potentially free, allowing the imidazopyridines and imidazothiazoles to readily reach the binding site.

At this point, the question arises spontaneously: why did the benzothiazole derivative show such as high affinity on both SPR and ThT assays?

An insight can be found in the work of Murugan *et al.*, where the radiotracer ^{11}C -PBB3 (a highly conjugated benzothiazole, Figure 13) was tested on molecular docking and molecular dynamics. ^{11}C -PBB3 revealed itself to be the most potent radiotracer among the molecules assessed in their work. ^{11}C -PBB3 showed high affinity for all the proposed binding-sites. In fact, it has been reported that ^{11}C -PBB3 is able to compete and displace T807.¹⁷⁴ Thus, due to its ability to bind all four binding sites, if some sites are shielded because of the position of the filament on the gold surface, the benzothiazole would still be able to bind other available sites and this would explain the high affinity observed in SPR. In our results from ThT assays the ability of the benzothiazole analogue to bind tau fibrils is even more striking, as it is shown in Figure 25.

This is supported by the structural analogies between our benzothiazole derivative and ^{11}C -PBB3, both compounds bearing a highly conjugated benzothiazole moiety.

In conclusion, the poor signal of imidazopyridines in SPR could be explained by the lower number of accessible binding sites on tau fibrils after the immobilization process, and a relative low density of mature tau fibrils on the sensor-chip.

1.3.9 Haemolysis assay

Radiotracers are typically intravenously injected simple alcoholic formulations administered as a bolus injection to monitor the *in vivo* behaviour of a functional molecule. Thus, understanding their haemolytic potential can provide an initial assessment of their safety for intravenous injection.

According to the Guidance for *in vitro* haemolysis prepared by Johnson & Johnson and Novartis,¹⁷⁵ compounds in parental formulations with a haemolysis value <10% are considered non-haemolytic, whereas those > 25% are considered to be at risk of haemolysis.

The haemolytic toxicity of imidazopyridines and imidazothiazole derivatives was evaluated at concentrations ranging between 0.05 μ M to 50 μ M, although only graphs corresponding to the highest and lowest concentrations tested are shown. Red Blood Cells (RBC) were incubated with compounds for 1 hour prior to measuring the released haemoglobin (Figure 40).

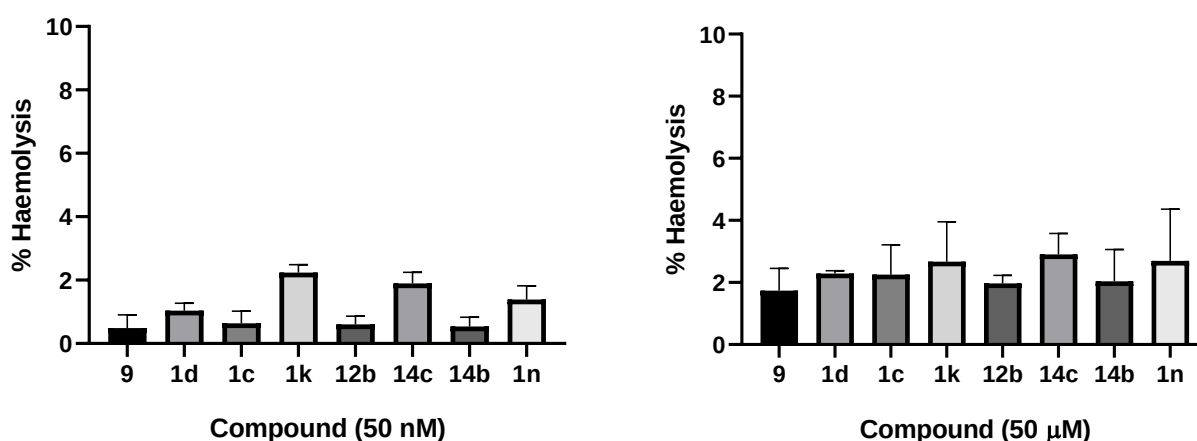


Figure 40 - Haemolysis (%) of selected imidazopyridines, imidazothiazole and pyridine derivatives, at 50 nM (left) and 50 μ M (right). Compounds were incubated with RBC for one hour at 37 $^{\circ}$ C. RBC treated with Triton 1% represented the 100% haemolysis.

All compounds produced haemolysis values below 10% even at high concentrations (50 μ M). Highest haemolytic values are found at 50 μ M, where compound **1n** and **1k** were the most haemolytic, with $3 \pm 1.1\%$ and $2.9 \pm 1\%$. At 50 μ M, the percentage of haemolysis among families was very similar, ranging from 1.9 to 3% (Figure 40). At 50 nanomolar, the differences between families were more noticeable. For example, **1k**, **1n** and **14c** were the most haemolytic ones, being ~2-fold more cytotoxic than the rest of analogues.

For PET imaging studies, the mass doses can range between 0.045 and 1.26 μ g.⁶⁸ Assuming the highest dose, 1.26 μ g, we can calculate the number of moles contained in such dose. For example, compound **1c**, whose molecular weight (MW) is 354.33 g/mol, would be dosed at 3.56 nmol (Equation 1.3)

$$\text{nmoles of compound} = \frac{\text{mass dose } (\mu\text{g}) \times 10^3}{\text{MW}}$$

Equation 1.3

The corresponding molarity of this amount of **1c** would be 3.56 nM. Assuming for example 1 mL of formulation, the administered concentration would be 0.0036 nM:

This concentration is quite below the 50 nM tested in the *ex vivo* haemolysis assay, where **1c** produced $0.63 \pm 0.3\%$ haemolysis. Another example could be the cold ligand **1n** (MW = 256.28 g/mol), which under the same assumption it would be dosed at 0.005 nM, 1000-fold less concentrated than 50 nM sample, whose percentage of haemolysis was 1.39 %. Both values are far away from the 10% haemolysis considered as the limit of non-haemolytic behaviour as established in the guidance for parenteral excipients.¹⁷⁵ However, haemolysis studies need to be repeated with the final formulation injected parentally, as excipients utilised to solubilise tracers might also add to the overall haemolysis risk. Based on our results, compounds developed and tested are non – haemolytic at concentrations that are likely to be utilised for PET imaging.

1.4 CONCLUSIONS

A new family of imidazo[1,2-*a*]pyridine and imidazo[2,1-*b*]thiazole derivatives has been successfully synthesised and characterised.

These families were designed for their potential application as selective tau radiotracers. The techniques employed for the assessment of the binding properties were Surface Plasmon Resonance and thioflavin T competition assay. Results obtained in the ThT assay demonstrated the ability of a series of compounds to bind tau aggregates, in contrast with poor outcomes from SPR experiments.

Molecular docking studies with tau fibrils supported the ThT assay results and gave important insights about where these compounds could preferably bind to the tau protofibrils. Interestingly, all the compounds clearly preferred the high-affinity binding site S1 over the rest of available sites.

It can be concluded that SPR is a suitable technique for drug discovery, however, when employed for the detection of potential radiotracers under the tested conditions, there is a high risk of false negatives. Further improvements should be done in this respect in order to obtain consistent results compared to the standard *in vitro* assays commonly employed to screen for the binding affinity of radiotracers.

The *ex vivo* haemolysis studies revealed that imidazo[1,2-*a*]pyridines and imidazo[2,1-*b*]thiazoles are not cytotoxic and can potentially be formulated for intravenous administration.

New synthetic routes suitable for radiosynthesis are currently being developed, as well as the determination of the pharmacokinetic profile of the most promising compounds. In addition to our studies, PET complementary experiments are planned to fully assess possibilities of these compounds as new tau radiotracers, which will enable the early detection of Alzheimer's disease.

1.5 MATERIALS AND METHODS

1.5.1 Materials and Equipment

Table 9 - Summary table of reagents utilised.

Chemical Name	Abbreviation	Purity %	Company
Heparin sodium salt porcine intestinal mucosa	Hep	IU>100/mg	Alfa Aesar
Insulin from porcine pancreas	Insulin	≥85% (GE), ~24 IU/mg	Sigma
Thioflavin T	ThT	>98%	Sigma
Sodium phosphate heptahydrate	Na ₃ PO ₄	ACS grade	Fisher Scientific
Sodium phosphate dibasic monohydrate	Na ₂ HPO ₄	ACS grade	Fisher Scientific
Glycine	-	Molecular biology grade	Fisher Scientific
Hydrochloric acid	HCl	32%	VWR
Tau ⁴⁴¹ protein	Tau	Recombinant human Tau – liquid > 90% purity	Abcam UK
Tau ⁴⁴¹ protein	Tau	Recombinant human Tau- lyophilised powder > 90% purity	rPeptide, GA, US
Dimethyl Sulfoxide	DMSO	Molecular Biology grade	Thermo Fisher
Sodium chloride	NaCl	ACS grade	Sigma
2-Amino-2-(hydroxymethyl)-1,3-propanediol	Tris base	Molecular biology grade	Fisher Scientific
Dithiothreitol	DTT	Molecular biology grade	Fisher Scientific
Triton X100	Triton	Molecular biology grade	Sigma

All reagents employed as starting materials were purchased from commercial suppliers with high purity and were used without further purification. Solvents were obtained from Sigma-Aldrich, Fisher Scientific, Acros and Scharlab and were employed with no additional pre-treatment. Reactions were monitored by Thin Layer Chromatography (TLC) whose spots were visualized by an UV lamp at 254 and 356 nm. In certain occasions, a solution of Vanillin or Phosphomolybdic acid, with subsequent heating, was used for better traceability of the reaction course. When needed, the purification of the synthesised compounds was performed by flash column chromatography with Silica Gel Merck-60 (230-400 mesh). All plastics for experiments and 96- well plates were obtained from Sarstedt Ltd. (Leicester, UK) or ThermoFisher Scientific. UV-VIS spectrometry assays were performed in a Multiskan Spectrophotometer (ThermoFisher Scientific).

Solvents were obtained from Sigma-Aldrich Chemical Co. (Dorset, UK) or Fisher Scientific (Loughborough, UK) with purity >99.0%. 96- well black plates were purchased from ThermoFisher Scientific (Loughborough, UK). Fluorimetry assays were performed with a SpectraMax i3x Fluorometer (Molecular Devices, ROM v16b45, UK).

NMR analysis

Nuclear magnetic resonance (NMR) spectra were recorded in deuterated solvents on Bruker AVANCE-300, Varian INOVA -300 Varian INOVA -400 and Varian INOVA-500 spectrometers. ^{13}C -NMR were registered with complete proton decoupling. The chemical shifts measured are reported in δ (ppm) and the residual signal of the solvent was used as the internal calibration standard. The multiplicity of the signals is reported as follows: s = singlet, d = doublet, t = triplet, q= quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, ddt = doublet of doublet of triplets, br = broad signal. The coupling constant J is reported in hertz (Hz).

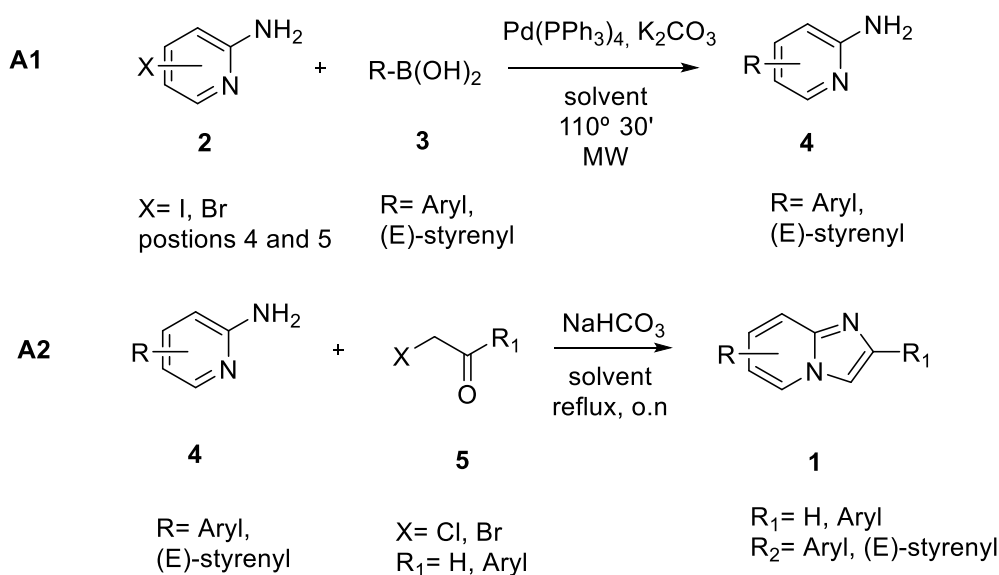
LC-MS analysis

HPLC-MS analyses were performed on a Waters (2695 HPLC system) apparatus, equipped with a quaternary pump and photodiode array detector (PDA), using a SunfireTM column (C18 stationary phase, 3.5 μm particle size, 4.6 $\times$ 50 mm). Mobile phase consisted of acetonitrile +0.08% formic acid (v/v) and H_2O +0.01% formic acid (v/v). The solvent gradient is specified for each compound in the following section. The molecular weight was determined by the nominal mass obtained from a single quadrupole mass spectrometer coupled to the HPLC system operating in positive electrospray ionization (ES^+).

1.5.2 Synthesis of Imidazo[1,2-*a*]pyridine and Imidazo[2,1-*b*]thiazole Derivatives

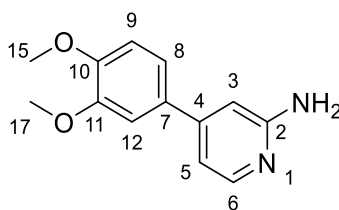
Experimental procedures A-C for the synthesis of imidazo[1,2-*a*]pyridine derivatives **1** and **9**.

Experimental procedure A: Suzuki's cross coupling reaction (**1**) and heterocyclization (**2**):

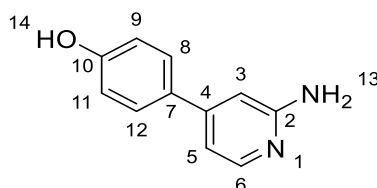


Suzuki's reaction: in a microwave vial, boronic acid (**3**, 1.2 eq) and K_2CO_3 (1.3 eq) were dissolved in 5 ml of DME/ H_2O (4:1). $\text{Pd}(\text{PPh}_3)_4$ (2 mmol%), was added and the mixture was purged with argon for one minute. Then, 4- or 5-halo-2-aminopyridine (**2**, 1 eq) was incorporated. The tube was sealed with a pressure cap and irradiated in a microwave reactor. After cooling to room temperature, the solvent was evaporated. The crude product was purified by flash column chromatography to afford substituted 2-aminopyridine-based intermediates, **4**.

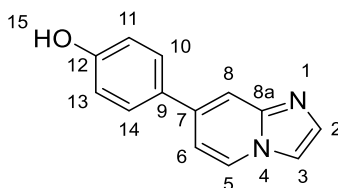
Heterocyclization: substituted 2-aminopyridine-based intermediates (**4**, 1 equiv), NaHCO_3 (0.1 g/5 ml EtOH) and 4 ml of EtOH were added into a sealed tube. Then, an α -halo-ketone (**5**, 1.5-2 eq) was incorporated to the reaction mixture. The tube was sealed and stirred at reflux overnight. After cooling to room temperature, the solvent was evaporated. The solid was suspended in concentrated K_2CO_3 (aq) and extracted with ethyl acetate (x3). The organic phases were dried over anhydrous MgSO_4 , filtered and evaporated under high vacuum to afford imidazo[1,2-*a*]pyridine derivatives, **1**.



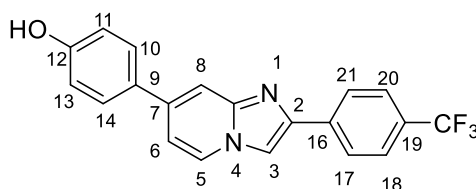
4-(3,4-dimethoxyphenyl)pyridin-2-amine, 4a : According to experimental procedure A, 3,4-dimethoxyphenylboronic acid (126 mg, 0.693 mmol), 4-bromo-2-aminopyridine (80 mg, 0.462 mmol), Pd(PPh₃)₄ (27 mg, 5mmol%) and K₂CO₃ (128 mg, 0.924 mmol) were dissolved in 4 mL of H₂O/EtOH/Toluene (1:2:8) mixture. The crude product was purified by flash column chromatography (DCM: MeOH) to afford **4a** (white solid, 100 mg, 93% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.92 (d, *J* = 5.4 Hz, 1H, **6**), 7.19 (d, *J* = 7.2 Hz, 2H, **8**, **12**), 7.04 (d, *J* = 8.8 Hz, 1H, **9**), 6.78 (dd, *J* = 5.4, 1.7 Hz, 1H, **5**), 6.69 (d, *J* = 1.7 Hz, 1H, **3**), 3.83 (s, 3H, -OCH₃), 3.79 (s, 3H, -OCH₃). ¹³C NMR (101 MHz, DMSO) δ 160.3 (C2), 149.4 (-C10-), 149.0 (C11), 148.2 (C6), 148.0 (C7 or C4), 130.9 (C7 or C4), 118.8 (C8), 112.0 (C9), 109.9 (C12), 109.9 (C5), 104.5 (C3), 55.5 (-OCH₃), 55.57 (-OCH₃). **HPLC-MS (ES⁺)**: CH₃CN/H₂O 5:95 to 95:5 (5 min), RT = 4.01 min, [M+H⁺] = 231.



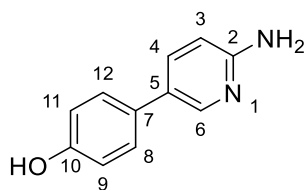
4-(2-aminopyridin-4-yl)phenol 4b: Experimental procedure A was used with 4-hydroxyphenylboronic acid (38 mg, 0.277 mmol) and K₂CO₃ (40 mg, 0.300 mmol), Pd(PPh₃)₄ (4 mg, 2 mmol%) and 4-bromo-2-aminopyridine (0.231 mmol) dissolved in 5 ml of dimethoxyethane and H₂O (4:1). The crude product was purified in DCM: MeOH to afford 36 mg of **4b** in 84% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.71 (s, 1H, -OH), 7.90 (d, *J* = 5.4 Hz, 1H, **6**), 7.48 (d, *J* = 8.6 Hz, 2H, **9**, **11**), 6.86 (d, *J* = 8.6 Hz, 2H, **12**, **8**), 6.71 (dd, *J* = 5.4, 1.7 Hz, 1H, **5**), 6.64 (s, 1H, **3**), 5.86 (s, 2H, -NH₂). ¹³C NMR (101 MHz, DMSO) δ 160.3 (C2), 158.1 (C10), 148.2 (C6), 148.0 (C7), 128.8 (C4), 127.5 (C9 y 11), 115.7 (C12, C8), 109.6 (C5), 104.0 (C3). **HPLC-MS (ES⁺)**: CH₃CN/H₂O 2: 98 to 30:70 (5 min), RT = 1.66 min, [M+H⁺] = 187.



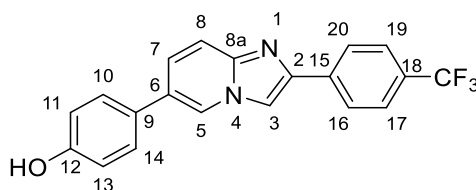
4-(imidazo[1,2-*a*]pyridin-7-yl)phenol 1a: According to procedure A, 4-(4-hydroxyphenyl)-2-aminopyridine (30 mg, 0.161 mmol), NaHCO₃ (83 mg, 0.1g/5ml EtOH) and 2-chloroacetaldehyde (20 μ L, 0.322 mmol) were stirred in 4 ml of EtOH to afford **1a** (25 mg, 74 % yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.53 (d, *J* = 7.2 Hz, 1H, **5**), 7.89 (s, 1H, **3**), 7.70 (s, 1H, **8**), 7.61 (d, *J* = 8.2 Hz, 2H, **11,13**), 7.54 (s, 1H, **2**), 7.20 (d, *J* = 6.9 Hz, 1H, **6**), 6.86 (d, *J* = 8.3 Hz, 2H, **10,14**). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 158.6 (C12), 145.2 (C8a), 136.2 (C7), 133.5 (C2), 127.9 (C9), 127.6 (C10, C14), 126.7 (C5), 116.0 (C11, C13), 112.4 (C3), 111.3 (C8), 111.0 (C6). HPLC-MS (ES⁺): CH₃CN/H₂O 2:98 to 95:5 (5 min), RT = 3.95 min, [M+H⁺] = 211.



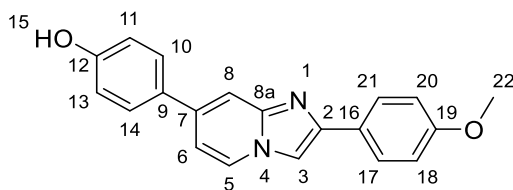
4-(2-(4-(trifluoromethyl)phenyl)imidazo[1,2-*a*]pyridin-7-yl)phenol, 1b: Following experimental procedure A, 4-(4-hydroxyphenyl)-2-aminopyridine **4b** (30 mg, 0.161 mmol), NaHCO₃ (83 mg, 0.1g/5 ml EtOH) and 4 ml of EtOH were combined with 2-bromo-1-(4-(trifluoromethyl)phenyl)ethan-1-one (30 mg, 0.322 mmol). The crude product was purified by flash column chromatography DCM: MeOH to give 31 mg of **1b** in 54% yield. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.74 (s, 1H, -OH), 8.56 (d, *J* = 7.1 Hz, 1H, **5**), 8.52 (s, 1H, **3**), 8.19 (d, *J* = 8.1 Hz, 2H, **17,21**), 7.80 (d, *J* = 6.4 Hz, 3H, **8, 18,20**), 7.68 (d, *J* = 8.6 Hz, 2H, **10,14**), 7.26 (dd, *J* = 7.2, 1.9 Hz, 1H, **6**), 6.89 (d, *J* = 8.6 Hz, 2H, **11, 13**). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 158.4 (C12), 146.2 (C8a), 143.6 (C2), 138.4 (C16), 137.5 (C7), 128.7 (C9), 128.2 (C10, C14), 127.3 (C5), 126.4 (C17, C21), 126.1 (q, *J*_{C-F} = 3.78 Hz, -CHCCF₃), 124.8 (q, *J*_{C-F} = 271 Hz, -CF₃), 116.3 (C11, C13), 112.2 (C8), 111.8 (C6), 110.5 (C3). HPLC-MS (ES⁺): CH₃CN/H₂O 30: 70 to 95:5 (10 min), RT = 1.96 minutes, [M+H⁺] = 355.



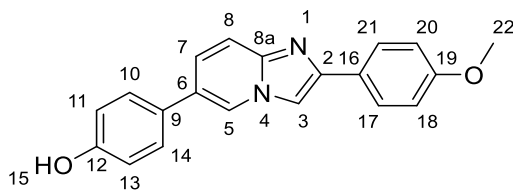
4-(6-aminopyridin-3-yl)phenol 4c: Following experimental procedure A, 4-hydroxyphenylboronic acid (138 mg, 0.999 mmol), NaHCO_3 (83 mg, 0.154 mmol), $\text{Pd(PPh}_3)_4$ (21 mg, 2 mmol%) and 5-iodo-2-aminopyridine (200 mg, 0.909 mmol) were dissolved in 9 ml of 1,4-dioxane / H_2O (3:1). Crude product was purified by flash column chromatography (DCM: MeOH) to afford 71 mg of **4c** as a yellow solid (42 % yield). $^1\text{H NMR}$ (500 MHz, $\text{DMSO-}d_6$) δ 9.38 (s, 1H, -OH), 8.13 (d, J = 2.4 Hz, 1H, **6**), 7.59 (dd, J = 8.6, 2.6 Hz, 1H, **4**), 7.35 (d, J = 8.6 Hz, 2H, **9**, **13**), 6.79 (d, J = 8.5 Hz, 1H, **10**, **12**), 6.49 (d, J = 8.6 Hz, 1H, **3**), 5.90 (s, 2H, $-\text{NH}_2$). $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO-}d_6$) δ 158.4 (C2), 156.1 (C10), 144.8 (C6), 134.9 (C4), 128.9 (C7), 126.5 (C12 and C8), 124.2 (C5), 115.6 (C11 and C9), 107.9 (C3). **HPLC-MS (ES $^+$):** $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 2:98 to 30:70 (5 min), RT = 1.55 min, $[\text{M}+\text{H}^+] = 187$.



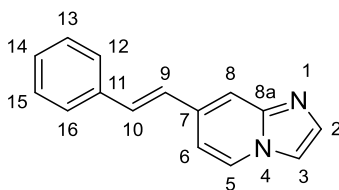
4-(2-(4-(trifluoromethyl)phenyl)imidazo[1,2-a]pyridin-6-yl)phenol, 1c: According to experimental procedure A, 5-(4-hydroxyphenyl)-2-aminopyridine **4c** (50 mg, 0.268 mmol), NaHCO_3 (0.1g/5ml EtOH) and 2-bromo-1-(4-(trifluoromethyl)phenyl)ethan-1-one (215 mg, 0.805 mmol, 3.5 eq) were dissolved in 4 ml of EtOH. Crude product was purified by flash column chromatography to afford 31 mg of **1c** (31% yield). $^1\text{H NMR}$ (500 MHz, $\text{DMSO-}d_6$) δ 9.65 (s, 1H, -OH), 8.77 (dd, J = 1.9, 1.0 Hz, 1H, **5**), 8.52 (s, 1H, **3**), 8.20 (d, J = 7.8 Hz, 2H, **20**, **16**), 7.80 (d, J = 7.9 Hz, 2H, **19**, **17**), 7.65 (d, J = 9.4 Hz, 1H, **8**), 7.58 (dd, J = 9.4, 1.8 Hz, 1H, **7**), 7.56 - 7.53 (m, 2H, **18**, **22**), 6.89 (d, J = 8.6 Hz, 2H, **11**, **13**). $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO-}d_6$) δ 157.4 (C12), 144.1 (C8a), 142.9 (C2), 137.9 (C15), 127.7 (C10, C14), 127.1 (C9), 126.0 (C16, 20), 125.7 (C6), 125.6 (q, $J_{\text{C-F}}$ = 3.8 Hz, CH-CCF_3), 125.6 (C7), 124.3 (q, $J_{\text{C-F}}$ = 272.5 Hz, CF_3), 122.9 (C5), 116.7 (C8), 115.9 (C11, C13), 110.8 (C3). **HPLC-MS (ES $^+$):** $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 30:70 to 95:5 (10 min), RT = 2.91 min, $[\text{M}+\text{H}^+] = 355$.



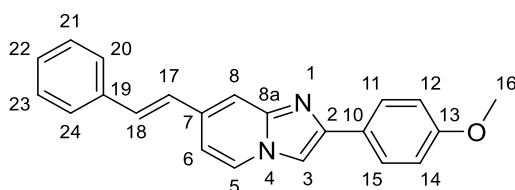
4-(2-(4-methoxyphenyl)imidazo[1,2-*a*]pyridin-7-yl)phenol, **1d:** According to experimental procedure A, 4-(2-aminopyridin-4-yl)phenol **4b** (69 mg, 0.386 mmol) and 2-bromo-1-(4-(methoxy)phenyl)ethan-1-one (103mg, 0.464 mmol, 1.2eq) were dissolved and refluxed in acetone (5 ml) over night. After cooling at room temperature, crude product formed a white precipitate that was separated by filtration (0.22uM) to afford **1d** (82 mg, 67% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.79 (s, 1H, -OH), 8.58 (d, *J* = 7.1 Hz, 1H, **5**), 8.33 (s, 1H, **3**), 7.89 (d, *J* = 8.7 Hz, 2H, **18,20**), 7.77 (s, 1H, **8**), 7.69 (d, *J* = 8.7 Hz, 2H, **11, 13**), 7.34 (d, *J* = 7.1 Hz, 1H, **6**), 7.05 (d, *J* = 8.8 Hz, 2H, **17, 21**), 6.91 (d, *J* = 8.6 Hz, 2H, **10, 14**), 3.81 (s, 3H, -OCH₃). ¹³C NMR (101 MHz, DMSO) δ 159.4 (C19), 158.2 (C12), 144.2 (C8a), 142.7 (C2), 138.2 (C7), 127.9 (C9), 127.9 (C11, C13), 127.0 (C5), 127.0 (C18, C20), 124.7 (C16), 115.9 (C10, C14), 114.3 (C17, C21), 112.1 (C6), 109.6 (C8), 108.0 (C3) 55.2 (-CH₃). HPLC-MS (ES⁺): CH₃CN/H₂O 15:85 to 95:5 (5 min), RT = 4.09 min, [M+H⁺] = 317.



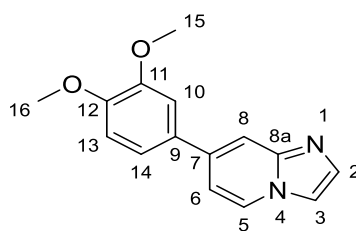
4-(2-(4-methoxyphenyl)imidazo[1,2-*a*]pyridin-6-yl)phenol, **1e:** According to experimental procedure A, 4-(6-aminopyridin-3-yl)phenol **4c** (70 mg, 0.375 mmol) and 2-bromo-1-(4-(methoxy)phenyl)ethan-1-one (103mg, 0.451 mmol, 1.2eq) were dissolved in acetone (5 ml). After cooling at room temperature, crude product formed a precipitated that was recovered by filtration (0.22uM) to give **1e** (71 mg, 60% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.64 (s, 1H, -OH), 8.76 (s, 1H, **5**), 8.30 (s, 1H, **3**), 7.90 (d, *J* = 8.7 Hz, 2H, **17, 21**), 7.61 (m, 2H, **7, 8**), 7.54 (d, *J* = 8.5 Hz, 2H, **10, 14**), 7.04 (d, *J* = 8.8 Hz, 2H, **18, 20**), 6.89 (d, *J* = 8.6 Hz, 2H, **11, 13**), 3.81 (s, 3H, -OCH₃). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 159.2 (C19), 157.4 (C12), 149.6 (C2) 143.1 (C8a), 127.7 (C10, C14), 127.0 (C9 or C16), 126.9 (C18, C20), 125.8 (C7), 125.7 (C9 or C16), 122.8 (C5), 115.8 (C11, C13), 115.6 (C8), 114.2 (C17 and C21), 108.5 (C3), 55.1 (CH₃). HPLC-MS (ES⁺): CH₃CN/H₂O 15:85 to 95:5 (5 min), RT = 4.13 min, [M+H⁺] = 317.



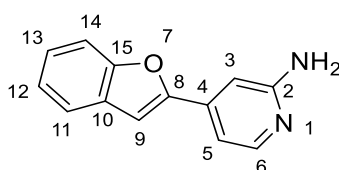
(E)-7-styrylimidazo[1,2-*a*]pyridine, **1f:** According to experimental procedure A, (*E*)-4-styrylpyridine-2-amine (50 mg, 0.254 mmol) and 2-chloroacetaldehyde (~50 wt. % in H₂O, 19 μ L, 0.305 mmol) were dissolved in acetone forming a suspension. Compound **1f** was obtained) as a brown solid without further purification (53mg, 94% yield). ¹H NMR (400 MHz, Chloroform-*d*) δ 8.00 (d, *J* = 7.1 Hz, 1H, **5**), 7.54 (apparent d, *J* = 8.0 Hz, 2H, **2, 8**), 7.46 (apparent d, *J* = 8.8 Hz, 3H, **3, 13, 15**), 7.31 (apparent t, *J* = 7.5 Hz, 2H, **12, 16**), 7.26 – 7.17 (m, 2H, **14**), 7.03 (dd, *J* = 16.5, 6.5 Hz, 3H, **6, 9, 10**). ¹³C NMR (101 MHz, CDCl₃) δ 145.8 (C8a), 136.6 (C11), 134.3 (C7), 134.1 (C2) 130.6 (C10 or C9), 128.9 (C16, C12), 128.3 (C14), 126.8 (C9 or C10, C13 and C15), 125.5 (C5), 115.7 (C8), 112.6 (C3), 110.2 (C6). **HPLC-MS (ES⁺)**: CH₃CN/H₂O 5:95 to 95:5 (5 min), RT = 4.59 min, [M+H⁺] = 221.



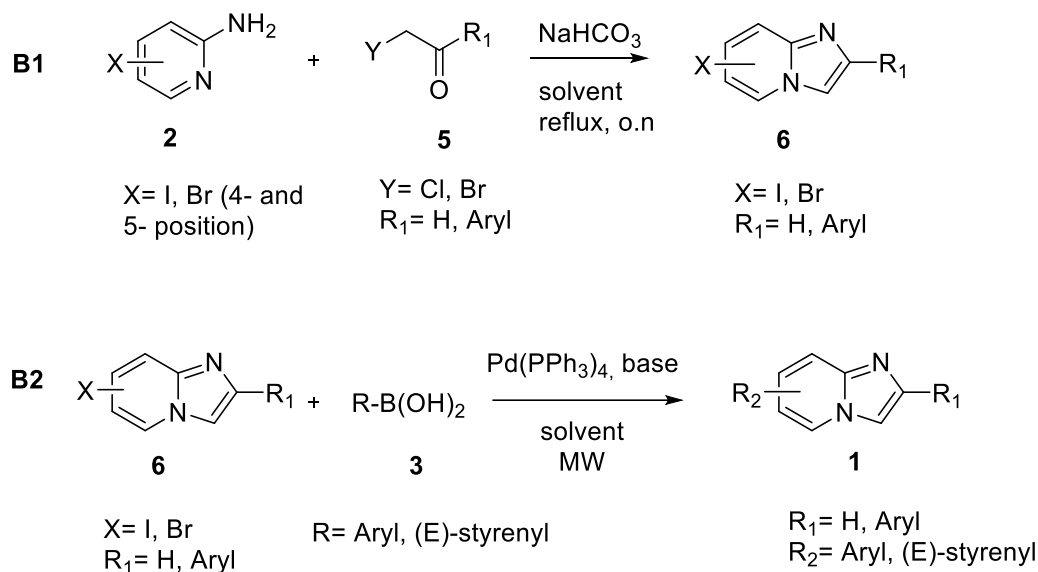
(E)-2-(4-methoxyphenyl)-7-styrylimidazo[1,2-*a*]pyridine, **1g:** According to experimental procedure A, to a mixture of (*E*)-4-styrylpyridine-2-amine (63 mg, 0.323 mmol) in 5 mL of acetone, 2-bromo-1-(4-(trifluoromethyl)phenyl)ethan-1-one (88 mg, 0.387 mmol,) was added. Crude product was filtrated in nylon filter (0.22 μ M) to afford 60mg of **1g** (47% yield). ¹H NMR (500 MHz, Chloroform-*d*) δ 7.98 (d, *J* = 7.0 Hz, 1H, **5**), 7.73 (d, *J* = 8.7 Hz, 2H, **11, 13**), 7.67 (s, 1H, **3**), 7.44 (s, 1H, **8**), 7.39 (d, *J* = 7.1 Hz, 2H, **20, 24**), 7.22 (t, *J* = 7.6 Hz, 2H, **21, 23**), 7.14 (t, *J* = 7.3 Hz, 1H, **22**), 7.01 (d, *J* = 16.3 Hz, 1H, **18**), 6.98 - 6.92 (m, 2H, **17, 6**), 6.81 (d, *J* = 8.8 Hz, 2H, **10, 14**), 3.69 (s, 3H, -OCH₃). ¹³C NMR (126 MHz, CDCl₃) δ 159.4 (C12), 145.5 (C8a), 145.3 (C2), 136.2 (C19), 134.3 (C7), 130.2 (C18), 128.5 (C21, C23), 127.9 (C22), 127.0 (C13, C11), 126.4 (C24, C20), 126.45 (C17), 125.7 (C9), 125.2 (C5), 114.3 (C8), 113.9 (C14, C10), 109.9 (C6), 107.5 (C3), 55.1 (-OCH₃). **HPLC-MS (ES⁺)**: CH₃CN/H₂O 15:85 to 95:5 (5 min), RT = 4.81 min, [M+H⁺] = 327.



7-(3,4-dimethoxyphenyl)imidazo[1,2-*a*]pyridine, **1h:** According to experimental procedure A, 4-(3,4-dimethoxyphenyl)pyridin-2-amine **4a** (50 mg 0.217 mmol), 2-chloroacetaldehyde (27 μ L, 0.434 mmol) and NaHCO_3 (50 mg, 0.1g/5ml EtOH) were dissolved in 2.5 ml of EtOH. The organic phases were dried over anhydrous MgSO_4 , filtered and evaporated under high vacuum to afford **1h** as a brown oil (52 mg, 94 % yield). $^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 8.13 (d, J = 7.1 Hz, 1H, **5**), 7.76 (s, 1H, **8**), 7.63 (s, 1H, **3**), 7.55 (s, 1H, **2**), 7.20 (dd, J = 8.3, 2.2 Hz, 1H, **14**), 7.15 (d, J = 2.2 Hz, 1H, **10**), 7.04 (dd, J = 7.1, 1.8 Hz, 1H, **6**), 6.95 (d, J = 8.3 Hz, 1H, **13**), 3.95 (s, 3H, -OCH₃), 3.92 (s, 3H, -OCH₃). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 149.5 (C11, C12), 146.1 (C8a), 137.4 (C7), 134.3 (C2), 131.6 (C9), 125.6 (C5), 119.3 (C14), 113.9 (C8), 112.3 (C6), 111.9 (C3), 111.7 (C13), 109.9 (C10), 56.1 (-OCH₃ x2). **HPLC-MS (ES⁺)**: $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ gradient 10:90 to 95:5 (5 min), RT = 1.46 min, $[\text{M}+\text{H}^+] = 255$.

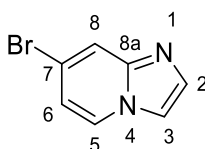


4-(benzofuran-2-yl)pyridin-2-amine, **4d:** According to experimental procedure A, benzofuran-2-yl boronic acid (0.65 mg, 0.405 mmol) and K_2CO_3 (52 mg 0.375 mmol,) were dissolved in 5 ml of DME/ H_2O (4:1). $\text{Pd}(\text{PPh}_3)_4$, (6 mg, 2 mmol%), was added followed by 4-bromo-2-aminopyridine (50 mg, 0.281 mmol) was incorporated. The crude product was purified by column chromatography (DCM: MeOH) to give 50 mg of **4d** in 82% yield. $^1\text{H NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ 8.00 (dd, J = 5.3, 0.8 Hz, 1H, **6**), 7.73 – 7.66 (m, 1H, **11**), 7.64 (dd, J = 8.3, 0.9 Hz, 1H, **14**), 7.50 (d, J = 1.0 Hz, 1H, **9**), 7.37 (ddd, J = 8.4, 7.2, 1.3 Hz, 1H, **13**), 7.29 (ddd, J = 7.9, 7.4, 1.0 Hz, 1H, **12**), 6.98 (dd, J = 5.3, 1.6 Hz, 1H, **5**), 6.93 (dd, J = 1.6, 0.8 Hz, 1H, **3**), 6.13 (s, 2H, -NH₂). $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO}-d_6$) δ 160.4 (C15), 154.3 (C10), 153.6 (C4), 148.6 (C6), 137.3 (C2), 128.3 (C8), 125.4 (C13), 123.4 (C12), 121.6 (C11), 111.2 (C14), 107.4 (C5), 104.4 (-C9), 102.3 (C3). **HPLC-MS (ES⁺)**: $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 15:85 to 95:5 (5 min), RT = 1.42 minutes, $[\text{M}+\text{H}^+] = 211$.

Experimental procedure B: Heterocyclization (B1) and Suzuki's cross coupling reaction (B2)

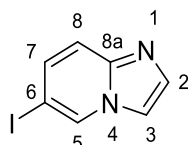
Heterocyclization (B1): halo-2-aminopyridine (**2**, 1 eq) and NaHCO_3 (0.1g/5ml if EtOH was used) were added into a sealed tube. The solvent used was either EtOH or acetone. Then, an α -halo-ketone (**5**, 1.5 to 2 eq) was added to the reaction mixture. The tube was sealed and stirred at reflux overnight. After cooling to room temperature, the solvent was evaporated. The solid was suspended in concentrated K_2CO_3 (aq) and extracted with ethyl acetate (x3). The organic phases were dried over anhydrous MgSO_4 , filtered and evaporated under high vacuum to afford imidazo[1,2-*a*]pyridine intermediates, **6**.

Suzuki's reaction (B2): in a microwave vial, boronic acid (**3**, 1.2 eq) and NaHCO_3 or K_2CO_3 (1.1 or 1.3 eq, respectively) were dissolved in 1,4-dioxane / H_2O (3:1) or DME/ H_2O (4:1). $\text{Pd}(\text{PPh}_3)_4$, (2 mmol%), was added and the mixture was purged with argon for one minute. Finally, halo-imidazo[1,2-*a*]pyridine (**6**, 1 eq) was added. The tube was sealed with a pressure cap and irradiated in a microwave reactor at 130 °C for 30'. After cooling at room temperature, the solvent was evaporated and the crude product was purified by flash column chromatography to afford **imidazo[1,2-*a*]pyridine derivatives, 1**.

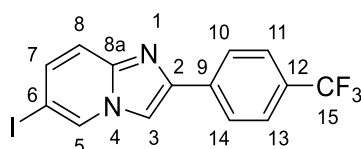


7-bromoimidazo[1,2-*a*]pyridine, 6a: According to experimental procedure B, a solution of 4-bromopyridin-2-amine (0.2 g, 1.15 mmol), and 2-chloroacetaldehyde (~50 wt. % in H_2O , 0.14 mL, 2.31 mmol) and NaHCO_3 (50 mg, 0.59 mmol) in ethanol (2.5 mL) was refluxed overnight. Crude product

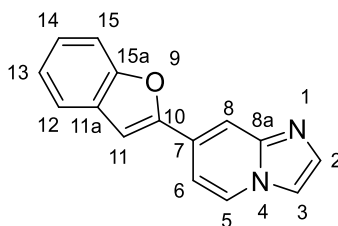
was purified by flash chromatography with a gradient (DCM: MeOH) give **6a** (196mg, 86%) as a brown solid. Spectral data are in accordance with those found in the litterarure.¹⁷⁶ **¹H NMR** (400 MHz, CDCl₃) δ 7.99 (d, J = 7.1 Hz, 1H, **5**), 7.81 (s, 1H, **3**), 7.59 (s, 1H, **2**), 7.56 (s, 1H, **8**), 6.88 (dd, J = 7.2, 1.6 Hz, 1H, **6**). **¹³C NMR** (101 MHz, CDCl₃) δ 145.5 (C8a), 134.2 (C7), 126.0 (C2), 120.0 (C5), 118.2 (C8), 116.5 (C3), 112.7 (C6).



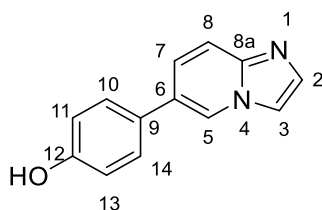
6-iodoimidazo[1,2-*a*]pyridine, 6b: According to experimental procedure B, 2-chloroacetaldehyde (50 percent wt. aqueous solution, 0.58 mL, 2 eq, 9.08 mmol,) was added to a reaction mixture of 5-iodo-2-aminopyridine (1g, 4.54mmol), NaHCO₃ (100 mg) and ethanol (5 ml). The final product was obtained without further purification as a brown solid (1g, 90% yield). Spectral data are in accordance with those found in the litterarure.¹¹⁹ **¹H NMR** (400 MHz, Methanol-*d*₄) δ 8.80 (dd, J = 1.7, 0.9 Hz, 1H, **5**), 7.81 (s, 1H, **3**), 7.53 (s, 1H, **2**), 7.48 (dd, J = 9.4, 1.7 Hz, 1H, **7**), 7.37 (d, J = 9.4 Hz, 1H, **8**). **¹³C NMR** (101 MHz, Methanol-*d*₄) δ 145.0 (C8a), 134.6 (C7), 133.6 (C2), 133.2 (C5), 118.4 (C8), 114.3 (C3), 76.2 (C6). **HPLC-MS (ES⁺):** CH₃CN/H₂O 5:95 to 95:5 (5 min), RT = 0.69 min, [M+H⁺] = 245.



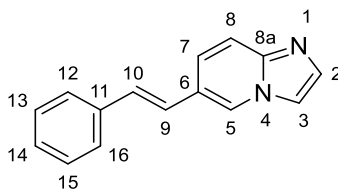
6-iodo-2-(4-(trifluoromethyl)phenyl)imidazo[1,2-*a*]pyridine, 6c: Following experimental procedure B (first step), 5-iodo-2-aminopyridine (41 mg, 0.136 mmol) was added to a mixture of 2-bromo-1-(4-(trifluoromethyl)phenyl)ethan-1-one (60 mg, 0.204 mmol,) and NaHCO₃ (80 mg, 0.99 mmol) in EtOH (4 ml). The crude product was purified by flash column chromatography (DCM: MeOH) to give 25 mg of **6c** in 47% yield. **¹H NMR** (400 MHz, Chloroform-*d*) δ 8.40 (s, 1H, **5**), 8.03 (d, J = 8.0 Hz, 2H, **10**, **14**), 7.85 (s, 1H, **3**), 7.68 (d, J = 8.1 Hz, 2H, **11**, **13**), 7.45 (d, J = 9.4 Hz, 1H, **8**), 7.37 (dd, J = 9.5, 1.6 Hz, 1H, **7**). **¹³C NMR** (101 MHz, CDCl₃) δ 144.6 (C2), 144.4 (C8a), 136.6 (C10), 133.4 (C5), 126.3 (C14, C10), 125.9 (q, J_{C-F} = 273 Hz, -CF₃), 124.3 (q, J_{C-F} = 3.81 Hz, CH-CCF₃), 118.7 (C8), 108.7 (C3), 75.8 (C6). **HPLC-MS (ES⁺):** CH₃CN/H₂O 40:60 to 95:5 (10 min), RT = 5.57, [M+H⁺] = 389.



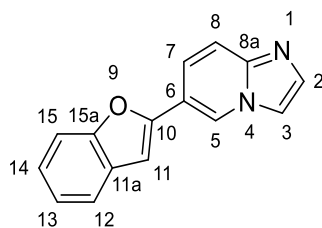
7-(benzofuran-2-yl)imidazo[1,2-*a*]pyridine, **1i** : According to experimental procedure B, benzofuran-2-yl boronic acid **4d** (62mg, 0.106 mmol) and K₂CO₃ (46 mg, 0.106 mmol) were dissolved in 3 ml of DME/H₂O (4:1) in a microwave tube. Pd(PPh₃)₄, (6 mg, 2mmol%), followed by 7-bromoimidazo[1,2-*a*]pyridine (50 mg, 0.082 mmol). The crude product was purified by flash column chromatography to yield **1i** (42 mg 70% yield). ¹H NMR (400 MHz, CDCl₃) δ 8.09 - 8.00 (m, 2H, **5**, **11**), 7.62 (s, 1H, **3**), 7.55 - 7.43 (m, 3H, **12**, **15**, **2**), 7.29 - 7.21 (m, 1H, **14**), 7.20 - 7.11 (m, 2H, **6**, **13**), 7.01 (s, 1H, **8**). ¹³C NMR (101 MHz, CDCl₃) δ 155.2 (C15a or C10), 153.8 (C15a or C10), 145.5 (C8a), 135.0 (C2), 129.0 (C11a or C7), 126.7 (C11a or C7), 125.8 (C5), 125.1 (C14), 123.3 (C13), 121.2 (C12), 112.96 (C3), 112.9 (C11), 111.4 (C15), 109.7 (C6), 103.2 (C8). **HPLC-MS (ES⁺)**: CH₃CN/H₂O 15:85 to 95:5 (5 min), RT = 1.39 min, [M+H⁺] = 235.



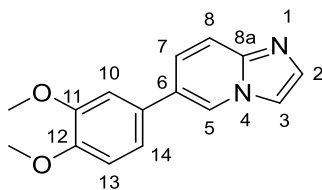
4-(imidazo[1,2-*a*]pyridin-6-yl)phenol **1j**: According to experimental procedure B, 4-hydroxyphenylboronic acid (20 mg, 0.147 mmol) and K₂CO₃ (22mg, 0.160 mmol) were dissolved in 3 ml of DME/H₂O (4:1). Pd(PPh₃)₄, (2mmol%), was added and then 6-iodoimidazo[1,2-*a*]pyridine **6a** (30 mg, 0.123 mmol). The crude product was purified by flash column chromatography (DCM: MeOH) to give the desired product, **1j** (28 mg, 70% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.60 (s, 1H, -OH), 8.78 (s, 1H, **5**), 7.93 (s, 1H, **3**), 7.63 - 7.55 (m, 2H, **2**, **8**), 7.55 - 7.48 (m, 3H, **12**, **14**, **7**), 6.87 (d, *J* = 8.6 Hz, 2H, **11**, **15**). ¹³C NMR (101 MHz, DMSO) δ 157.2 (C12), 143.5 (C8a), 133.3 (C2), 127.6 (C11, C13), 127.3 (C6), 125.1 (C9), 124.4 (C7), 122.9 (C5), 116.7 (C8), 115.8 (C10, C14), 113.3 (C3). **HPLC-MS (ES⁺)**: CH₃CN/H₂O 2:98 to 95:5 (5 min), RT = 3.92 min, [M+H⁺] = 211.



(E)-6-styrylimidazo[1,2-*a*]pyridine, 1k: According to experimental procedure B, in a microwave tube, styrylboronic acid (22mg, 0.147 mmol) and NaHCO₃ (11mg, 0.135 mmol) were dissolved in 3 ml of 1,4-dioxane and H₂O (3:1). Pd(PPh₃)₄, (2 mg, 2 mmol%), followed by 6-iodoimidazo[1,2-*a*]pyridine **6b** (30 mg, 0.123 mmol) were added. The crude product was purified in DCM: MeOH to afford 25 mg of **1k** (91% yield). ¹H NMR (400 MHz, Chloroform-*d*) δ 8.05 (s, 1H, **5**), 7.57 - 7.50 (m, 2H, **3**, **8**), 7.47 (s, 1H, **2**), 7.46 - 7.36 (m, 3H, **7**, **13**, **15**), 7.34 - 7.25 (m, 2H, **16**, **12**), 7.25 - 7.17 (m, 1H, **14**), 6.99 (d, *J* = 16.3 Hz, 1H, **10**), 6.92 (d, *J* = 16.3 Hz, 1H, **9**). ¹³C NMR (101 MHz, CDCl₃) δ 145.1 (C8a), 136.9 (C11), 134.1 (C2), 129.5 (C9), 128.9 (C12, C16), 128.1 (C14), 126.5 (C13, C15), 124.3 (C5), 124.2 (C10), 123.5 (C6), 122.4 (C7), 117.9 (C8), 112.9 (C3). HPLC-MS (ES⁺): CH₃CN/H₂O 10:90 to 95:5 (5 min), RT = 4.13 min, [M+H⁺] = 221.

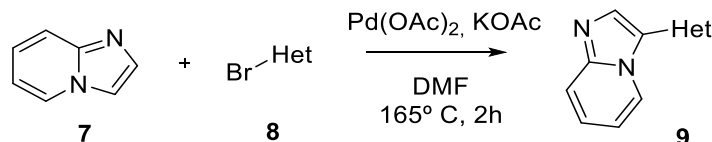


6-(benzofuran-2-yl)imidazo[1,2-*a*]pyridine 1l: According to experimental procedure B, benzofuran-2-yl boronic acid (16 mg, 0.106 mmol, 1.2 eq), K₂CO₃ (15 mg, 0.106 mmol) Pd(PPh₃)₄, (2 mg, 2mmol%) and 6-iodoimidazo[1,2-*a*]pyridine (20 mg 0.082 mmol) were dissolved in 3 ml of DME/H₂O (4:1). The crude product was purified by semipreparative HPLC (gradient 10: 90 to 40: 60 CH₃CN/H₂O for 30 min) to give 10 mg **1l** in 52% yield. ¹H NMR (400 MHz, Acetone-*d*₆) δ 9.07 (s, 1H, **5**), 8.02 (s, 1H, **3**), 7.74 (dd, *J* = 9.5, 1.8 Hz, 1H, **7**), 7.70 – 7.60 (m, 3H, **2**, **8**, **15**), 7.56 (d, *J* = 8.1 Hz, 1H, **12**), 7.41 – 7.32 (m, 2H, **11**, **14**), 7.27 (apparent t, *J* = 7.4 Hz, 1H, **13**). ¹³C NMR (101 MHz, Acetone) δ 155.6 (C15a), 153.9 (C10), 145.4 (C8a), 135.0 (C2), 130.04 (C11a), 125.6 (C11), 124.1 (C13), 123.9 (C5), 122.5 (C7), 121.9 (C15), 118.5 (C8), 117.0 (C6), 114.8 (C3), 111.7 (C12), 103.2 (C12). HPLC-MS (ES⁺): CH₃CN/H₂O 10:90 to 95:5 (5 min), RT = 4.40 min, [M+H⁺] = 235.

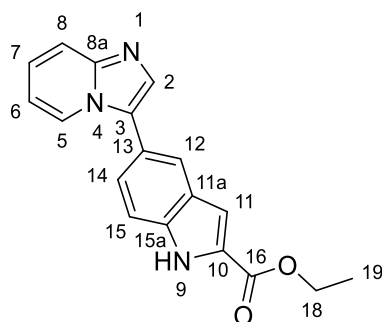


6-(3,4-dimethoxyphenyl)imidazo[1,2-*a*]pyridine, **1m:** According to experimental procedure B, 3,4-dimethoxyphenylboronic acid (33 mg 0.184 mmol) and Na₂CO₃ (20 mg, 0.246 mmol) were dissolved in 3 mL of H₂O/MeOH/Toluene (1:2:8). Pd(PPh₃)₄ (7 mg 5mmol%), 6-iodoimidazo[1,2-*a*]pyridine (30 mg, 0.123 mmol) was incorporated. The crude product was purified in DCM: MeOH to give product **1m** (30 mg, 96% yield). ¹H NMR (400 MHz, Chloroform-*d*) δ 8.25 (s, 1H, **5**), 7.64 (*apparent t*, *J* = 10.9 Hz, 3H, **2,3,8**), 7.39 (dd, *J* = 9.3, 1.9 Hz, 1H, **7**), 7.09 (dd, *J* = 8.2, 2.2 Hz, 1H, **14**), 7.03 (d, *J* = 2.1 Hz, 1H, **10**), 6.95 (d, *J* = 8.2 Hz, 1H, **13**), 3.95 (s, 3H, -OCH₃), 3.92 (s, 3H, -OCH₃). ¹³C NMR (101 MHz, CDCl₃) δ 149.5 (C12), 149.2 (C11), 144.7 (C8a), 134.0 (C2), 130.3 (C9), 126.9 (C6), 125.4 (C7), 122.6 (C5), 119.5 (C14), 117.7 (C8), 112.8 (C3), 111.8 (C13), 110.3 (C10), 56.1 (-OCH₃), 56.1 (-OCH₃). **HPLC-MS (ES⁺):** CH₃CN/H₂O 10:90 to 95:5 (5 min), RT = 1.39 min, [M+H⁺] = 255.

Experimental procedure C: Heck's reaction

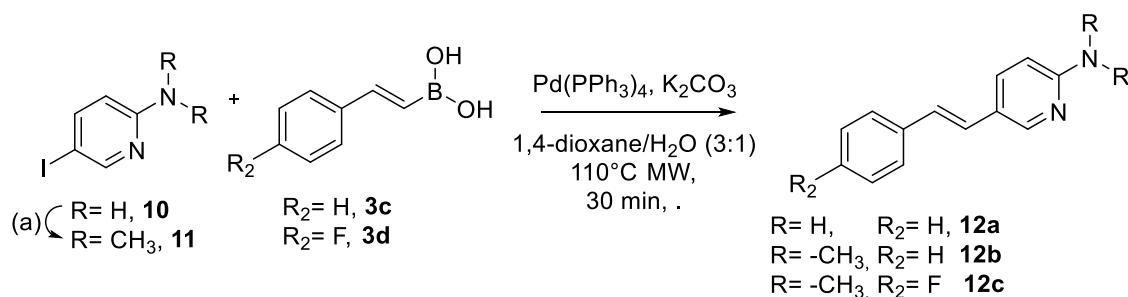


In a microwave vial, imidazo[1,2-*a*]pyridine (**7**, 1 eq), haloheteroaryl (**8**, 1.3 eq) and KOAc (2 eq) were dissolved in 4 mL DMF. The mixture was purged with argon for 1 minute prior to addition of the metal catalyst Pd(OAc)₂ (10 mmol%). The tube was sealed and irradiated in a microwave reactor for two hours at 165 °C. After cooling to room temperature, 1 mL KOH (aq) concentrated was added, and the mixture was stirred for 1 h prior to removing the solvent. Crude reaction was diluted in water, extracted with AcOEt (3x 10 mL) and dried over anhydrous MgSO₄. The crude product was purified by flash column chromatography (hexane: ethyl acetate) to afford **imidazo[1,2-*a*]pyridine derivative, 9**.



Ethyl 5-(imidazo[1,2-*a*]pyridin-3-yl)-1*H*-indole-2-carboxylate 9: According to experimental procedure C, imidazo[1,2-*a*]pyridine (80 mg, 0.677 mmol), ethyl 5-bromo-1*H*-indole-2-carboxylate (0.235 mg, 0.880 mmol), KOAc (86 mg, 0.880 mmol) and Pd(OAc)₂ (15 mg, 10 mmol%) were dissolved in 4 mL DMF. Crude product was purified by flash column chromatography (hexane: ethyl acetate) to yield **9** in good yield (130 mg, 62%). ¹H NMR (400 MHz, Chloroform-*d*): δ 9.61 (s, 1H, -NH), 8.33 (d, *J* = 6.9 Hz, 1H, **5**), 7.86 (s, 1H, **12**), 7.70 (d, *J* = 11.5 Hz, 2H, **2**, **8**), 7.58 (d, *J* = 8.4 Hz, 1H, **15**), 7.48 (d, *J* = 8.5 Hz, 1H, **14**), 7.29 (s, 1H, **11**), 7.20 (t *apparent*, *J* = 7.9 Hz, 1H, **7**), 6.80 (t *apparent*, *J* = 6.9 Hz, 1H, **6**), 4.44 (q, *J* = 7.1 Hz, 2H, -CH₂), 1.43 (t, *J* = 7.1 Hz, 3H, -CH₃). ¹³C NMR (101 MHz, CDCl₃): δ 161.9 (CO), 145.8 (C8a), 136.7 (C15a), 132.1 (C2), 128.8 (C10), 128.1 (C11a), 126.4 (C13), 126.0 (-C14), 124.2 (C7), 123.5 (C5), 122.6 (C12), 121.8 (C3), 118.2 (C8), 113.0 (C15), 112.6 (C6), 108.7 (C11), 61.3 (-CH₂), 14.5 (-CH₃). **HPLC-MS (ES⁺):** CH₃CN/H₂O 10:90 to 90:10 (5 min), RT = 4.24, [M+H⁺] = 306.

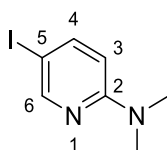
Experimental procedure D for the synthesis of 2-aminopyridines derivatives



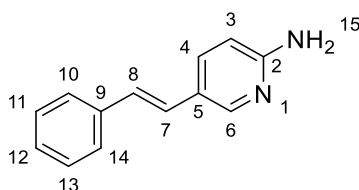
N-methylation (a): NaH, MeI, DMF (anh) from 0°C to r.t, 1.5 hours

N-methylation: NaH (10.6 eq) and the commercial 5-iodo-2-aminopyridine (**10**, 1 eq) were stirred at 0 °C under inert atmosphere for 30 min in DMF. Next, methyl iodide (2.5equiv) was added dropwise and stirred for another 10 min. Then, the mixture was allowed to reach room temperature for 1.5h, prior to quenching with water. After extraction with ethyl acetate (x3), the combined organic layers were dried over MgSO₄, and then the solvent was evaporated under vacuum. The obtained crude mixture was then purified by flash silica-gel column chromatography to give the corresponding N-N-dimethylated product, **11**.

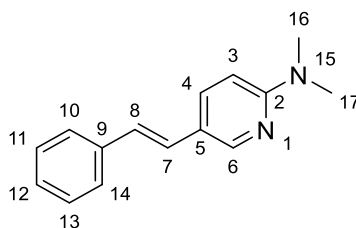
Suzuki's cross coupling reaction (2): In a microwave tube, styrylboronic acid (**3c**, 1.2 equiv) and K_2CO_3 (1.3 equiv) were dissolved in 4 ml of 1,4-dioxane/ H_2O (3:1). $Pd(PPh_3)_4$ (2 mmol%), was added and the mixture was purged with Ar for one minute. Then, 1 eq of 5-iodo-2-aminopyridine (**10** or **11**, depending on example,) was added. The tube was irradiated in a microwave reactor for 30 minutes at 110°C. After cooling to room temperature, the solvent was filtered and evaporated. The crude product was purified by flash column chromatography to afford 2-aminopyridines derivatives, **12**.



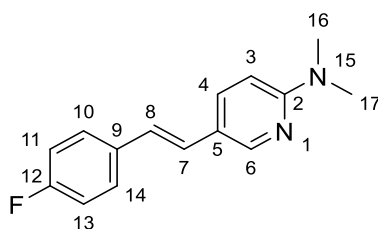
5-iodo-N,N-dimethylpyridin-2-amine, 11: According to experimental procedure D for N, N-methylation, product 14 was obtained by mixing NaH (34 mg, 1.14 mmol) 5-iodo-2-aminopyridine (100 mg, 0.454 mmol) and MeI (161 mg, 1.14 mmol). Crude product was purified by column chromatography (hexane: AcOEt) to afford 70 mg of product **14** (60% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 8.28 (d, J = 2.3 Hz, 1H, **6**), 7.61 (dd, J = 9.0, 2.3 Hz, 1H, **4**), 6.35 (d, J = 9.0 Hz, 1H, **3**), 3.05 (s, 6H, - CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.15 (C5), 153.58 (C6), 144.60 (C4), 108.31 (C3), 75.52 (C2), 38.20 (- CH_3). **HPLC-MS (ES $^+$)**: CH_3CN/H_2O 15-95 (5 min), RT = 1.32 min, $[M+H]^+$ = 249.



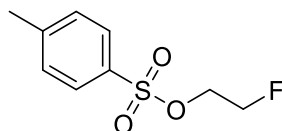
(E)-5-styrylpyridin-2-amine, 12a: Following experimental procedure D, second step, styrylboronic acid (73mg, 0.499 mmol) $Pd(PPh_3)_4$ (10mg, 2 mmol%), 5-iodo-2-aminopyridine (100 mg, 0.454 mmol) and K_2CO_3 (81 mg, 0.590 mmol) were dissolved in 4 ml of 1,4-dioxane/ H_2O . The crude product was purified by column chromatography (DCM: MeOH) to give 52 mg of **12a** as a white solid (58% yield). ^1H NMR (400 MHz, Acetone- d_6) δ 8.12 (d, J = 2.5 Hz, 1H, **6**), 7.74 (dd, J = 8.7, 2.5 Hz, 1H, **4**), 7.53 (d, J = 7.3 Hz, 2H, **10**, **14**), 7.33 (t, J = 7.7 Hz, 2H, **11**, **13**), 7.21 (t, J = 7.4 Hz, 1H, **12**), 7.11 (d, J = 16.4 Hz, 1H, **7**), 7.00 (d, J = 16.4 Hz, 1H, **8**), 6.58 (d, J = 8.6 Hz, 1H, **3**), 5.55 (s, 2H, - NH_2). ^{13}C NMR (101 MHz, Acetone) δ 160.2 (C2), 148.6 (C6), 138.9 (C9), 134.7 (C4), 129.4 (C4), 127.7 (C13, C11), 126.8 (C12), 126.7 (C10, 14), 125.6 (C7), 123.4 (C8), 109.0 (C3). **HPLC-MS (ES $^+$)**: CH_3CN/H_2O 10:90 to 95:5 (5 min), RT = 4.36 min, $[M+H]^+$ = 197.



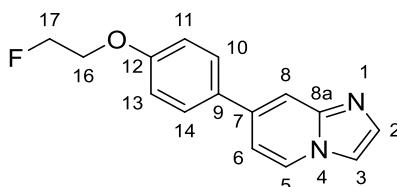
(E)-N,N-dimethyl-5-styrylpyridin-2-amine, 12b: According to procedure D, styrylboronic acid (38 mg 0.262 mmol) and K_2CO_3 (36mg, 0.262 mmol) were dissolved in 4 ml of 1,4-dioxane and H_2O (3:1). $Pd(PPh_3)_4$ (4 mg, 2 mmol%) followed by *N,N*-dimethyl-5-iodo-2-aminopyridine (50 mg, 0.136 mmol). The crude product was purified by column chromatography (DCM: MeOH) to yield a pale-yellow solid (35 mg, 74% yield). ^1H NMR (500 MHz, Chloroform-*d*) δ 8.30 (s, 1H, **6**), 7.74 (d, J = 9.0 Hz, 1H, **4**), 7.52 (dd, J = 8.0, 2.2 Hz, 2H, **10, 14**), 7.38 (td, J = 7.8, 2.3 Hz, 2H, **11, 13**), 7.29 - 7.23 (m, 1H, **12**), 7.04 (d, J = 16.3 Hz, 1H, **7**), 6.93 (d, J = 16.3 Hz, 1H, **8**), 6.57 (d, J = 8.9 Hz, 1H, **3**), 3.16 (s, 3H, $-CH_3$, **16** or **17**), 3.15 (s, 3H, $-CH_3$, **16** or **17**). ^{13}C NMR (126 MHz, $CDCl_3$) δ 158.7 (C2), 147.6 (C6), 137.9 (9), 133.9 (4), 128.7 (C11, C13), 127.1 (C12), 126.1 (C14, C10), 125.8 (C7 or C8), 125.1 (C8 or C7), 121.3 (C5), 106.0 (C3), 38.3 ($-CH_3$ x2). HPLC-MS (ES^+): CH_3CN/H_2O 10:90 to 95:5 (5 min), RT = 4.54 min, $[M+H]^+$ = 225.



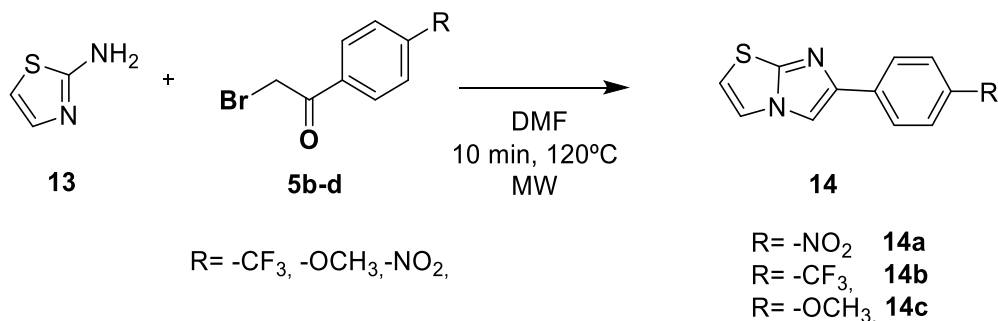
(E)-5-(4-fluorostyryl)-N,N-dimethylpyridin-2-amine 12c: According to procedure D, styrylboronic acid (30 mg, 0.123 mmol) and K_2CO_3 (17 mg, 0.123 mmol) were dissolved in 4 ml of 1,4-dioxane and H_2O (3:1). $Pd(PPh_3)_4$ (2 mg, 2 mmol%) followed by *N,N*-dimethyl-5-iodo-2-aminopyridine (15.2 mg, 0.062 mmol). The crude product was purified by column chromatography (DCM: MeOH) to yield a pale-yellow solid (70% yield). ^1H NMR (400 MHz, DMSO-*d*₆) δ 8.22 (d, J = 2.4 Hz, 1H, **6**), 7.82 (dd, J = 8.9, 2.5 Hz, 1H, **4**), 7.61 – 7.52 (m, 2H, **10, 14**), 7.23 – 7.13 (m, 2H, **11, 13**), 7.08 (d, J = 16.5 Hz, 1H, **8**), 7.01 (d, J = 16.4 Hz, 1H, **7**), 6.68 (d, J = 8.9 Hz, 1H, **3**), 3.05 (s, 6H, 2x $-CH_3$). ^{13}C NMR (101 MHz, DMSO-*D*₆) δ 161.7 (d, J_{C-F} = 243.8 Hz, C12), 158.8 (C2), 147.8 (C6), 134.7 (d, J_{C-F} = 3.2 Hz, C9), 134.7 (C4), 128.2 (d, J_{C-F} = 8.0 Hz, C10, C14), 126.2 (d, J_{C-F} = 2.5 Hz, C8), 123.75 (C7), 121.29 (C5), 116.0 (d, J_{C-F} = 21.5 Hz, C11, C13), 106.5 (C3), 38.2 ($-CH_3$ x2).

Experimental procedure E for the synthesis of cold ligand 1n:**Synthesis of the fluorinated lateral chain:**

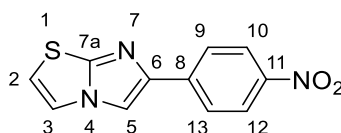
2-fluoroethyl-4-methylbenzenesulfonate: A solution of 2-fluoroethanol (1 g, 15.6 mmol, 1eq) in 5 mL of pyridine was stirred for 5 min under inert atmosphere. Next, *p*-toluenesulfonyl chloride (3.57 g, 18.7 mmol, 1.2 eq) was added dropwise and the mixture was stirred at room temperature for 24 hours. The mixture was dilute in DCM and extracted with an aqueous solution of 10 % (w/v) CuSO₄ (3 x 50 mL). The organic phases were dried over anhydrous MgSO₄, filtered and evaporated under high vacuum. The solid was purified by column chromatography (hexane: ethyl acetate) to afford the desired product (1.185 g, 35% yield). The spectral data is in accordance with that found in the literature.¹⁷⁷ **¹H NMR** (300 MHz, CDCl₃): δ 7.81 (d, *J* = 8.3 Hz, 2H, CH₃CCCHCHCCHCH-), 7.36 (d, *J* = 8.4 Hz, 2H, CH₃CCCHCHCCHCH-), 4.57 (dm, *J*_{HF} = 47.1 Hz, 2H, -OCH₂CH₂F), 4.26 (dm, *J*_{H-F} = 27.2 Hz, 2 H, -OCH₂CH₂F), 2.45 (s, 3 H, -CH₃). **¹³C NMR** (100 MHz, CDCl₃) δ: 145.2 (-CSO₃), 132.7 (-C-), 130.0 (-CH- Ar), 128.0 (CH- Ar), 80.6 (d, *J*_{C-F} = 173.8 Hz, -CH₂F), 68.5 (d, *J*_{C-F} = 21 Hz, -OCH₂-), 21.7 (-CH₃).



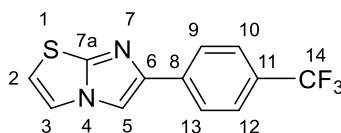
7-(4-(2-fluoroethoxy)phenyl)imidazo[1,2-*a*]pyridine, 1n: To a stirring solution of 4-(imidazo[1,2-*a*]pyridin-7-yl)phenol **1a** (41 mg, 0.195 mmol), KI (48 mg, 0.292 mmol), K₂CO₃ (107 mg, 0.78 mmol) and nBu₄NI (14 mg, 0.039 mmol) in anhydrous DMF (4 ml) were added 120 mg (0.580 mmol) of 2-fluoroethyl 4-methylbenzenesulfonate. After a reaction time of 3 h at 70 °C, the solvent was evaporated under reduced pressure. The mixture was suspended in water and extracted 3 times with ethyl acetate. The combined organic layers were dried over MgSO₄ and dried under vacuum. The crude product was purified by flash column chromatography (DCM: MeOH) to yield **1n** (36 mg, 72 %). Purification was also possible by means of precipitation using a mixture of DCM and ether. **¹H NMR** (400 MHz, Methanol-*d*₄) δ 8.45 (d, *J* = 7.1 Hz, 1H, **5**), 7.82 (s, 1H, **3**), 7.72 – 7.64 (m, 3H, **8**, **10**, **14**), 7.59 (s, 1H, **2**), 7.22 (d, *J* = 7.1 Hz, 1H, **6**), 7.11 – 7.03 (m, 2H, **11**, **13**), 4.34 – 4.28 (dm, *J*_{H-F} = 47.7 Hz, 2H, -CH₂CH₂F), 4.27 – 4.20 (dm, *J*_{H-F} = 28.9 Hz, 2 H, -OCH₂CH₂F). **¹³C NMR** (101 MHz, Methanol-*d*₄) δ 159.1 (C12), 138.1 (C7), 132.4 (C2), 130.9 (C9), 127.7 (10,14), 126.5 (C5), 114.8 (C11, C13), 112.6 (C3), 112.0 (C6), 111.5 (C8), 81.8 (d, *J*_{C-F} = 168.9 Hz, CH₂F), 67.3 (d, *J*_{C-F} = 20.1 Hz, -OCH₂-).

Experimental procedure F for the synthesis of 6-arylimidazo[2,1-*b*]thiazole derivatives, 14.

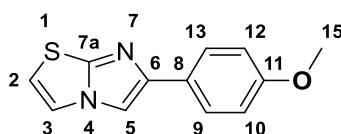
2-aminothiazol (**13**, 1eq) and a α -bromoketone (**5**, 1eq) were added to a microwave vial and solved with 4 ml of DMF. The tube was sealed with a pressure cap and irradiated in a microwave reactor for 10 minutes at 120 °C. After cooling at room temperature, the solvent was evaporated. The crude was suspended in water, basified with concentrated K_2CO_3 aqueous solution and extracted with dichloromethane (10 mL x 3). The organic phases were dried over anhydrous MgSO_4 , filtered and evaporated under high vacuum. The solid product was purified by flash column chromatography to afford imidazothiazole derivatives **14**.



6-(4-nitrophenyl)imidazo[2,1-*b*]thiazole , 14a. The experimental procedure F was used with 2-aminothiazol (100mg, 0.999 mmol), K_2CO_3 (83 mg, 0.6 mmol) and 2-bromo-1-(4-nitrophenyl)ethan-1-one (243mg, 0.999 mmol) in 4 mL of DMF. Crude product was purified in DCM: MeOH, to give product **14a**. (127mg, yield 52%). $^1\text{H NMR}$ (500 MHz, $\text{DMSO-}d_6$) δ 8.48 (s, 1H, **5**), 8.25 (d, $J = 9.0$ Hz, 2H, **10, 12**), 8.09 (d, $J = 9.0$ Hz, 2H, **9, 13**), 7.98 (d, $J = 4.5$ Hz, 1H, **3**), 7.33 (d, $J = 4.5$ Hz, 1H, **2**). $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO-}d_6$) δ 150.1 (C7a), 145.8 (C11), 144.0 (C6), 140.7 (C8), 125.2 (C13, C9), 123.9 (C12, C10), 119.9 (C3), 114.1 (C2), 112.0 (C5). **HPLC-MS (ES^+)**: $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 30:70 to 95:5 (5 min), RT = 3.95 min, $[\text{M}+\text{H}^+] = 246$.



6-(4-(trifluoromethyl)phenyl)imidazo[2,1-*b*]thiazole, **14b.** The experimental procedure F was used to obtain compound **14b** by mixing 2-aminothiazol (100mg, 0.99 mmol) and 2-bromo-1-(4-(trifluoromethyl)phenyl)ethan-1-one (266mg, 0.99mmol) in 4 mL of DMF. Crude product was purified in DCM, to give 133 mg of the desired product in 50% yield. **¹H NMR** (400 MHz, Chloroform-*d*) δ 7.91 (d, J = 8.1 Hz, 2H, **10**, **12**), 7.79 (s, 1H, **5**), 7.63 (d, J = 8.1 Hz, 2H, **9**, **13**), 7.42 (d, J = 4.5 Hz, 1H, **3**), 6.84 (d, J = 4.5 Hz, 1H, **2**). **¹³C NMR** (101 MHz, CDCl₃) δ 150.7 (C7a), 146.5 (C6), 137.6 (C8), 129.2 (q, J_{C-F} = 32.4 Hz, -C-CF₃), 125.7 (q, J_{C-F} = 3.8 Hz, CH-CCF₃), 125.3 (C13, C9), 124.4 (q, J_{CF} = 272.5 Hz, -CF₃), 118.6 (C3), 113.3 (C5) 109.1 (C2). **HPLC-MS (ES⁺):** CH₃CN/H₂O 15:85 to 95:5 (5 min), RT = 5.31 min, [M+H⁺] = 269.



6-(4-methoxyphenyl)imidazo[2,1-*b*]thiazole, **14c:** According to procedure F, 2-aminothiazol (70 mg, 0.718 mmol) and 2-bromo-1-(4-(methoxyl)phenyl)ethan-1-one (165mg, 0.718mmol) with 4 ml of DMF. Crude product was purified in DCM: MeOH, to give product **14c**. (95 mg, yield 57%). **¹H NMR** (300 MHz, DMSO-*d*₆) δ 8.45 (s, 1H, **5**), 8.24 (d, J = 4.2 Hz, 1H, **3**), 7.74 (d, J = 8.8 Hz, 2H, **12**, **10**), 7.66 (d, J = 4.2 Hz, 1H, **2**), 7.05 (d, J = 8.8 Hz, 2H, **9**, **13**), 3.80 (s, 3H, -CH₃). **HPLC-MS (ES⁺):** CH₃CN/H₂O 15:85 to 95:5 (5 min), RT = 2.61 min, [M+H⁺] = 231. Spectral data was in accordance with that found in the literature.¹⁵⁸

1.5.3 Thioflavin T Competition Assays with Amyloid Proteins

Affinity studies with porcine insulin

Insulin aggregates preparation

Insulin aggregates were obtained by incubating the protein at 2 mg/mL in Glycine-HCl buffer,¹⁷⁸ pH = 2, for 8 h at 60 °C under mild and constant shaking (70 rpm) as describe elsewhere.^{164,179} Final concentration of aggregates = 346 μ M.

ThT and compound stocks preparation

Thioflavin T shows a dramatic increase in fluorescence when its rotational freedom is restricted in confined spaces within proteins. These dyes possess a flat structure and the ability to bind β -sheets such as those present in amyloid aggregates. Thus, binding affinity of small molecules to amyloid aggregates can be assessed by the ThT competition assay. In this test, changes in the ThT fluorescence are monitored at 440 nm and 482 nm (excitation and emission wavelengths). If the affinity for the fibrils is high enough, the competitor may displace ThT, so the emitted fluorescence of ThT would drop. The bigger the reduction of ThT fluorescence, the higher the affinity of the competitor for the aggregates.

ThT stock solution was freshly prepared at 1 mM (0.636 mg in 2 mL in sodium phosphate buffer 10 mM, pH= 7.4). The solution was filtered (0.22 μ m) and diluted further for a final diluted stock at 2.42 μ M. In the assay, 142 μ L of 2.42 μ M ThT stock were added to each test well, so the final concentration of ThT employed was 2.15 μ M (Table 10).

Compounds stock solutions at 10 mM in pure DMSO were used to prepare diluted stock solutions at 1 mM (10 % v/v DMSO) by mixing 30 μ L of compound stock and 270 μ L of sodium phosphate buffer 10 mM, pH 7.4. The 1 mM stock was used to prepare 10-fold serial dilutions of compound ranging 2.15 μ M to 215 μ M, by diluting with the mixture 10% DMSO v/v in PBS. Compounds were tested in a range of concentrations from 0.215 μ M to 21.5 μ M (16 μ L of diluted compound stocks in 160 μ L total volume).

Table 10 - Stock solutions and volume employed in the ThT competition assay.

Stock solution (μ M)	Volume (μ L)	Final concentration per well (μ M)
Insulin aggregates – 340	2	4.32
Competitor – 2.15-215	16	0.215-21.5
ThT - 2.42	142	2.15
Total volume	160	-

ThT binding assay

In a 96-well black plate, sample test contained 2 μ L of insulin aggregates, 16 μ L of competitor stock solution and 142 μ L of ThT stock solution. The final concentration of each component is shown in Table 10, and the final percentage of DMSO was 1% (v/v) per well. Molar ratios [competitor: protein]

were [5: 1], [3:1], [2: 1], [0.5: 1], [0.25: 1] and [0.05: 1]. As positive control, insulin aggregates were added with only ThT (insulin- ThT molar ratio 2:1). Fluorescence of insulin aggregates with every concentration of competitor was also calculated.

Plates were covered in foil and incubated at room temperature for 2 h under low agitation in a rotatory shaker. Fluorescence emission spectra were then taken using excitation at 440 nm, emission at 482 nm. The excitation and emission slit widths were set as 9 nm and 15 nm, respectively. Tests were performed in 6 replicates.

Data were obtained from SoftMax Software 7.0 (Molecular Devices, San Jose, CA, USA) and processed in Microsoft Excel. The percentage (%) Th T displacement induced by competitors was obtained by normalizing the values according to Equation 1.4.

$$\% \text{ ThT displacement} = \frac{\text{Th T fluorescence Sample} - \text{background}}{\text{Th T fluorescence Control}} \times 100$$

Equation 1.4

Baseline was corrected by subtracting intrinsic fluorescence of compounds to sample test values. After background subtraction, the average fluorescence of the positive control (insulin aggregates + ThT samples) was used to normalize all experimental data points. Finally, each well value was multiplied by 100 to calculate the % ThT displacement that occurred in each individual well relative to control.

Hence, the remaining ThT fluorescence (%) was calculated by subtracting the % ThT displacement value to 100, according to Equation 1.5

$$\text{Remaining ThT fluorescence (\%)} = 100 - \text{ThT displacement (\%)}$$

Equation 1.5

Affinity studies with Tau⁴⁴¹ protein

Formation of Tau protein aggregates

Assays were performed with two different suppliers of Tau protein

- *Abcam, UK*: Tau protein (0.2 mg/mL) was dissolved in aggregation buffer composed by 20 mM Tris-HCl, 50 mM NaCl and 1 mM DTT (final concentrations). The pH was adjusted with HCl to 7.4 and buffer solution was filtered (0.22 µm filter). In order to prone the aggregation of the peptide, heparin was added into the aggregation mixture dissolved in aggregation buffer,^{44,165} for a final ratio Tau: Hep = 1.87: 1.¹⁶⁵ Tau protein (2.18 µM) was allowed to aggregate at 37 °C for 13 days with no agitation.
- *rPeptide*: The manufacturer provided tau protein as lyophilized powder.

Initial conditions: 100 µg Tau⁴⁴¹ were dissolved in aggregation buffer for a final concentration of 11 µM. Heparin was included in the aggregation buffer so that the tau: heparin molar ratio was 4:1.⁴⁴ Aggregation buffer included: 50 mM MES, 100 mM NaCl and 100 mM

ammonium acetate pH 7. Tau protein was incubated under these conditions for 8 days at 37°C.

Final conditions: Tau protein from rPeptide was resuspended from lyophilized powder with filtered deionized water containing 2.3 μM of heparin and 1 mM of DTT. The final concentration of Tau was 200 $\mu\text{g/mL}$ (4.36 μM), so that the molar ratio of tau: heparin was 1.87:1. The composition of the aggregation buffer was: 100 mM MES, 200 mM NaCl, 0.5 mM EGTA, 1 mM DTT (pH 6).¹⁴² Aggregation conditions were 13 days at 37 °C with no agitation.

ThT stock solution

ThT stock solution was freshly prepared at 1 mM in sodium phosphate buffer (10 mM, pH= 7.4). The solution was filtered (0.22 μm) and diluted further in phosphate buffer for a final 2.2 μM stock.

ThT competition assay - Competition assay with 12b:

The assay was performed in 96-well black plates, final volume per well was 60 μL . Tau and ThT were added separately for final concentrations of 1.09 μM and 0.55 μM , respectively (molar ratio 2:1). Compound: tau molar ratios were and [1:1], [5:1], [10:1]. Negative and positive controls were also included. Final DMSO percentage was 1% (v/v) in aggregation buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM DTT). Firstly, the compound was added followed by tau aggregates and then the ThT solution. Plate was incubated for 2.5 h inside the fluorometer at room temperature, and fluorescence values were obtained every 15 min under a slow intensity shake between reads in an orbital mode (orbital, low intensity for 800 seconds). Fluorescence spectra were taken using excitation at 440 nm, emission at 482 nm. The excitation and emission slit widths were set as 9 nm and 15 nm, respectively (Height 14.9mm, PTM and Optics: 6 flashed/read, read from top, read height 1.00mm). Tests were performed in 3 replicates. Data were obtained from SoftMax Software 7.0 (Molecular Devices, San Jose, CA, US) and processed in Microsoft Excel. Results were normalized by dividing the ThT fluorescence in the presence of the competitor divided by the ThT fluorescence value of the control (without compound) expressed in percentage (%) as per equation 1.4 above.

ThT competition assay - Screening of compounds

Tau protein (rPeptide) was aggregated as described above in the “final conditions” tau aggregation in tau aggregates formation section. In a 96-well plate, 15 μL of tau aggregates were added, followed by 15 μL of compound and 30 μL of ThT stock solution. Compound stocks were prepared at 4% DMSO in aggregation buffer. The total volume per well was 60 μL , and final concentration of tau, competitor and ThT was 1.09 μM , 5.45 μM and 0.5 μM , respectively. Buffer contained 50 mM MES and 150 mM NaCl in deionized water. (For fluorimeter setup, refer to the section above).

1.5.4 Morphological Studies

Atomic Force Microscopy (AFM)

Tau aggregates were prepared as described previously (section 3.3b), and visualized in a AFM microscope (Park NX10 enclosed in a Park NX10 acoustic enclosure, Park Systems Europe GmbH, Mannheim, Germany) with triangular silicon cantilevers with integrated tips [$t = 3.0 - 5.0 \mu\text{m}$, $l = 115 - 135 \mu\text{m}$, $w = 22.5 - 37.5 \mu\text{m}$, $V_0 = 204 - 497 \text{ kHz}$, $k = 10 - 130 \text{ N m}^{-1}$, $R < 10 \text{ nm}$; PointProbe® Plus Non-Contact Tapping Mode High Resonance Frequency Reflex Coating (PPP-NCHR-20) Nanosensors, Neuchâtel, Switzerland]

Sample preparation:

Muscovite disk was cut (1 cm x 1 cm) and the surface was clean by stripping off with scotch tape. Immediately after, a drop of 5 μL of tau stock suspension (4.36 μM) in aggregation buffer was placed in the surface of the disk, incubated at room temperature for 5 min and dried under air prior to being attached to a nickel disk (1 cm^2) using double-side adhesive tape and placed on the AFM microscope.

Samples were analysed at scan rate 1 Hz, amplitude $\sim 40 \text{ nm}$. The images were processed, and dimensions were measured using Gwyddion 2.55 software (Department of Nanometrology, Czech Metrology Institute).

Transmission Electron Microscopy (TEM)

Tau aggregates were also characterized by TEM. A drop of tau stock suspension (4.36 μM) was located on the coated side of a copper coated grid and allowed to dry for 5 minutes. Then, samples were negatively stained with uranyl acetate aqueous solution (2% w/v) for 30 sec. The excess of dye was removed, and samples were immediately loaded for their visualization on a JEM-1400 electron microscope (Jeol, Herts, UK).

1.5.5 Surface Plasmon Resonance (SPR)

Surface plasmon resonance experiments were accomplished at a Biacore X100 instrument at 25 °C. Tau aggregates were prepared as specified previously on section 3.3b and immobilised on a CM5 sensor chip by standard amine coupling reactions. Buffer acetate (pH 5.0, 90 μL) was used to immobilize 7800 resonant units (RUs) of tau aggregates (10 μL) in the second channel, whereas channel 1 was block as reference. One RU represents approximately 1pg protein/ mm^2 of chip surface^{180,181} or angle shift of 10^{-4} deg. Chip was inactivated by a consecutive injection of ethanolamine 1.0 M pH 8.5.

All the solutions and the running buffer had 50 mM MES, EGTA 1mM, NaCl 100 mM, 0.005% Tween and 2% DMSO, pH 6.8.

Compounds were weighted and dissolved in pure DMSO to obtain stock solutions at 10 mM, which were used further diluted stock solutions. Thus, 4 μ L of 10 mM stock solution were dissolved in 196 μ L of running buffer to obtain a 200 μ M diluted stock solution, with the desired percentage of DMSO: 2% (v/v). The 200 μ M stock solution was used to prepare serial dilutions (2-100 μ M) diluting with running buffer enriched with 2% (v/v) DMSO.

The flow rate was 30 μ L/min. As contact and dissociation times, 50 sec were selected, total time of experiments 300 sec. After each injection, an extra wash with 50% DMSO (v/v) in water was completed. No chip regeneration was needed.

1.5.6 Modelling Studies

Docking simulations

The protofibril unit from the paired helical filament was used as the target structure for the molecular docking (this dodecamer can be found in the protein database, PDB, reference ID 5O3L). Each unit of the paired helical filament corresponds to a protofilament comprising residues 306–378 of the tau protein.

Docking simulations of various imidazopyridine analogues were performed using the four high-affinity binding sites (S1-S4) recently described in tau protofibrils by Murugan and co-workers.¹⁷⁰ Hence, compounds were subjected to successive docking simulations on each binding site with the program GOLD version 5.7.2 (CCDC; Cambridge, UK; <http://www.ccd.cam.ac.uk>). A semi-flexible docking was done with the polar hydrogen atoms of protein left free to rotate, while ligands were set fully flexible. For each ligand, 50 poses were generated and rescored using the HINT scoring function (see below).

Re-scoring methodology

Each pose generated by docking underwent the re-scoring procedure using the HINT function to better predict the protein-ligand association. Hydrophatic INTERations is a post-docking tool that re-evaluates the scores obtained by docking algorithms.¹⁸² A positive and high HINT score (HS) correlates with favourable binding free energy, thus allowing the estimate of the thermodynamic benefit of certain predicted complexes (i.e. the higher the score, the higher the thermodynamic benefit).^{171,173} For each site, only the best scored pose of each ligand was considered for the analysis as it best represents the binding architecture, as previously shown.¹⁸³

1.5.7 Ex Vivo Red Blood Cell Haemolysis Assay

Haemolysis was undertaken as previously published with minor modifications.¹⁸⁴ Briefly, sodium chloride 0.9% (w/v) was prepared, adjusted to pH 7.4 and the final solution was filtered (0.22 μ m). Vehicle used to solubilise the tested compounds contained 5% DMSO (v/v) in 0.9% NaCl. Triton X-100 (1% w/w) was used as a positive control.

Blood was obtained from 6-8 weeks old C57BL/6 male mice in EDTA spray coated K6 vacutainers (BD Biosciences). Blood was centrifuged (2,500 g, 4°C, 15 minutes). Plasma was removed and RBC suspension was washed twice with sodium chloride 0.9% solution and centrifuged. The final RBC suspension was diluted to 4.45 w/v with sodium chloride as prepared above or Triton X-100 (1% w/w) for the positive control. Compounds were tested at six different concentrations ranging from 0.05 μ M to 150 μ M in triplicate. 10-Fold stock solutions of compounds were prepared, so when diluted with 4.45% RBC suspension the DMSO concentration never exceeded 0.5% v/v. Thus, 20 μ L of each compound stock was added to each well followed by 180 μ L of diluted erythrocytes (RBC suspension). Sodium chloride with DMSO (20 μ L) or Triton X-100 (1%, 20 μ L) were diluted with 180 μ L of RBC suspension and served as controls. Plates were incubated for 1 h at 37 °C, after which they were centrifuged at 1200g for 5 min. The supernatant (100 μ L) was transferred into a new 96-well clear flat bottom plate without disturbing the pellet, and the absorbance was measured at 405 nm using a UV spectrophotometer.

$$\% \text{ Haemolysis} = \frac{OD \text{ sample} - OD \text{ mean neg control}}{OD \text{ mean positive control}} \times 100$$

Equation 1.6

Background average absorbance values from the negative control was subtracted from all other samples. After background subtraction, the average absorbance of the positive control (Triton samples) was used to normalize all experimental data points. Finally, each well value was multiplied by 100 to calculate % haemolysis that occurred in each individual well relative to the detergent control. (Optical density, OD)

1.5.8 Statistics

Experimental data was analysed with Excel (Microsoft®) or GraphPad Prism 8 (GraphPad Inc., California, USA) software. Results were expressed as mean $\pm$ SD using One-way ANOVA or Two-ANOVA to evaluate any statistically significant differences, and Dunnett's and/or Tukey's post-hoc tests for perform multiple group comparisons. Confidence interval= 95%. Data was considered statistically significant when P value (p) was < 0.05.

CHAPTER 2

Synthesis and evaluation of novel LDH-A inhibitors.

2.1 INTRODUCTION

2.1.1 Cancer and the Warburg effect

Cancer cells are recognised by an aberrant glucose metabolism. Their altered activity is characterized by an upregulated glycolysis pathway to obtain the energy from glucose, instead of using the cellular respiration (the oxidation-phosphorylation mechanism, “OxPhos”).¹⁸⁵

Cellular respiration is a highly efficient process that involves three main phases, where glucose is used as energy and carbon source. The first phase is known as glycolysis, and it takes place in the cytoplasm. This process does not need oxygen, and includes ten different reactions, all of them catalysed by enzymes.¹⁸⁶ The final products of glycolysis are pyruvate, a key intermediate in the energetic metabolism of cells, 2 ATP molecules and nicotinamide adenine dinucleotide in the reduced form (NADH).

If oxygen is available, in the second phase cells continue getting energy introducing the glucose-derived pyruvate into the mitochondrial matrix, where it is oxidised into acetyl-coenzyme A (CoA) by the pyruvate dehydrogenase complex. CoA enters into the Krebs cycle or tricarboxylic acid cycle (TCA), which is a series of redox and decarboxylation reactions that remove high-energy electrons and carbon dioxide. The electrons temporarily stored in molecules of NADH and flavin adenine dinucleotide (FADH₂) are used to generate ATP in a subsequent pathway. ATP or GTP are also produced on in each turn of the cycle.

Last phase of cellular respiration is the oxidative phosphorylation. This process involves a series of protein complexes that move electrons through redox reactions (the electron transport chain). Here, atmospheric oxygen is the final electron acceptor of the electrons removed from the intermediate molecules in glucose catabolism. The final products of the electron transport chain are water and ATP.¹⁸⁷

The full oxidation of one molecule of glucose produce, ideally, up to 38 ATP molecules (36 molecules + 2 ATP from glycolysis).¹⁸⁵ However, the yield of ATP varies. Intermediate compounds generated in previous pathways are used for different purposes. For example, five-carbon sugars, such as ribose, that form nucleic acids are made from intermediates in glycolysis. This metabolic pathway, named the pentose phosphate pathway, is parallel to glycolysis (Figure 2.1). Certain amino acids and lipids, such as triglycerides, are built or broke down for energy through these pathways. Overall, these alternative pathways extract about 34% of the energy obtained by a molecule of glucose.¹⁸⁸

In absence of oxygen, pyruvate is metabolized by the enzyme lactate dehydrogenase (LDH). This enzyme is required to maintain glycolysis and ATP production by regenerating NAD⁺ from NADH and pyruvate. This reaction is known as lactic acid fermentation, because lactate is obtained as a by-product. The net production of ATP is two molecules per molecule of glucose (Figure 41).

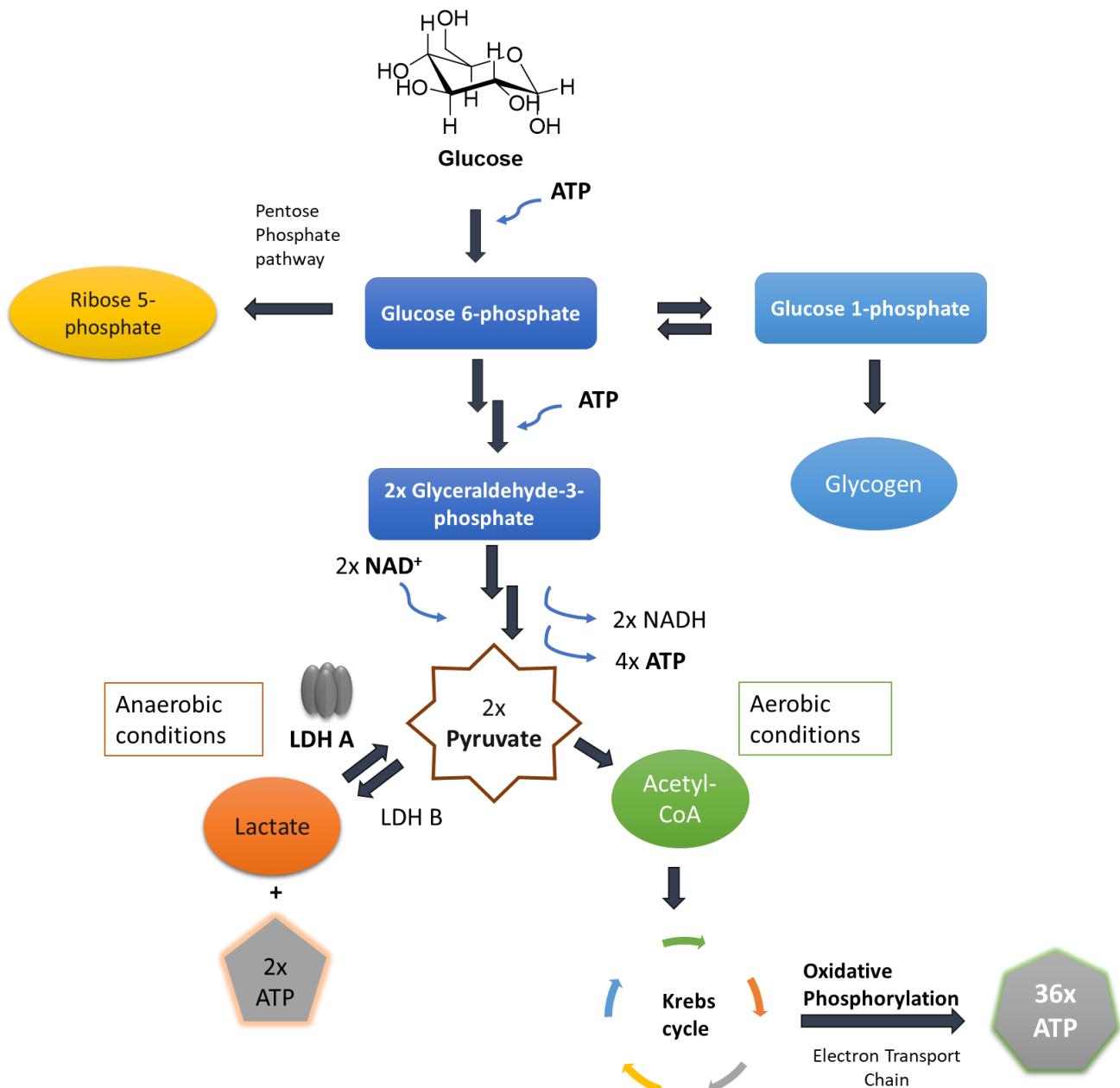


Figure 41 - Glycolysis is the first route in the breakdown of glucose to extract energy for cellular metabolism. Glycolysis is the source of metabolic intermediates that are used in parallel metabolic pathways, such as the pentose phosphate pathway (yellow) and the glycogenesis (light blue pathway).

Compared to the lactic acid fermentation, OxPhos is a more effective pathway for cells to get the energy necessary for their normal activity. However, cancer cells present an extensive reliance on glycolysis and lactate production, even under normoxic conditions and fully functioning of mitochondria. This deviation in metabolism is known as the “Warburg effect”, in honour of its

discovery by Otto Warburg in 1956.¹⁸⁹ Although human cancers display a wide range of metabolic profiles, the Warburg effect is present in most, if not all, types of cancer.¹⁹⁰

Solid tumours are characterised by the presence of hypoxic regions, which originate from an imbalance between blood supply and consumption of oxygen. Cancer cells possess an elevated rate of growth, so their metabolic requirements exceed the vasculature ability to provide oxygen and nutrients.¹⁹¹

Hypoxia provokes an overexpression of a big number of genes implicated in the cell survival, thus, hypoxia acts as a promoter of tumour progression. This phenomenon translates into changes in cellular behaviour and metabolism. For instance, hypoxic tumours overexpress the transcription factor HIF-1 α (hypoxia-inducible factor 1 α) as an adaptive response to hypoxia. This transcription factor up-regulates a series of downstream target genes, such as Glucose Transporter-1 (GLUT-1), Lactate Dehydrogenase A (LDH-A), together with Carbonic Anhydrase IX (CA IX), Vascular Endothelial Growth Factor (VEGF) and Lysyl Oxidase (LOX)^{192,193} (Figure 42).

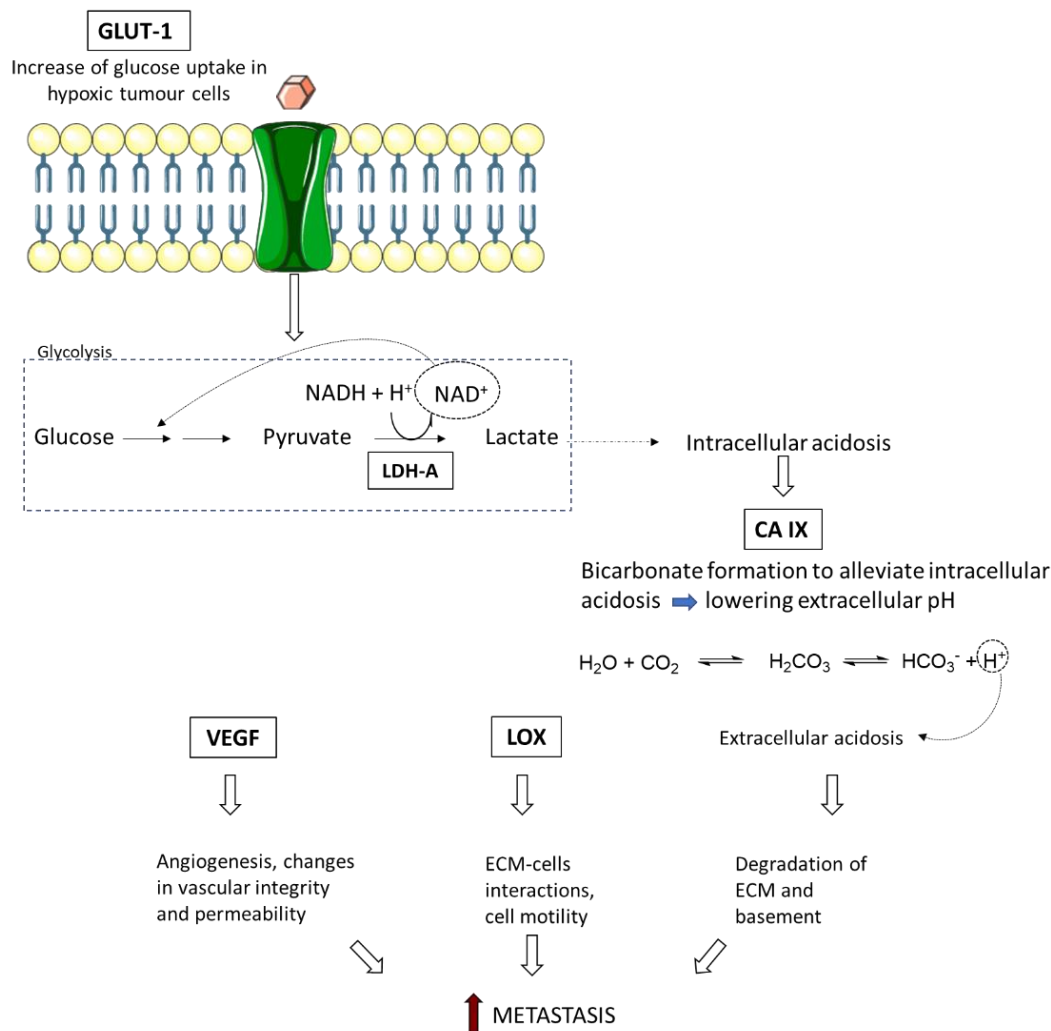


Figure 42 - Proteins (squared) up-regulated by HIF-1 α and their effect on cell metabolism in hypoxic environment. Key: ECM: extracellular matrix. Adapted from reference 191.

The principal objective of these proteins is to protect cells from the adverse hypoxic environment and to help them to proliferate and survive under hostile conditions. As a result, tumour cells can overcome the nutritive privation by increasing their vascularization through angiogenesis, boosting the glucose uptake, and optimizing their sugar metabolism. Furthermore, the overexpressed proteins mentioned above contribute to cell invasiveness because they provide a selective growth advantage over other competing cell populations. Taking all together, hypoxia triggers the development of tumours with a more malignant and aggressive phenotype.

Tumour cells found in hypoxic areas are highly dependent to glucose-fuelled anaerobic glycolysis, whereby they oxidize glucose to pyruvate and/or lactate. Likewise, oxidative tumour cells, present in the highly vascularized areas, can choose among several precursor substrates, depending on their availability (for example, lactate is oxidized to pyruvate), with the aim of fuelling OxPhos process.

One of the most important adaptive responses to hypoxia is the metabolic switch from oxidative to glycolytic pathway (Warburg effect), and here LDH-A plays a key role.

2.1.2 Human lactate dehydrogenase

*h*LDH isoforms

Human LDHs (*h*LDH) are a family of tetrameric isozymes, that is, enzymes which are coded by different genes, but phenotypically, they are very similar to each other in terms of protein sequence and activity.¹⁹⁴

In mammals, each tetramer is assembled from two different subunits, the M and H-type. These subunits merge originating 5 possible combinations, corresponding to five forms of LDH.¹⁹¹ Thus, there are two homotetramers: LDH-1 (H_4) and LDH-5 (M_4), which are commonly referred as LDH-H (or LDH-B) and LDH-M (or LDH-A), respectively, and three hybrid tetramers: LDH-2 (M_1H_3), LDH-3 (M_2H_2) and LDH-4 (M_3H_1) (Figure 43).

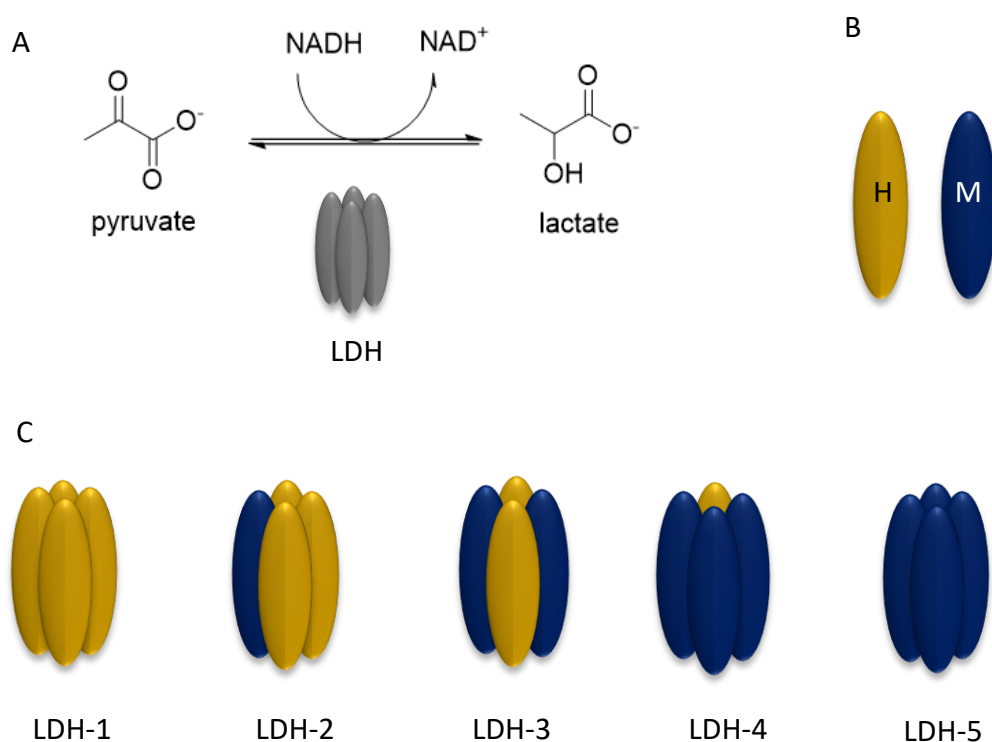


Figure 43 - LDH activity and isoforms. (A) LDH catalyses the redox interconversion between pyruvate and lactate with NADH as cofactor. (B) human LDH subunits H and M. (C) The functional LDH is a tetramer containing different ratios of the M and H subunits, which associate randomly. The five isozymes of LDH are shown.

The name of the subunits is derived from the tissues where they are most expressed. In humans, LDH-5 and LDH-4 are found mainly in anaerobic tissues such as skeletal muscle (M stands for muscle), liver and neoplastic tissues; LDH-3 is present in lymphatic tissues, platelets and malignant tissues. LDH-1 and LDH-2 predominate in tissues with aerobic metabolism as the main source of energy, such as spleen, heart, brain, kidney or erythrocytes.

These five isozymes are localized in the cytosol of somatic cells, although they also can be found in mitochondria. The distribution of each isozyme between cytosol and mitochondria also depends on the type of isozyme. For instance, the heart LDH-1 is mostly found in the mitochondrial matrix. By contrast, LDH-5 is equally present in mitochondria and cytosol, whereas the same isoform in liver is mainly present in mitochondria.

The catalytic activity of LDH

The general accepted catalytic mechanism of LDH involves the initial binding of NADH to its binding site in the enzyme followed by binding of the substrate, the pyruvate, forming a ternary complex LDH-NADH-pyruvate. This complex undergoes a rate-limiting conformational change, where a substrate specificity loop (residues 98-107) closes in order to bring the catalytical residue Arg109 into the active site. LDH possesses a hydrophobic active site which is quite buried inside the protein; thus,

it is not incongruous supposing that a considerable portion of protein does unfold so that the substrate reaches the binding pocket. Then, Arg109 can polarise the ketone moiety of pyruvate, promoting hydride transfer to the substrate (Figure 44).^{195,196}

Other important residues in the active site are Asp168, His195, Gln102, Arg171, and Thr246. They are involved in substrate discrimination and recognition, for example, by enclosing the methyl side chain of the substrate.¹⁹¹

The catalytic mechanism of pyruvate reduction into lactate involves a direct and stereospecific transfer of a hydride ion from the C4 carbon of the dihydronicotinamide ring of NADH to the ketone group of the pyruvate, with simultaneous proton donation from the catalytic pair Asp168/His195 to the carbonyl oxygen of pyruvate, yielding lactate (Figure 43A). His195 imidazole ring functions as proton donor-acceptor as well as it orientates the substrate in the proper position. Asp168, for its part, stabilises the cationic form of His195 through an H-bond between its carboxylic group and the protonated imidazole ring of His195. The residue Ile250 has been found to provide an environment suitable for the nicotinamide ring of NADH due to its hydrophobic side chain.¹⁹¹

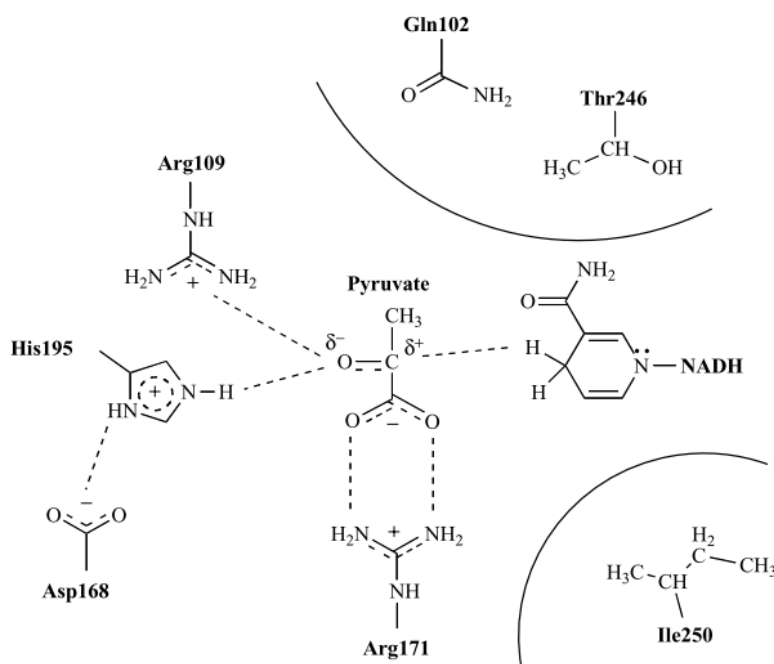


Figure 44 - Hydride and proton transfer occurring in the LDH active site. The cofactor NADH binds first and prepares the binding site for pyruvate binding. Arg109 polarises the carbonyl residue of pyruvate, which facilitates the hydride transfer between NADH and pyruvate, and the proton transfer from His195 and the substrate. Taken from reference 191.

All *h*LDH isoforms can catalyse the transformation of pyruvate into lactate, however, when the ratio H-M increases in favour of H subunits, the isozymes present higher efficiency in catalysing the reaction in the opposite direction, this is, the conversion of lactate into pyruvate via reduction of NAD⁺. If we recall the aerobic pathway to obtain ATP *via* cellular respiration, this modality of energy

production introduces the pyruvate into the Krebs cycle, which yields high energy molecules that are further transformed into ATP in the oxidation-phosphorylation. Thus, the presence of isozymes LDH-1 and LDH-2 (with four and three H subunits, respectively) in aerobic-dependent tissues is not casual: these two isoforms contribute to obtaining energy by catalysing the conversion of lactate into pyruvate.

*h*LDH-5 (or LDH-A) contains approximately 330 amino acids adopting a bilobal structure¹⁹⁷ consisting in a large and a small domain. Each monomer in the tetramer has full catalytic function. The NADH cofactor binding site is form by the large domain that adopts a Rossmann fold.¹⁹⁸ The small domain constitutes the substrate binding site, and it is characterised by a mixed α/β structure.

LDH and metabolic reprogramming in cancer

As we have discussed before, glycolysis is a catabolic pathway that depletes nicotinamide adenine dinucleotide (NAD⁺). For example, the oxidation of glyceraldehyde 3-phosphate requires NAD⁺ as an electron acceptor—it reduces to NADH (GAPDH step).¹⁸⁵ A recent study shows that low availability of NAD⁺ attenuates glycolysis at the GAPDH step, resulting in the accumulation of glycolytic intermediates before this step and a decrease of glycolytic intermediates after the step.¹⁹⁹ Cells contain a limited supply of NAD⁺, and the constant high-rate glycolysis found in tumoral tissues will deplete the NAD⁺ pool. Glycolysis cannot continue unless NAD⁺ is regenerated.

LDH-mediated reduction of pyruvate into lactate is coupled to the oxidation of NADH into NAD⁺, thus efficiently refilling NAD⁺. Typical traits of the glycolytic phenotype are high rate of conversion of glucose into lactate with an elevated glucose uptake. Both mechanisms ensure adequate ATP levels and constant supply of intermediates essential for cell growth and division.²⁰⁰

This behaviour in metabolism is maintained even when cells derived from hypoxic tumours are located under normoxic conditions, confirming that the conversion of pyruvate into lactate is not only a simple adaptive mechanism to hypoxia for energy obtaining, but most importantly, a hallmark of hypoxic tumours involving epigenetic transformations that deeply affect some cellular pathways.¹⁹¹ Therefore, cancer cells may limit glucose oxidation in order to maximally increase lactate conversion and NAD⁺ recycling, allowing the continuation of high-rate glycolysis.

In summary, a high glycolytic rate has several advantages for rapidly proliferating cells: ²⁰¹

First, despite its low efficiency at producing ATP compared to OxPhos in the mitochondria, anaerobic glycolysis can generate ATP at a faster rate when the supply of glucose is abundant.

Second, the carbon building blocks necessary for *de novo* synthesis of nucleotides, lipids, and non-essential amino acids required from proliferating cells are provided by glycolytic intermediates.

Finally, the maintenance of biosynthesis in fast-growing cells requires the regeneration of NAD⁺, which is partially supported by the conversion of pyruvate to lactate.

Hypoxia as an Obstacle to Medical Treatment of Cancer

“Oxygen enhancement effect” is the name given to the phenomenon observed in poor oxygenated tissues, which are resistant to standard therapies such as radiotherapy.²⁰² The link between hypoxia and radio-resistance can be explained considering that DNA damage is usually induced by oxygen radicals such as hydroxyl radical, superoxide anion etc., or provoked by direct ionization from ionizing radiations. In the presence of oxygen, O₂ reacts with the broken DNA forming stable organic peroxides which are hardly repaired by the cell, leading to important chromosome aberrations. Basically, oxygen fixes the DNA damage, making it permanent. By contrast, under hypoxia conditions, the damage is more easily repaired: the broken DNA can be restored to its original form, thanks to reparative processes based upon reductions by –SH containing intracellular components. This mechanism explains why tumours with low levels of oxygen present a reduced effect on response to radiotherapy, compared with normal oxygenated tissues.^{202–204}

Some anticancer drugs fail in defeating hypoxic cells because their mechanism of action: chemotherapy is usually more efficient against cells with high rates of proliferation. However, hypoxic tissues present a slower rate of division. Moreover, hypoxia selects cells that overexpress genes encoding for proteins involved in drug resistance.^{203,205} A very recent study demonstrated that LDH-A is also responsible for the resistant of cells to TMZ therapy.²⁰⁶ In that study, tests performed on glioblastoma cell lines showed that the combination of LDH-A knockdown with TMZ treatment significantly reduced cell viability of tumoral cells.

In addition to radiotherapy and chemotherapy resistance, hypoxic cancer cells possess more malignant phenotypes, which predispose to the formation of metastases, thus compromising curability of tumours by surgery.²⁰⁷

Nevertheless, hypoxic tumours present a unique feature that is common to many cancer tissues and it lacks in healthy tissues: the metabolic switch associated to the Warburg effect. This phenomenon can be exploited in targeted cancer therapy.

Taking advantage of Tumour Hypoxia: LDH-A inhibition

LDH-A enzyme is overexpressed in many tumours such as pancreatic cancer, prostate cancer, gastric cancer, gliomas and cutaneous melanoma metastases.^{208–211}

When LDH-A levels are knocked down *via* shRNAs (short hairpin RNA) in tumour cell lines that show high glucose dependency and high LDH-A expression under both normal and hypoxic conditions, the ability of these cell populations to proliferate shows a noticeable reduction.²¹²

Furthermore, LDH-A silencing enhances oxygen consumption via OxPhos metabolism, which results in elevated level of mitochondrial reactive oxygen species (ROS) (cellular respiration generates ROS even under physiological conditions). Exposure to cellular stressors such as ROS can trigger p53 tumour suppressor (a sequence-specific transcription) which induces cell growth arrest or apoptosis.²¹³

ROS are powerful regulators of Ca^{2+} signalling, and as a consequence, knocking down the LDH-A causes an increase of intracellular levels of Ca^{2+} (~2.9-fold; $p < 0.001$), which may be involved in triggering apoptosis, among other mechanism, via an activation of apoptotic endonucleases.^{214,215} Simultaneously, humans suffering from complete LDH-A deficiency due to an inherited 20-bp deletion in the LDHA gene, which results in the loss of LDH-A protein, have been studied. This condition has only relatively mild symptoms of exertional myopathy and myoglobinuria after intense anaerobic exercise, without showing any symptoms under ordinary circumstances.^{216–218}

In summary, LDH-A constitutes a crucial checkpoint in the bifurcation point of glycolysis, and it can be blocked with a limited risk of affecting “normal” glucose metabolism through OxPhox. Therefore, LDH-A constitutes a valid target for the development of antiglycolytic agents against cancer.

2.1.3 Lactate dehydrogenase A inhibitors

The interest on LDH inhibitors as therapeutic agents rose after *Plasmodium falciparum*, the parasite that produces malaria, developed resistance against almost all the existing anti-malarial drugs, especially against chloroquine.

Plasmodial metabolic pathways are quite different from human's, if not absent. During its life cycle in human beings, the parasite lacks a functional Krebs cycle, and presents a total dependence on the glycolytic pathway for the obtention of ATP.

The LDH in the parasite, *pf*LDH, possesses key structural features that make it different from human LDH. A wide range of chemical structures has been developed aiming the selective inhibition of *pf*LDH over hLDH.¹⁹¹ Many of them presented activity against both enzymes, if some showed higher affinity for the human dehydrogenase. Their chemical structures served as starting point for designing new LDH-A inhibitors.^{219–221}

Gossypol and its derivatives

Gossypol is a polyphenolic binaphthyl disesquiterpene compound (Figure 45) obtained from the *Gossypium* species (cotton plant). It participates on redox reactions catalysed by NAD^+/NADH -based enzymes such as many dehydrogenases, which possess a common structural homology showing the typical dinucleotide “Rossmann fold”. It possesses antifertility action due to the inhibition of LDH- C_4 ²²² and antimalarial activity by inhibition of *pf*LDH. This toxicity and low selectivity questioned its use as therapeutic agent, and some derivatives have been developed.¹⁹¹

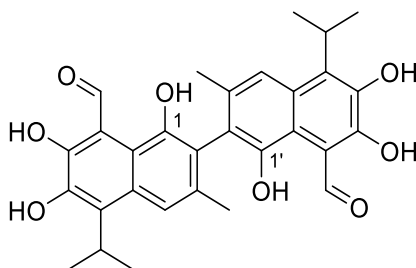


Figure 45 - Gossypol chemical structure.

Some structural modifications include the removal of the dimeric fragment of gossypol and/or acylation of the 1 and 1' (peri) positions, or simply removal of these hydroxy groups. The CHO functional group has been also replaced by nitriles, obtaining peri-acylated gossylic nitriles derivatives. Despite of having K_i in the nano and low micromolar range, none of these derivatives has shown a better selectivity than gossypol and most part of them are better inhibitors of the *pf*LDH.

Naphthoic acids

Another important group of LDH inhibitors are the naphthoic acid family, composed by a naphthalene scaffold substituted by carboxylic acid or sulphonic acid groups.²²³ This family showed very low potency for both human and *pf*LDH. Compound FX-11²²⁴ was an exception (Figure 46), reaching IC_{50} values of 50 nM against LDH-A on enzymatic assays. However, Ward *et al.*²²⁵ fail to find significant binding affinity by NMR and Surface plasmon resonance.

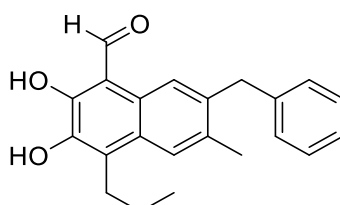


Figure 46 – Structure of naphthoic acid derivative FX-11.

Piperidinone derivatives

Genentech (CA, USA) reported a family of piperidinone derivatives with potent LDH-A inhibitory activity (IC_{50} in the nanomolar range).²²⁶ Some examples are shown in the next figure:

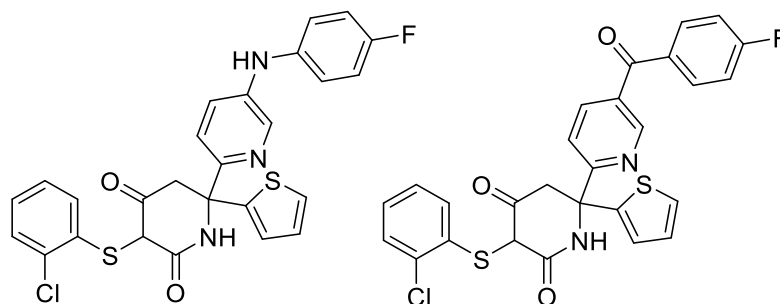


Figure 47 - Examples of piperidone-based LDHA inhibitors.

Pyrazole and indole derivatives

The research group lead by F. Minutolo reported a series of glucose-conjugated methyl esters of *N*-hydroxyindole-2-carboxylates that were able to inhibit LDH-A (Figure 48).²²⁷ Among them, the glycosylated analogue of NHI-2, showed good cellular activity. Although having a higher K_i constant in enzymatic assays with isolated enzyme ($K_i = 37.8 \mu\text{M}$), than NHI-2 ($K_i = 5.1 \mu\text{M}$), the glucose fragment enhanced its cellular uptake, via GLUT-1 transporters, obtaining higher cytotoxic activity on HeLa cells.²²⁸

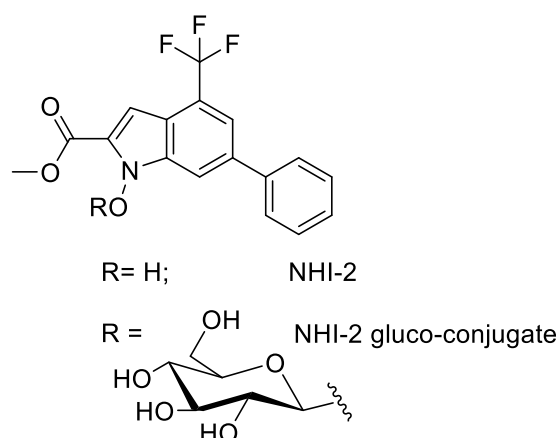


Figure 48 - Structures of NHI-2 and its glucose-conjugated analogue.

Other types of pyrazolo- and indole-based analogues have been discovered (Figure 49) by screening of large compound databases.²²⁹ These derivatives showed high inhibitory activity against LDH-B. However, these compounds showed also good selectivity for other dehydrogenases, such as glyceraldehyde 3-phosphate.

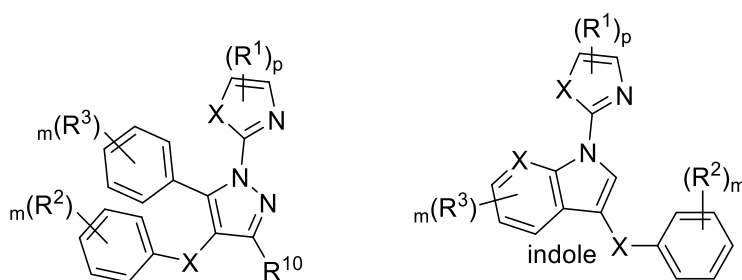


Figure 49 - General chemical structure of pyrazole (left) and indole derivatives (right).

Pyrazolidine derivatives

A family of pyrazolidine derivatives showed IC_{50} between 10 and 50 μM in carcinoma cell lines (Figure 50). These compounds showed affinity for both LDH-A and LDH-B.²³⁰

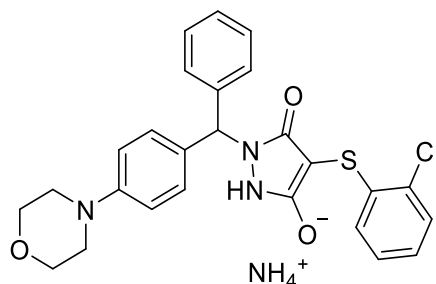


Figure 50 - Example of pyrazolidine analogue with LDH inhibitory activity.

Pyrazolylthiazole derivatives

Very recently, novel LDH inhibitors containing a 2-(1*H*-pyrazol-1-yl)thiazole-4-carboxylate scaffold have been reported. An example is shown in Figure 51. This compound was active in biological assays for the determination of inhibitory activity on LDH. It showed an IC_{50} value lower than 100 nM in LDH-A inhibition assays and an IC_{50} value lower than 1 μM in the cellular inhibition of lactate production.²³¹

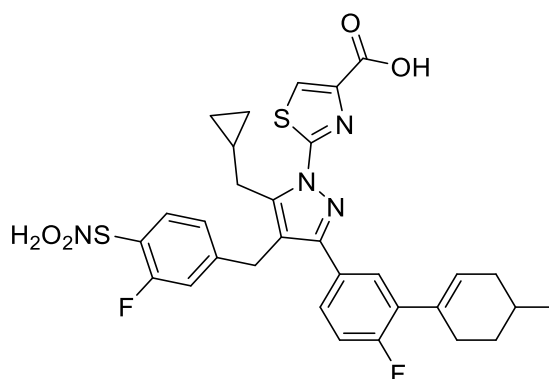


Figure 51 - Pyrazolylthiazole derivative active as LDHA inhibitor.

4-aminoquinoline derivatives

A patent application of GSK for LDH inhibitors contained compounds with the 4-aminoquinoline scaffold.²³² One of the most potent derivatives is shown in Figure 52, where the quinoline motif has a sulphonamide group at position 3, and the nitrogen atom at position 4 carries an aryl ring containing a carboxylic acid. This compound showed a certain level of selectivity for LDH-A over LDH-B. Assays with this compound in Snu398 cells (hepato-carcinoma cell line) resulted in promotion of apoptosis.

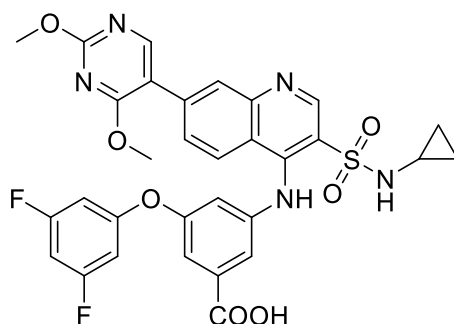


Figure 52 - 4-aminoquinoline derivative.

Malonic derivatives

The diacid malonate-derivative AZ-33 was discovered by NMR fragment-based approaches at AstraZeneca UK as LDH-A inhibitor. It showed an IC_{50} of 0.5 μ M on enzymatic assays with isolated enzyme, however, this compound lacked any cellular activity.²²⁵

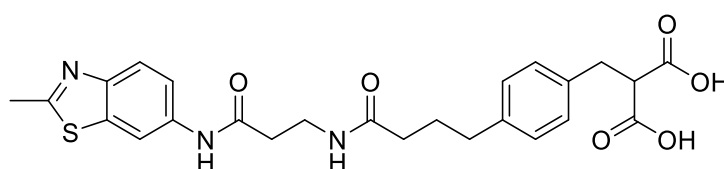


Figure 53 - Chemical structure of the *h*LDH5 inhibitor AZ-33.

Nicotinic acid derivatives

With a similar approach to AZ-33, scientists from ARIAD Pharmaceuticals followed a similar fragment-based approach to discover a family of LDHA inhibitors by linking fragments of different picolinic acid analogues.²³³ Methyl esters analogues were also evaluated. Acid derivatives showed better activity than their ester counterparts and reached the low IC_{50} values in micromolar range in the enzymatic assay, as well as the K_D on SPR assays.

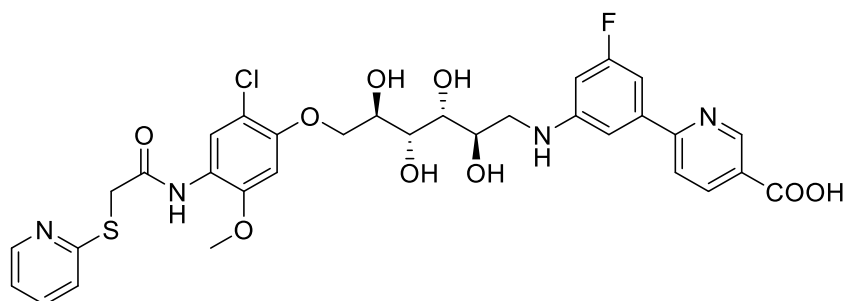


Figure 54 - Example of nicotinic acid derivative developed as LDH-A inhibitor.

2.1.4 Enzymology: a short introduction

Enzymes are macromolecules that catalyse the chemical transformation of one molecule, named substrate, into a different molecular entity, the product. This reaction implies that enzyme and substrate must meet and form a binary complex through the binding of the substrate to a specific site on the enzyme macromolecule, the active site.²³⁴

All the equations presented in this section and their corresponding dissertation have been adapted from several books written by R. Copeland.^{235,236} We will start this short review of key concepts in enzymology by introducing the **equilibrium dissociation constant, K_D** .

K_D describes the equilibrium between free and ligand-bound receptor molecules. Assuming, for now, that the receptor has a single binding site for the ligand, and the only states of any ligand molecule are either free or bound to a receptor molecule, we can obtain a pair of mass conservation equations to describe it:²³⁴

$$[R] = [RL] + [R]_f \quad \text{Equation 2.1}$$

$$[L] = [RL] + [L]_f \quad \text{Equation 2.2}$$

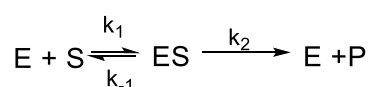
where $[R]$ and $[L]$ are the total concentrations of receptor and ligand, respectively, $[R]_f$ and $[L]_f$ are the free concentrations of the two molecules, and $[RL]$ is the concentration of the binary receptor—ligand complex. Under any specific set of solution conditions, an equilibrium will be established between the free and bound forms of the receptor. The position of this equilibrium is most commonly quantified in terms of the dissociation constant, K_D :

$$K_D = \frac{[R]_f [L]_f}{[RL]} \quad \text{Equation 2.3}$$

The strength of binding, or as Robert Copeland states in his book, “the relative affinities of different receptor-ligand complexes are inversely proportional to their K_D values: the tighter the ligand binds, the lower the value of the dissociation constant”.²³⁴

The rapid equilibrium model of enzyme kinetics

Since early in the 20th century, it is accepted among enzymologists that the reaction catalysed by an enzyme can be described by the following reaction scheme:



Scheme 2.1- General scheme of enzyme-catalysed reactions provided by Brown in 1902.²³⁷

The equilibrium between two components, the enzyme E and substrate, S, forming a complex, ES, will be governed by the rate of complex formation (association rate constant, k_1) and by the rate of dissociation of the formed complex, (dissociation rate constant k_{-1}).²³⁴

The equilibrium dissociation constant, K_D is given by the ratio k_{-1} to k_1 :

$$K_D = \frac{k_{-1}}{k_1} \quad \text{Equation 2.4}$$

Equilibrium constants k_{-1} and k_1 are also referred to with the general terms k_{off} and k_{on} , respectively.

$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}} \quad \text{Equation 2.5}$$

Thus, K_D is giving us information about the affinity of the substrate for an enzyme and ES complex formation.

When illustrating the rate of enzyme activity versus increasing substrate concentrations, rather than observing a linear relationship, enzymologists found that the velocity approached asymptotically to maximum velocity, V_{max} . The velocity slowed down at high substrate concentrations (Figure 55).

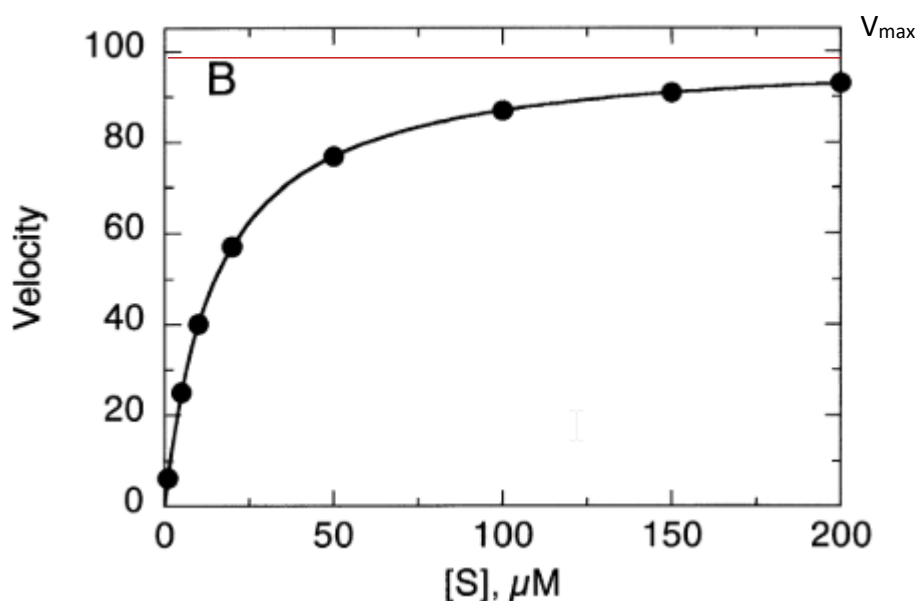


Figure 55 - Enzyme activity rates measured at different substrate concentrations. Data are fitted by a non-linear regression. Taken from reference 234.

The model initially proposed in scheme 2.1, and the observed behaviour of the enzyme in the reaction progress need of an equation that could be used by experimental scientist for studying

enzymes kinetics. This was first achieved by Henri (1903) and Michaelis-Menten ten years later (1913).²³⁷ The Henri—Michaelis—Menten approach assumes that a rapid equilibrium is established between the reactants ($E + S$) and the ES complex, followed by slower conversion of the ES complex back to free enzyme and product(s); this is, this model assumes that $k_2 \ll k_{-1}$ (Scheme 2.1). Based in this assumption, the mathematical expression that describes the enzyme behaviour was:

$$v = \frac{V_{max}[S]}{K_d + [S]} = \frac{V_{max}}{1 + \frac{K_d}{[S]}}$$

Equation 2.6

Equation 2.6 was derived independently by Henri and Michaelis and Menten to describe enzyme kinetic data in the rapid equilibrium state.²³⁴ It possesses a remarkable similarity with the equation and the forms of equation firstly derived by Langmuir (Figure 56).

$$[RL] = \frac{[R][L]_f}{K_d + [L]_f} = \frac{[R]}{1 + \frac{K_d}{[L]}}$$

Equation 2.7

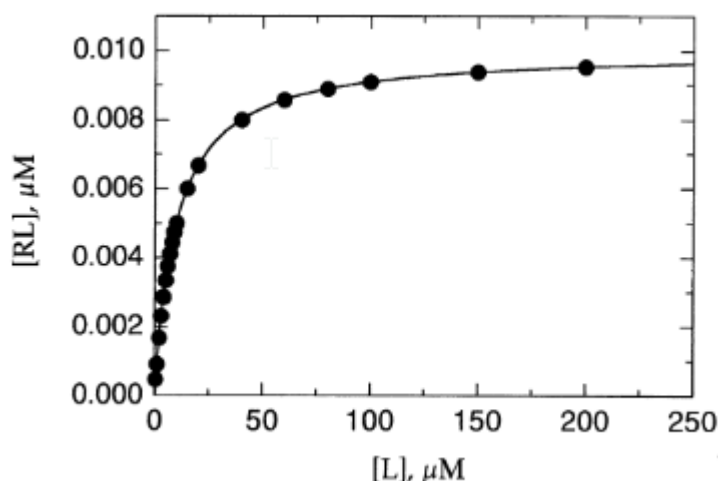


Figure 56 - Langmuir equation (Equation 2.7) and its representation (right graph, the Langmuir isotherm) for the formation of the binary complex, RL. Taken from reference 234.

The equation describes a square hyperbola that is typical of saturable binding in a variety of physical, chemical, and biochemical situations. Langmuir derived this equation to describe adsorption of gas molecules as function of pressure under constant temperature, this is, under isothermal. In his honour, the equation is known as the *Langmuir isotherm equation*.

The steady state model of enzyme kinetics

The Henri-Michaelis-Menten model was based on a rapid equilibrium approach to enzyme reactions. However, most of experimental measurements of enzyme reactions take place when the ES complex is present at a constant, steady state concentration.

As described by Copeland, “steady state refers to a time period of the enzymatic reaction during which the rate of formation of the ES complex is exactly matched by its rate of decay to free enzyme and products”.²³⁷

To achieve a steady state, certain conditions must be met:²³⁷

- a) During the initial phase of the reaction progress, this is, conditions which measures the linear initial velocity) there is no appreciable formation of any intermediates other than the ES complex. Thus, all the enzyme molecules possibly found are either free enzyme [E] or enzyme-substrate complex [ES].

$$[E]_t = [E] + [ES]$$

- b) The total amount of S can be obtained by the free substrate plus the substrate forming the ES complex

$$[S]_t = [S] + [ES]$$

The enzyme works catalytically, so in the rapid equilibrium established between S and E, the concentration of substrate is very high compared to that of the enzyme ($[S] \gg [E]$). The formation of the ES complex barely decreases the concentration of free substrate, so the approximation $[S]_t \sim [S]$ can be assumed.

- c) At the start of the reaction will be fast burst of formation of the ES complex followed by a kinetic phase where the formation of the ES complex is balance by the dissociation of such complex into free enzyme and products. This is the steady state, defined by

$$\frac{d[ES]}{dt} = 0$$

With these assumptions made, we can now elaborate an expression for the enzyme velocity under the steady state conditions as a function of the [S].²³⁷ Recalling our scheme of the simplest of enzymatic reactions (Scheme 2.1), the pseudo-first-order progress curve for an enzymatic reaction (this is, the velocity, V_0) can be described as:

$$V_0 = k_2[ES] \quad \text{Equation 2.8}$$

The rate of the reaction depends on the concentration of the ES complex. At the same time, [ES] is dependent on the rate of formation and dissociation of the complex.

The rate of ES complex formation is given by equation 2.9:

$$[ES] = k_1[E][S] \quad \text{Equation 2.9}$$

ES complex can dissociate in two directions, either to form E + S or E + P (Scheme 2.1). The rate of dissociation of ES is described as follows:

$$ES = k_{-1}[ES] + k_2[ES] \quad \text{Equation 2.10}$$

The steady state requires that the formation of ES complex is balanced by its dissociation into free enzyme and products and substrate.²³⁷ In other words, a moment in which the rate of ES formation (Equation 2.9) is equal to the rate of ES dissociation (Equation 2.10). Thus:

$$k_1[E][S] = k_{-1}[ES] + k_2[ES] \quad \text{Equation 2.11}$$

This can be arranged to give the equation:

$$k_1[E][S] = (k_{-1} + k_2)[ES] \quad \text{Equation 2.12}$$

and therefore,

$$\frac{[E][S]}{ES} = \frac{k_{-1} + k_2}{k_1} \quad \text{Equation 2.13}$$

Instead using these three constants ratio, we use a new constant named Michaelis constant, K_m :

$$\frac{[E][S]}{ES} = \frac{k_{-1} + k_2}{k_1} = K_m \quad \text{Equation 2.14}$$

And now we can rearrange the equation as follows:

$$ES = \frac{[E][S]}{K_m} \quad \text{Equation 2.15}$$

Bearing in mind the conditions assumed in the steady state, this is, $[E]_t = [E] + [ES]$ and $[S] \sim [S]_t$, we can rewrite equation 2.16:

$$ES = \frac{([E]_t - [ES]) [S]}{K_m} \quad \text{Equation 2.16}$$

Which can be simplified as:

$$ES = \frac{[E]_t [S]}{K_m + [S]} \quad \text{Equation 2.17}$$

If we now combine equation 2.17 with the expression of the velocity expression in equation 2.8, we obtain:

$$v_0 = k_2 \frac{[E]_t [S]}{K_m + [S]} \quad \text{Equation 2.18}$$

Recalling now the reaction reaches a maximum velocity, V_{max} , where all the enzyme's active sites are occupied, the equation that describes that enzymatic state is:

$$V_{max} = k_2 [ES]_{max} \quad \text{Equation 2.19}$$

If all the enzyme's active sites are occupied, this means that no free enzyme is found, so the equation $[E]_t = [E] + [ES]$ can be simplified as $[E]_t = [ES]$ and therefore:

$$V_{max} = k_2 [E]_t \quad \text{Equation 2.20}$$

Rearranging equation 2.20, the total enzyme concentration is:

$$[E]_t = V_{max} / k_2 \quad \text{Equation 2.21}$$

Including this expression in equation 2.19, we finally obtain the central expression for steady state enzymatic kinetics that describe our curve represented in Figure 56:

$$v_0 = V_{\max} \frac{[S]}{K_m + [S]} = \frac{V_{\max}}{1 + \frac{K_m}{[S]}}$$

Equation 2.22

Although equation 2.22 differs from the original equilibrium expression derived by Henri and by Michaelis-Menten (equation 2.6), this equation is nevertheless universally referred to as the Michaelis—Menten equation.

Furthermore, if we compare these equations, 2.22 and 2.6, the former for steady state conditions and the latter for the rapid equilibrium treatment, we realise that the equations are identical except for the substitution of K_D for K_m . However, the two constants are not equal. Recall that K_d is defined by the ratio of the reverse and forward reaction rate constants ($K_D = k_{-1} / k_1$; equation 2.4).

This value is different from the expression for K_m given in equation 2.7. Only when $k_2 \ll k_{-1}$, K_m and K_D are equivalent. Otherwise, depending on the enzymatic system, K_m value can be greater than, equal to or less than K_D . Therefore, K_m should be considered as a kinetic, not thermodynamic constant.²³⁷

The significance of K_m – Interpretation of the Michaelis-Menten equation

We have just derivate the equation 2.22 that explains the behaviour of an enzyme and how its velocity catalysing a reaction changes as the concentration of substrate increases.

When plotting the V_0 as function of $[S]$, we obtained a curve as the one represented in the next figure:

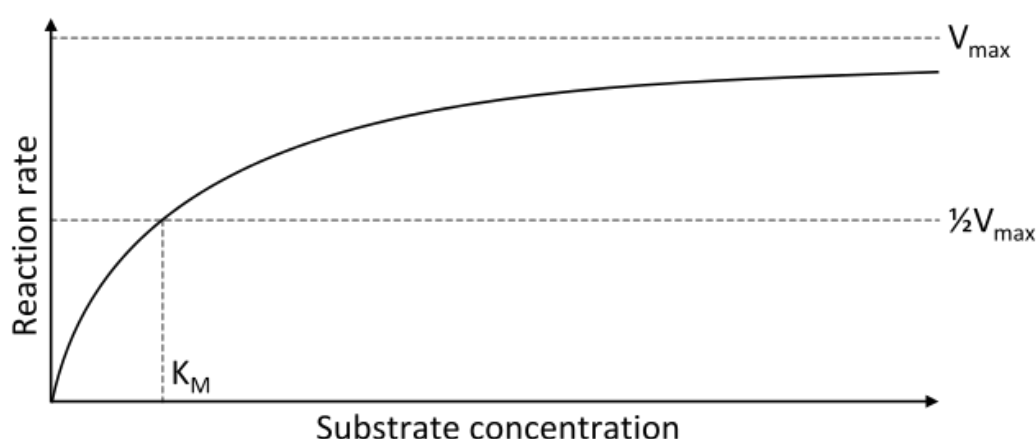


Figure 57 - Saturating curve representing the variation of the reaction rate of an enzyme as function of substrate concentration $[S]$. Michaelis-Menten constant (K_M) and $V_{\max}$ values are also indicated. At

the beginning of the reaction, there is a relative straight line and then, the velocity starts to decrease and approach asymptotically to the V_{max} value.

What value do K_m add to the understanding of the enzyme under study?

First, we will simplify the Michaelis-Menten equation. Because K_m has the same units as $[S]$, we will assume that K_m value is equal to $[S]$:

$$K_m = [S] \quad \text{Equation 2.23}$$

Now we can substitute this value on the Michaelis-Menten equation (equation 2.22), and obtain:

$$V_0 = V_{max} \frac{[S]}{[S] + [S]}$$

Equation 2.24

Which can be simplify as follows:

$$V_0 = V_{max} \frac{[S]}{2[S]} = \frac{V_{max}}{2}$$

Equation 2.25

This simplification has a relevant physiological meaning: when the K_m constant is equal to that particular substrate concentration $[S]$, the velocity of the enzyme is exactly half of the maximum velocity of that particular enzyme (Figure 16).²³⁷

In other words, the K_m is the substrate concentration $[S]$ at which the velocity of that enzyme's activity is exactly half of its maximum velocity, V_{max} .

K_m describes the situation in which exactly half of all the active site of the enzyme are occupied with the substrate in the steady state.

K_m value varies considerably from one enzyme to another, and for a specific enzyme with different substrates. Although K_m is not an equivalent to K_D (the constant that measures the formation of the ES complex), it can nevertheless be used as a relative measure of substrate binding affinity.²³⁷

“Apparent” Michaelis-Menten constant, K_m'

A competitive inhibitor obstructs binding of substrate to enzyme, hence raising the K_m value (this new K_m value is the “apparent” Michaelis-Menten constant, K_m'), without affecting the V_{max} .

The relationship between K_m and K_m' (or K_{mapp}) is described by equation 2.26:²³⁷

$$K_{m_{app}} = K_m \left(1 + \frac{[I]}{K_i} \right) \quad \text{Equation 2.26}$$

where I is the concentration of inhibitor and K_i is the dissociation constant for the enzyme-inhibitor complex.

Increasing concentrations of inhibitor displace the midpoint of the binding isotherm towards the right (Figure 58). This change is indicative of a “loss” of affinity of the S for the enzyme, however, competitive inhibition can be overcome by increasing substrate concentration.

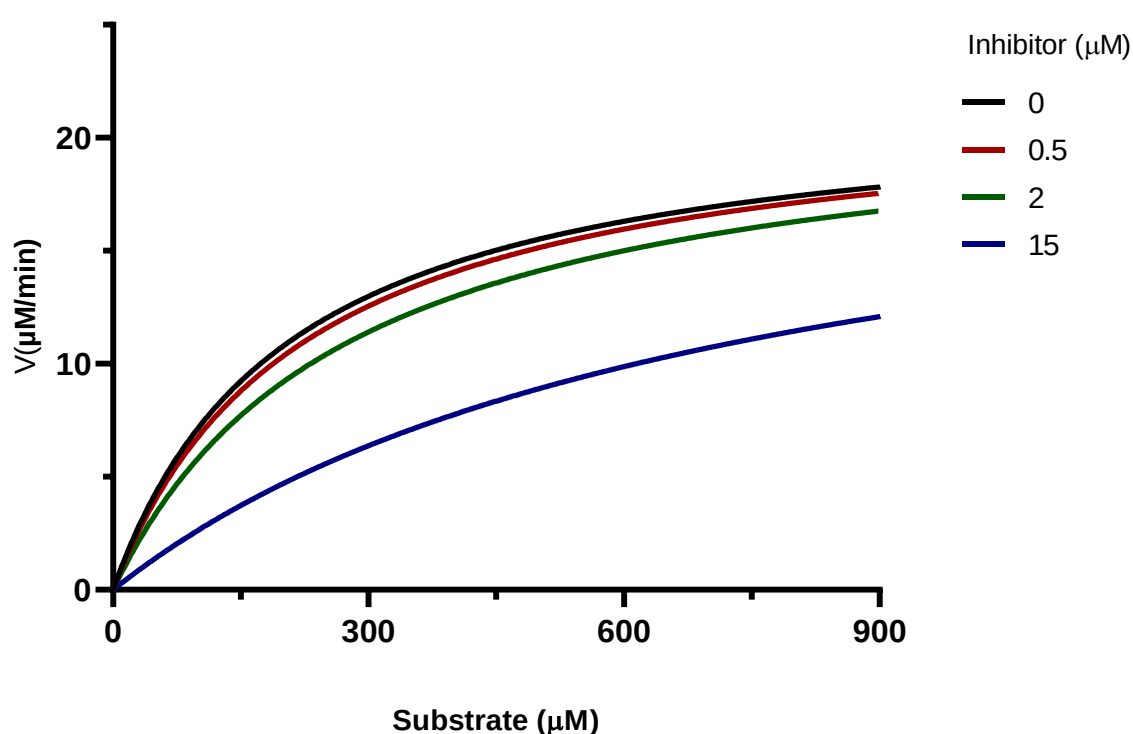


Figure 58 - Substrate titration of steady state velocity for LDH-A in the presence of an inhibitor at varying concentrations.

In the present chapter we will study to what extent the compounds under study will alter the K_m values of pyruvate and NADH, both the natural substrate and cofactor of LDH-A enzyme, to exert inhibitory activity with the enzyme.

2.2 HYPOTHESIS, AIM AND OBJECTIVES

Cancer cells differ from healthy tissues because of their metabolism. They preferentially obtain energy through glycolysis rather than oxidative phosphorylation. This so-called Warburg effect is based on the over expression and strong dependence of tumoral cells on LDH-A to obtain energy. This deviation from normal metabolism is a common feature of cancer processes and makes of LDH-A a perfect target for antitumoral therapies.

The aim of the present study was to discover novel human Lactate Dehydrogenase A inhibitors for their use as antitumoral agents.

In particular, the objectives pursued in this chapter were the following:

- 1- To screen the library of compounds present in the research group and unveil potential new LDH-A inhibitors.
- 2- To synthesize and characterize new derivatives of the selected compounds, with the goal to improve their inhibition activity.
- 3- To perform enzymatic based assays and Surface Plasmon Resonance experiments to assess the potency of each inhibitor via derivation of the kinetics parameters (K_i , K_d , IC_{50}).
- 4- To evaluate the cytotoxic activity against a specific type of cancer cells: human glioblastoma cell line.
- 5- To determine the Structure-Activity relationships of the target compounds based on the outcomes of the enzymatic assays, as well as to apply chemical modifications for improving the inhibition potency.

2.3 RESULTS AND DISCUSSION

The compound library belonging to our research group was screened via enzymatic assays to detect potential novel active LDH inhibitors. As a result, several compounds with a quinone-based core structure showed good inhibitory activity, and were selected for their further development and characterization as LDH-A inhibitors.

2.3.1 Biologically active quinones

Quinones derivatives are part of the chemical structure of many compounds with a great variety of functions, some of them naturally occurring. They play key roles in the metabolism of cells, including respiration and photosynthesis. For instance, coenzyme Q works as electron carrier in the respiratory chain, whereas vitamin K (Figure 59) is fundamental for blood coagulation and carboxylation of glutamate.²³⁸ Quinones and naphthoquinones can function as cellular mechanism of defence. For example, juglone (from *Juglans nigra*) and plumbagin (from *Plumbago rosea*) are naturally produced in plants against fungi and bacteria.²³⁹ Quinones are even active against parasite (e.g. as trypanocidal agents).²⁴⁰ Very recently, a series of naphthoquinone derivatives has been found to avoid aggregation of amyloid proteins such as β -amyloid, tau protein and insulin.²⁴¹ Naphthoquinones have shown anti-inflammatory and antipyretic activities,²⁴² as well as potent antitumoral agents approved for clinical use (doxorubicin, mitomycin C and mitoxantrone, among others).^{243,244}

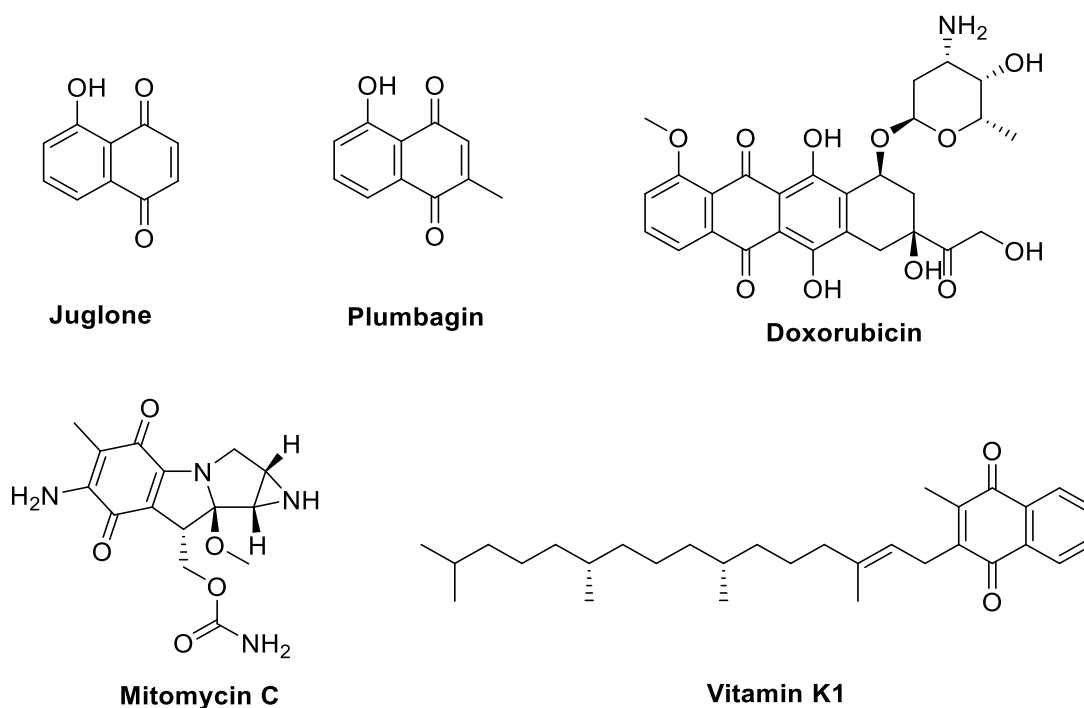


Figure 59- Chemical structures of some quinone-base compounds with different physiological and pharmacological activities.

In the present work, naphtho- and anthraquinones have been conjugated to different *L*-amino acids with the aim of exploring their potential activity as human LDH inhibitors, which, to our knowledge, it remains unreported in the literature.

2.3.2 Synthesis of Quinone-Amino acid Conjugates

The synthesis of the quinone-amino acids hybrids family pivots on the idea of using *L*-tryptophan as the amino acid fragment in combination with two commercially available quinone derivatives. The selected quinone fragments were the 1,4-naphthoquinone and the 1,4-anthraquinone (Figure 60).[‡]

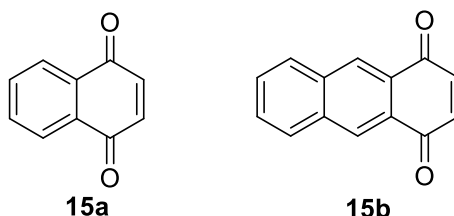
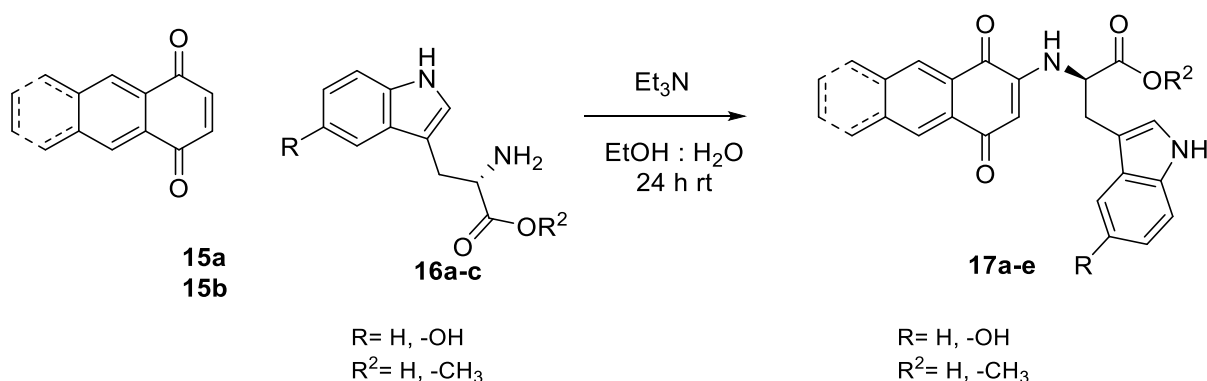


Figure 60 - Quinone-based fragments used in this study: the 1,4-naphthoquinone, **15a**, and the 1,4-anthraquinone, **15b**.

The reaction involves two steps: the conjugate addition of the amine on the quinone analogue and the subsequent aromatization of the product to yield the quinone-amino acid conjugate with general formula **17** (Table 10).

The experimental conditions for these reactions were previously described by Katritzky and co-workers.²⁴⁵ Briefly, the amino acid and the quinone were dissolved in a mixture of EtOH and water and stirred for 24 h at room temperature. Table 10 summarises the conditions and general structure of naphtho- and anthraquinone-*L*-tryptophan conjugates:

Table 11 - Synthesis of quinone- *L*-tryptophan conjugates (**17a-e**) via Michael addition and re-oxidation.



[‡] Many of the compounds here described has been previously presented in the Master's project entitled "Síntesis de nuevos radiotrazadores 18F-PET con esqueletos heterocíclicos novedosos para el diagnóstico in vivo de la enfermedad de Alzheimer y otras tauopatías" (Álvaro González Molina, 2017)

Entry	15	16	R	R ²	17	Yield (%)
1	15a	16a	H	H	17a*	61
2	15a	16b	CH ₃	H	17b	63
3	15a	16c	H	OH	17c	57
4	15b	16a	H	H	17d	68
5	15b	16c	H	OH	17e	52

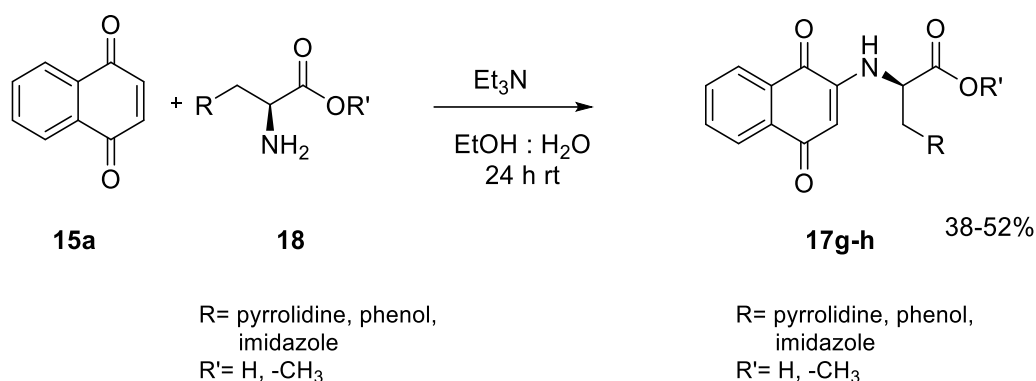
**17a has been previously described by Kratritzky et al.²⁴⁵*

The quinone-amino acid conjugates were obtained in moderated yields, due to the arduous extraction and purification steps. Amino acids are zwitterionic species, and the determination of the pH value at which the isoelectric point was reached was hard to determine.

The extraction procedure was optimized from the conditions described in the literature.²⁴⁵ Those conditions included, first, direct purification by column chromatography eluting with hexane: ethyl acetate under gradient conditions; and second, extraction of the yielded naphthoquinone-amino acid triethyl ammonium salts with dichloromethane and acidified water (HCl 4M) and then with brine. This methodology was improved by altering the order, this is, firstly the extraction (starting with a saturated aqueous solution of Na₂CO₃) and then, washing the organic phases with HCl 4M. Finally, the crude was purified by column chromatography affording the desired compounds in yields indicated above (Table 10).

More amino acids were employed for their conjugation with 1,4-naphthoquinone, **2a**. The selected amino acids were those which possess a cycle in their side chain. Hence, new naphthoquinone hybrids were obtained with tyrosine, proline, and histidine.

The applied conditions for the obtaining of these new quinone-amino acids hybrids were equal to those described in Table 10 and are summarised in scheme 2.2:

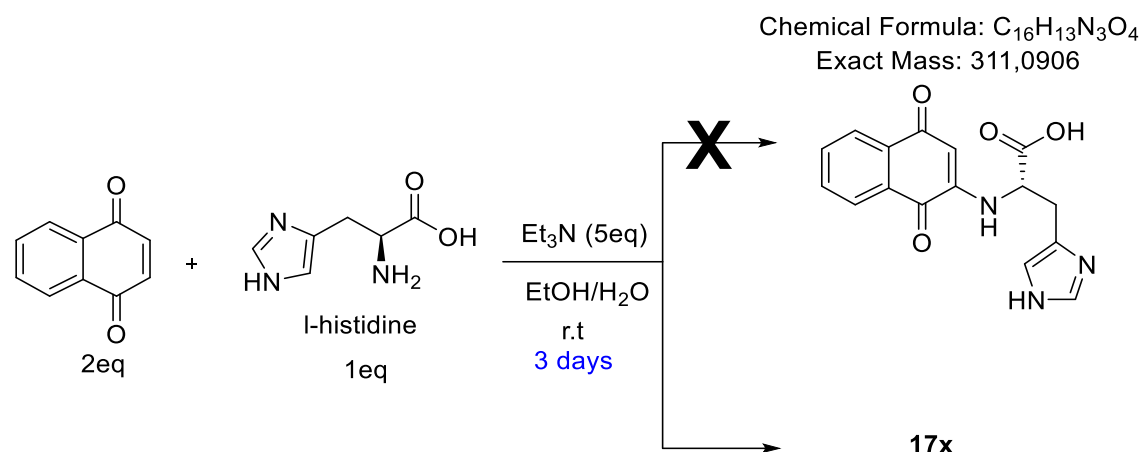


Scheme 2.2- Reaction between 1,4-naphthoquinone **2a** and the corresponding amino acid **3** to afford quinone conjugates **17g-h**.

The yields of the products **17g-h** were moderate. When the amino acid *L*-proline was used, the reaction proceeded in a different manner affording unexpected products, probably due to the steric hindrance on the secondary amine.

Structural elucidation of compound **17x**

Interestingly, when the 1,4-naphthoquinone and *L*-histidine were incidentally left to react for a longer period (i.e. 3 days rather than 1 day) the reaction led to a new compound, **17x** (Scheme 2.3).



Scheme 2.3 - Reaction conditions under which compound **17x** was formed.

The HPLC-MS analysis of **17x** after purification on silica revealed a base-peak in the chromatogram whose m/z was 512 ($M+H^+$), which did not match with the expected naphthoquinone-histidine hybrid (m/z 312, $M+H^+$).

The ¹H NMR spectrum of **17x** product is shown in Figure 61:

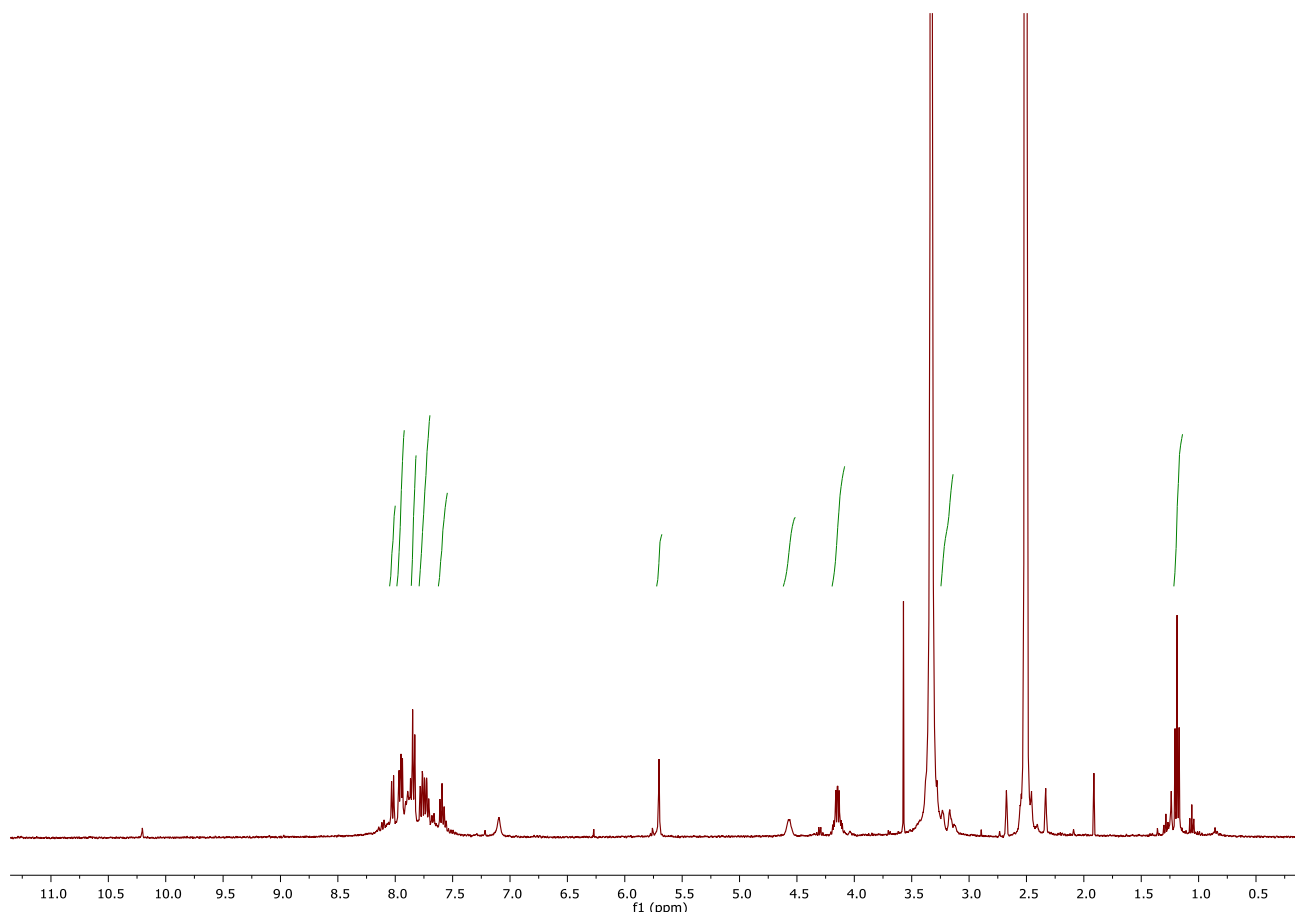


Figure 61: ^1H NMR spectrum of crude product **17x**, the deuterated solvent of choice was DMSO. This spectrum was acquired in a 400 MHz Bruker instrument.

Some of the most important features presented in this series of quinone amino acids conjugates were visible. For example, the single proton corresponding to the quinone fragment presented a chemical shift of 5.70 ppm. The chiral CH appeared at 4.57 ppm and the vicinal CH_2 appeared at 3.17 ppm, partially hidden by the signal corresponding to residual water of the deuterated solvent (3.33 ppm). The COOH group was also visible at 10.20 ppm as well as a very congested aromatic zone. Particular signals were the *quartet* at 4.15 ppm and the *triplet* at 1.19 ppm, which initially could be interpreted as residuals of AcOEt as solvent, but the singlet at 1.90 ppm in this spectrum did not correlate with the expected chemical shift typical of AcOEt (1.99 ppm), also its integral did not correspond to the theoretical value (expected for AcOEt = 3, found = 0.46). The aromatic area of the spectrum was very rich in signals, with integral values superior to the expected ones for the quinone-histidine hybrid.

To facilitate the structural elucidation of **17x**, an Ion Mobility-High Resolution Mass Spectrometry (IM-HRMS) analysis was undertaken using electrospray ionization operating in positive and negative ion mode (ESI+/-).

The base-peak ion chromatogram (BPI) shows a peak with a $\text{RT} = 6.48$ min, whose purity (blank subtracted) was 88% (Figure 62).

Item name: 200312_001_StdX_POS
 Channel name: 2: HD TOF MSe (50-1000) 6eV ESI+ (BPI)

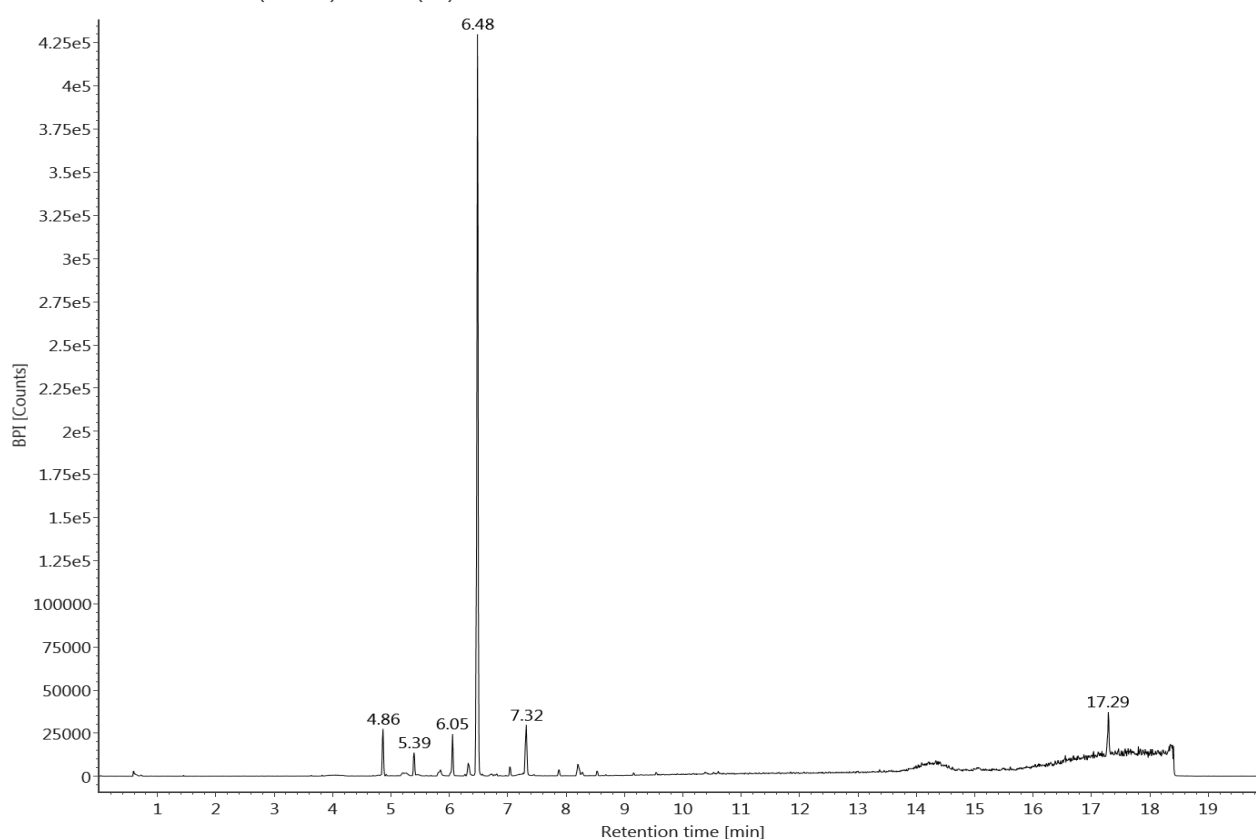


Figure 62- Base-peak ion chromatogram (BPI) of compound **17x**.

MS raw data were processed on UNIFI Scientific Information System which uses a 3D-peak detection algorithm that takes into account the three dimensions: retention time (RT), mass-to-charge ratio (m/z) and arrival time (t_A). A component list is automatically generated by the software. In ESI+, by sorting the components for their absolute response (i.e. detector counts for the monoisotopic ions) the component with the 3 coordinates: RT = 6.48 min, t_A = 6.93 ms, m/z = 512.1467 was recognised as the most intense ion in the chromatogram.

The low energy spectrum (A) presented the quasi-molecular ion at m/z 512.1467 (Figure 63), whilst the drift time-aligned high energy spectrum (B) shows the product ions originated in the collision cell from the fragmentation of the precursor ion (Collision Induced Dissociation, CID).

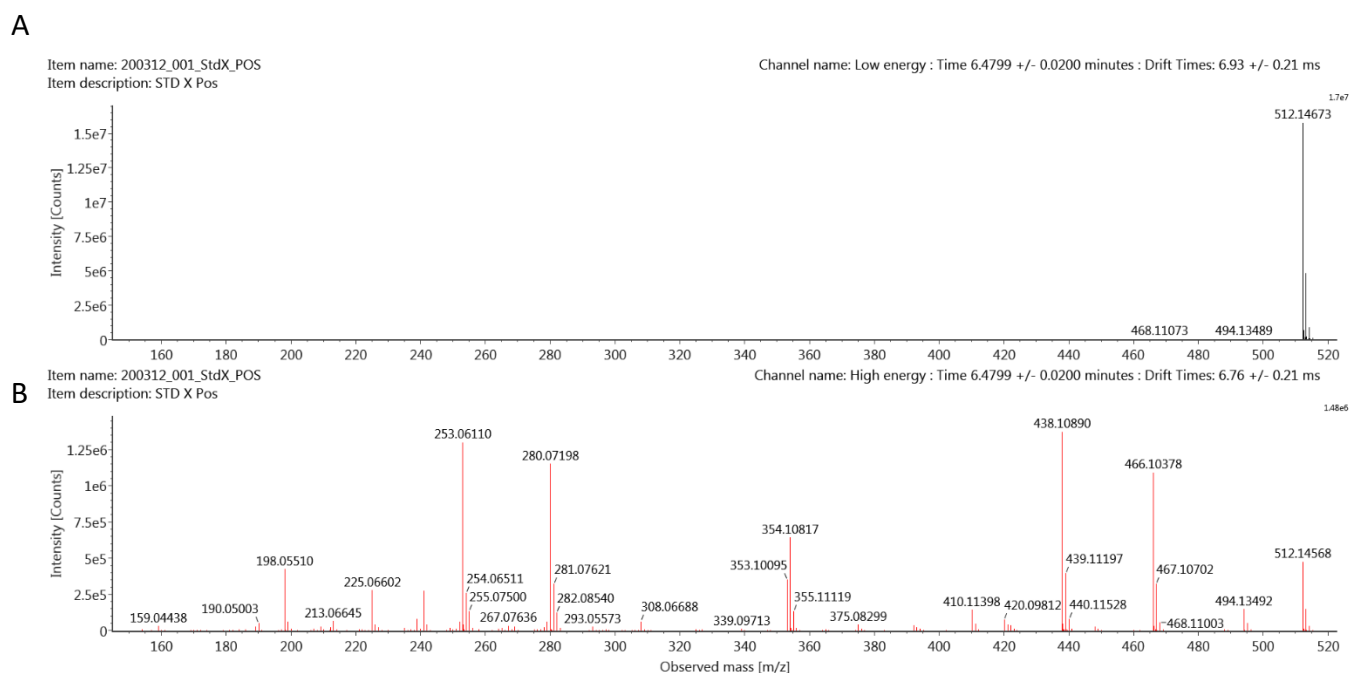


Figure 63 - Low energy (**A**) and high energy spectrum (**B**) of the compound **17x** in ESI+.

It is important to note that by enabling the ion mobility separation prior to the Q/ToF mass analyser, the alignment of fragment ions with the respective precursor is performed. In this way, the ion mobility cell acts as an ion-filter, thus the low energy spectrum presents only the ions with a $t_A = 6.93 \pm 0.21$ ms (i.e. the component of interest), while the high energy spectrum presents exclusively the fragments of the precursor ion at the above-mentioned arrival time. This allowed us to perform a comprehensive fragmentation study of the candidate molecule, as all major product ion peaks are attributed to the fragmentation of the ion m/z 512.1467, whilst the fragments coming from potential co-eluting compounds and/or background contaminants with a different drift time are discarded.

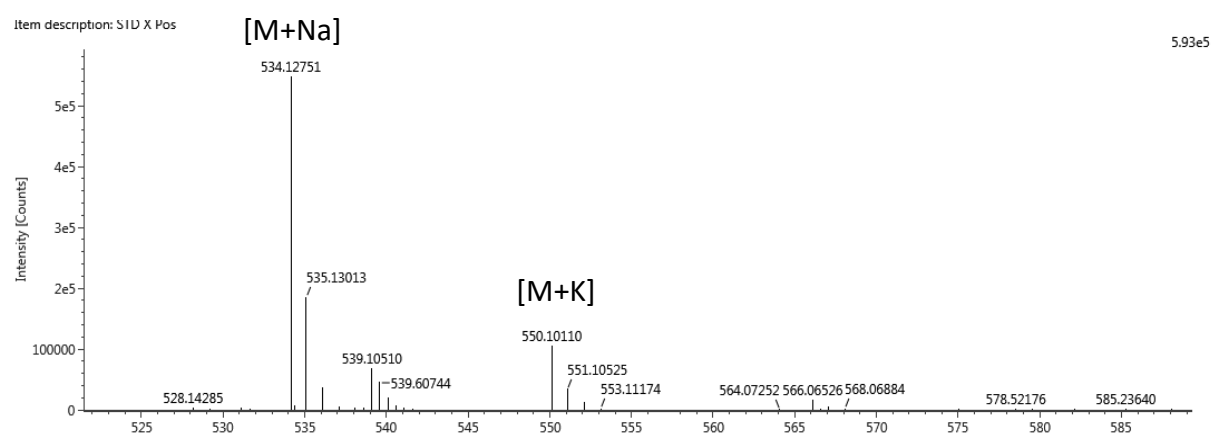


Figure 64 - Low energy spectrum of compound **17x** showing its $[M+Na]^+$ and $[M+K]^+$ adducts.

The m/z 512.1467 was attributed to the $[M+H]^+$ adduct. This was confirmed by the presence of the ions m/z 534.1275 and 550.1011 in the low-energy (non-drift aligned) spectrum, being the $[M+Na]^+$ and $[M+K]^+$ adducts, respectively (Figure 64).

The elemental composition of all adducts was automatically calculated by UNIFI software (Waters Corp.), keeping the mass error threshold at 2 mDa. The most probable calculated molecular formula was $C_{28}H_{21}N_3O_7$.

A first hypothesis for the chemical structure of compound **17x** was that illustrated in Figure 65. This structure would be consistent with a further addition of the amino acid moiety to a second naphthoquinone molecule:

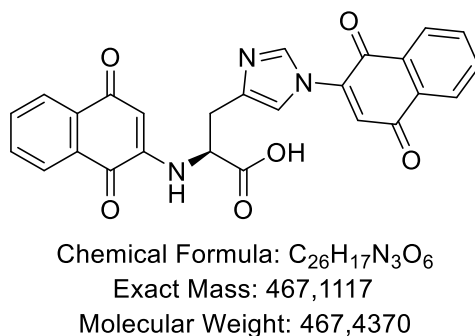


Figure 65- First hypothesised chemical structure for compound **17x**.

Due to an excess of base and naphthoquinone, in addition to a longer time of reaction, a second Michael addition would have taken place on a second molecule of naphthoquinone from one of the imidazole nitrogen atoms. However, this structure didn't match the hypothesised molecular formula by the mass analysis mentioned above ($C_{28}H_{21}N_3O_7$). But the double bond equivalent (DBE) for this structure was 20 (based on the sum of molecule insaturations and rings). A value that fully agrees with that calculated for the main ion peak.

Therefore, the previously hypothesised molecule (Figure 65) missed a chemical group consisting of C_2H_4O . This fragment highly resembled an ethoxide moiety, that is $-OCH_2CH_3$, or ethanol: $HOCH_2CH_3$. It can be assumed that the solvent of the reaction, ethanol, added to one of the quinone rings via Michael addition, followed by aromatization of the product, possibly via oxidation by atmospheric oxygen or other quinone molecules present in the reaction mixture, as previously observed for the naphthoquinone analogues.

This led us to a second possible structure, which is shown in figure 66.

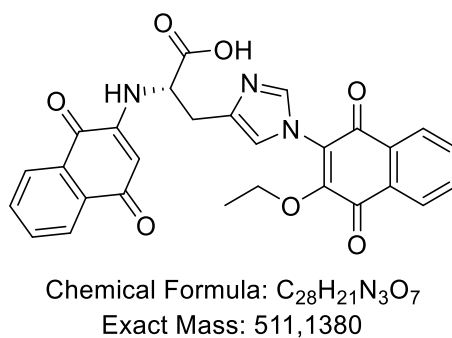


Figure 66 - Proposed chemical structure of compound **17x**.

This new chemical structure matched the predicted molecular formula, the calculated mass and the DBE.

From a deeper analysis of the ^1H -NMR spectrum in Figure 60, we can speculate that the addition of an ethoxide group can be explained by the presence of the quartet at 4.66 ppm and the triplet at 1.19 ppm. It is important to notice that the addition of this fragment to the naphthoquinone directly linked to the imidazole ring has been based on the more electrophilic nature of the C3 carbon in this quinone compared to the C3 carbon of the second quinone fragment. The imidazole ring can potentially reduce the electron density in the double bond on the quinone, making of it a more reactive Michael's acceptor. Nevertheless, NOE studies are required to confirm the location of the ethoxyl moiety.

Summary of the synthesised candidate LDH inhibitors

A total of 9 quinone-conjugates hybrids were selected for the enzymatic and SPR studies as potential LDH-A inhibitors (Figure 67):

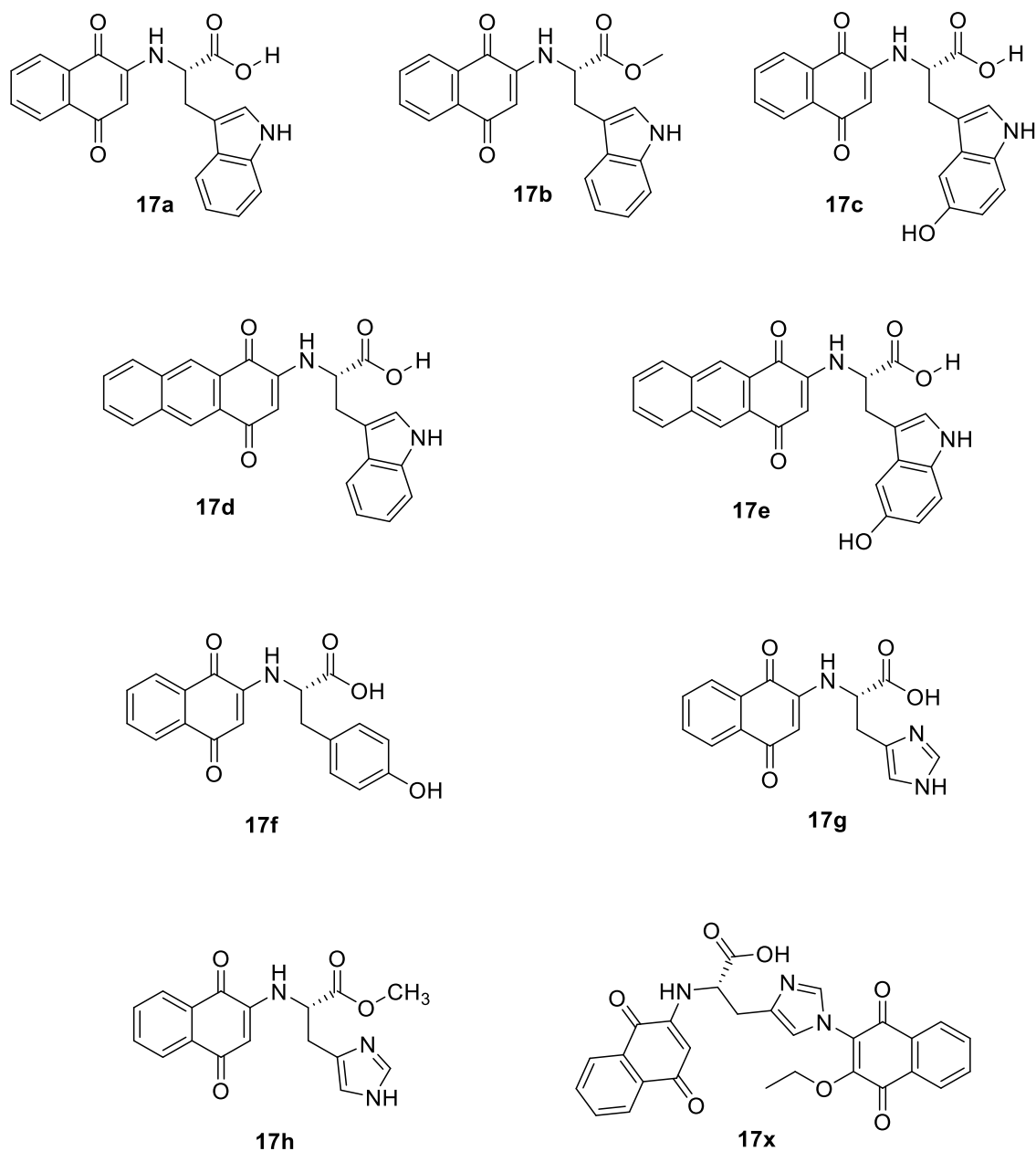


Figure 67 - Quinone-amino acids hybrids proposed in the present study as candidates for LDH-A inhibitors.

2.3.3 Assessment of the Inhibitory Activity of Quinone Activity of Quinone-Amino Acid Conjugates

The inhibitory potency of quinone hybrids was initially explored by two screening assays: first, evaluating the strength of interactions in the inhibitor-protein complex by Surface Plasmon resonance (SPR). Second, measuring the consumption of NADH as a direct indicator of the enzyme activity.

Next, kinetic studies were performed, for the obtention of dissociation constants K_D and K_i .

Screening with Surface Plasmon Resonance (SPR)

SPR is a surface technique which allows real-time study and monitoring of binding events between entities such as proteins and small molecules. Additionally, it provides kinetics and affinity parameters. In brief, the protein – or more technically named, the ligand- is immobilised in the surface of a gold sensor chip. Then, solutions of different analytes flow over the surface allowing the study of the interactions between both ligand and analyte. (Please refer to Chapter 1, Results and Discussion section 2.3, for further technical details of SPR).

In the present study, SPR was initially used as screening tool for determining interactions between the protein LDH-A and the synthesised compounds (the analytes). Next, SPR was employed to perform kinetic studies and determination of the dissociation constant, K_D .

With the aim of discerning the compounds that bind to the enzyme from those that present no interaction, LDH-A enzyme was immobilized in the gold sensor-chip by means of amine coupling, and compounds were injected one at a time. The compounds flow over the functionalised chip with the enzyme (Figure 68). Compounds were injected at 100 μ M dissolved in running buffer.

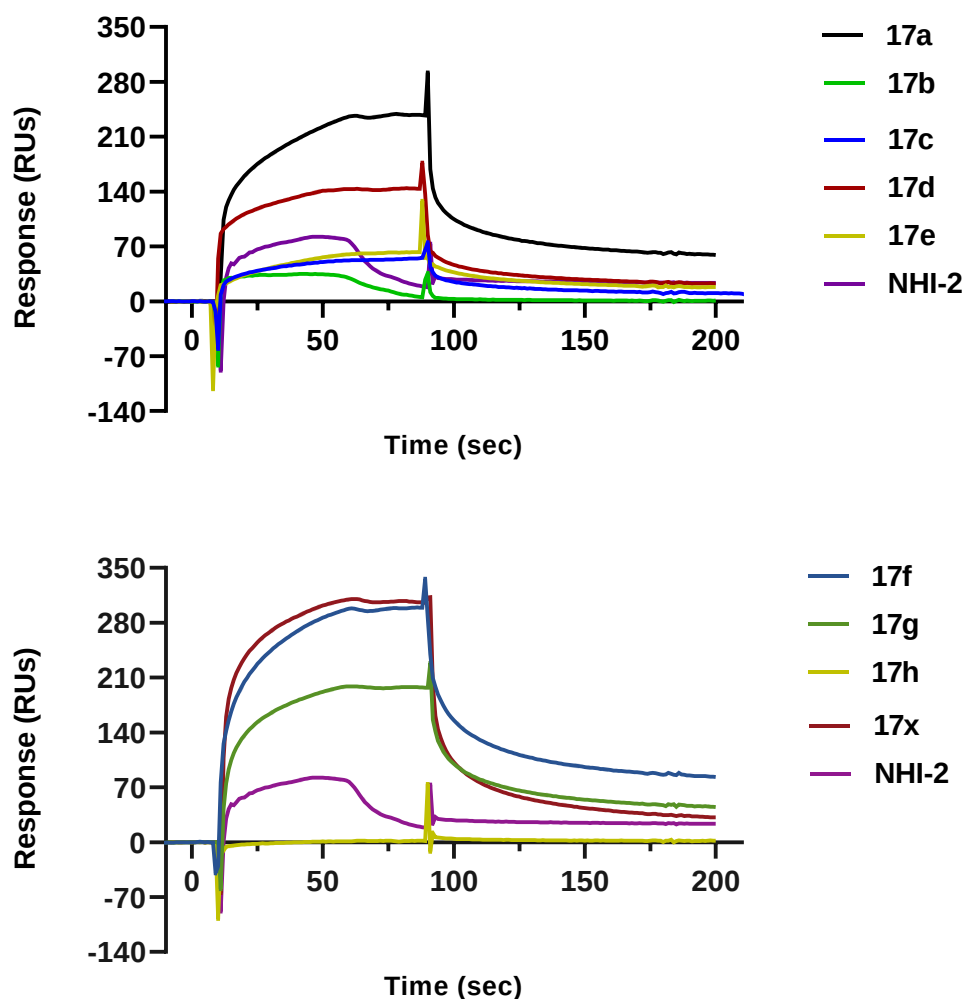


Figure 68 - SPR Sensorgrams for quinone-amino acids hybrids and NHI-2 binding to the surface with immobilised LDH-A.

According to the principle of the SPR assay, the response (which is measured as resonance units, RUs) is a direct indicator of the binding events on the surface of the sensor chip, and the higher the response registered, the higher the number of molecules of analyte bound the ligand. However, it is important to remember that under these experimental conditions what we are observing is just a *yes/no* answer to the question *Is there any interaction with the enzyme?*

As it can be appreciated in Figure 68A and 68B, most of the quinone-amino acids conjugates bound the enzyme. Compounds **17a**, **17f**, **17g** and **17x**, with more than 200 RUs, appeared again as the best candidate LDH inhibitors, closely followed by **17d** (~140 RUs). By contrast, the histidine methyl ester compound, **17h**, barely bound the enzyme

Compounds **17c**, **17e** and the positive control NHI-2 gave a response ranging between 40 to 70 RUs. NHI-2 presented a more irregular profile, partly due to the high lipophilicity of this compound. Compound **17b** behaved similarly to NHI-2, reaching up to 40 RUs. (Figure 68A).

Kinetic studies with SPR

The next step was the assessment of the compound's affinity for LDH-A enzyme by obtaining the dissociation constant (K_D) with respect to the enzyme-inhibitor (EI) complex. The assay was performed in two stages: 1) the enzyme was fixed on the surface of the sensor-chip; 2) each compound was injected in a range of scaled concentrations.

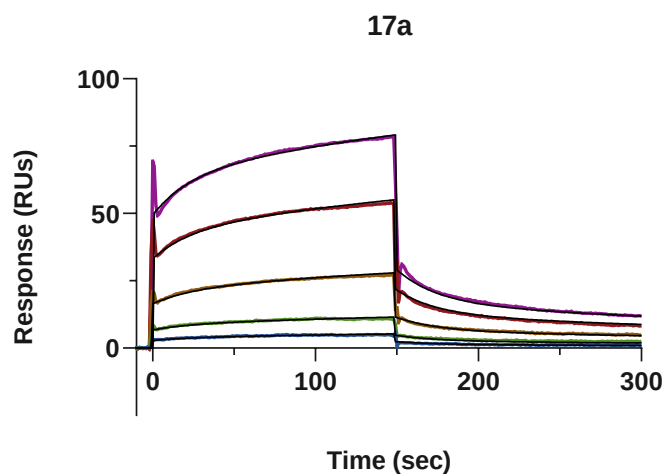
The compounds showed an interaction ratio enzyme: inhibitor of 1:1. Experimental data fitted the kinetic model (based on k_{on}/k_{off} measurements), according to Equation 2.27, which describes a two-state reaction model. This model addresses the situation where the analyte binds to immobilized ligand followed by a conformational change that stabilizes the complex.

$$A + B \rightleftharpoons AB \rightleftharpoons AB^*$$

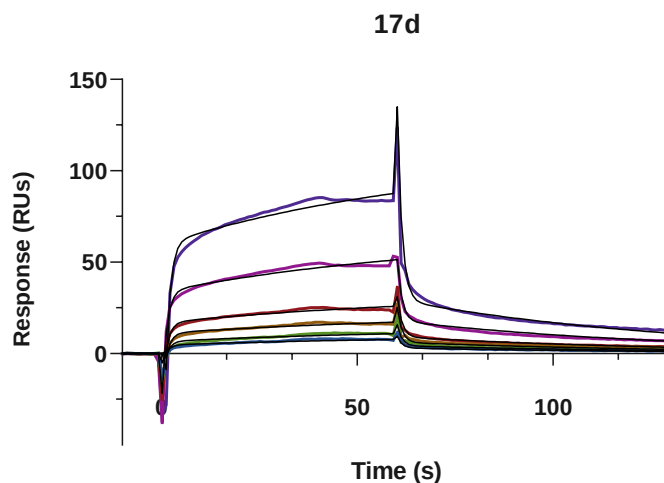
$$K_D = \frac{k_{d1}}{k_{a1}} \times \frac{k_{d2}}{k_{a2} + k_{d2}} \quad \text{Equation 2.27}$$

k_{d1} corresponds to the dissociation constant of the first state whereas k_{d2} is the dissociation constant for the second state. Likewise, k_{a1} described the association rate in first state and k_{a2} described the association rate in the second state.

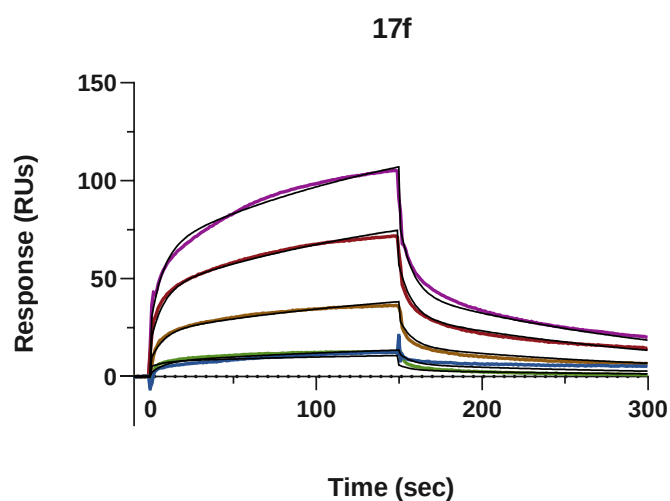
The K_D of compounds was calculated (Table 11).



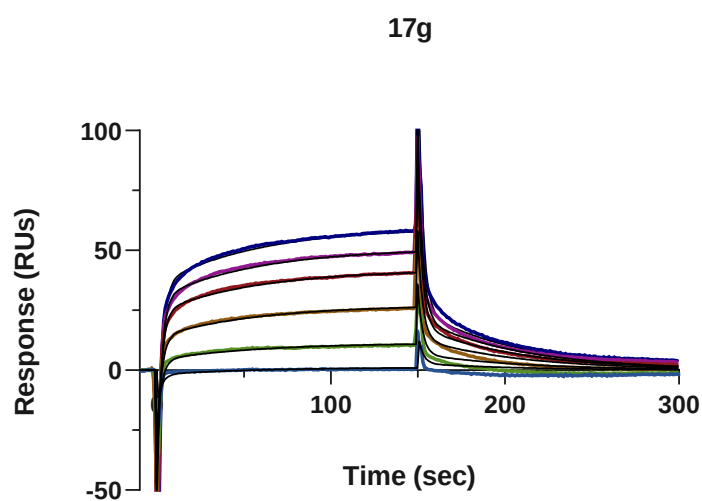
$K_D = 189 \mu\text{M}$ $\text{Chi}^2 = 1.64$



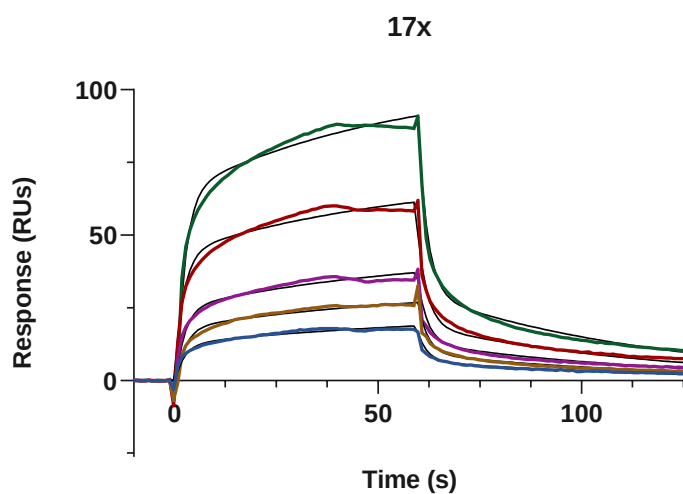
$K_D = 575 \mu\text{M}$ $\text{Chi}^2 = 4.1$



$K_D = 1100 \mu\text{M}$ $\text{Chi}^2 = 2.18$



$K_D = 682 \mu\text{M}$ $\text{Chi}^2 = 6.46$



$K_D = 117 \mu\text{M}$ $\text{Chi}^2 = 1.46$

Figure 69 – SPR sensorgrams and kinetic fitted curves (black lines) of synthesised compounds. Sensorgrams were obtained using dilution series of the tested compounds (5-100 μM). Curve fit and error (Chi^2) values were obtained with Biacore Software.

Table 12 - K_D values obtained in SPR for quinone-amino acids hybrids.

Compound	K_D (μM)
17a	189
17d	575
17f	1100
17g	682
17x	117

Compound **17x** showed the highest affinity, with a K_D value of 117 μM , closely followed by compound **17a** (Table 11). Despite presenting a high level of binding in the previous screening, compound **17f** showed the lowest affinity, possibly due to additional off-target interactions with the enzyme.

The shape of the sensorgram can provide useful information. As we already introduced in Chapter 1, sensorgrams can be divided in three main regions, corresponding to association, equilibrium and dissociation phases (Figure 70).

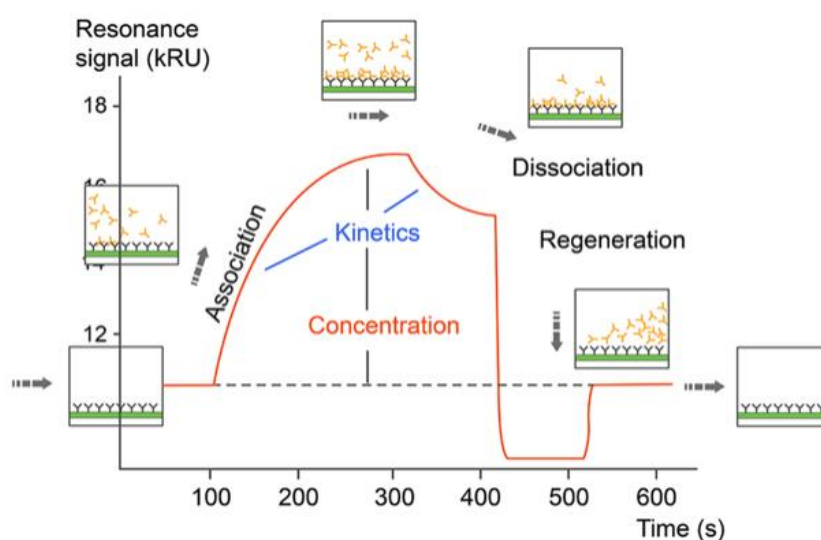


Figure 70 – Typical curve shape of a SPR sensorgram. Taken from reference ¹⁴⁶.

A pronounced slope in the association phase indicates a quick interaction between ligand and analyte, which rapidly leads to the steady state, where binding and dissociation events are in equilibrium. This phase is identified when the curve reaches *plateau*. Finally, the ease by which the analyte separates from the ligand is also reflected in the curve shape. The more pronounced the slope, the faster is the dissociation, and *vice versa*.

In general, all the tested compounds dissociated rapidly from the ligand, as it can be appreciated by their curve shape. Fast dissociation phase implied a reversible interaction, which means that our

compounds bind the enzyme in a reversible manner. This fact gave us valuable insights of the enzyme-inhibitor complex formation, suggesting that our quinone-amino acid compounds could behave as reversible inhibitors of LDH-A.

Compounds **17f**, **17g** and **17x** presented very good profiles, reaching equilibrium and then a fast dissociation (Figure 69). In particular, compound **17g** rapidly reached equilibrium and kept the steady state for a longer period compared to the other compounds. Compounds **17d** and **17a** showed good profiles as well, with quick association and dissociation phases.

Encouraged by these preliminary results and with the aim to better characterise the mechanism of action of the quinone- amino acid hybrids as LDH-A inhibitors, compounds were subjected to a second screening via enzymatic assays.

Enzymatic assays

The oxidation of NADH is a direct measure of the consumption of pyruvate and its transformation into lactate. The reaction rate of LDH-A was determined by a decrease in absorbance of NADH at 340 nm in a UV-VIS spectrophotometer. In all experiments described herein, the activity of LDH was observed under competitive conditions between the compounds and the natural substrate (pyruvate) or the cofactor (NADH). Thus, two experimental designs were applied:

1. *NADH competition assay* - the ability of inhibitor candidates to interact with the active site of NADH is assessed by using competitive concentrations of NADH and a fixed (and saturating) concentration of pyruvate (Figure 71).
2. *Pyruvate competition assay* - by using competitive concentrations of pyruvate and fixed and saturating concentration of NADH, the ability of compounds to bind the pyruvate site of LDH was evaluated.

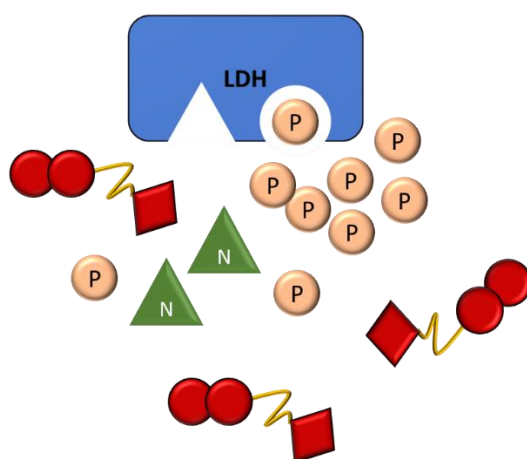


Figure 71 - Schematic representation of the NADH competition assay. In this experiment, the natural substrate, pyruvate (P), is added at high concentrations, so the inhibitor (illustrated as red circles and diamond figures) is forced to compete with NADH (N) for binding the NADH site.

Screening of inhibitory activity of quinone-amino acid hybrids on enzymatic assays

The assessment of the inhibitory capacity of the compounds relative to the negative control was verified by using 125 μ M of each candidate under standard enzyme kinetics experiments (refer to materials and methods for more details). Compound *N*-hydroxyindole-2 (NHI-2) is a known small molecule with potent inhibitory activity against human LDH-A that was included in the screening assays as positive control.²⁴⁶

The screening was carried out in two independent experiments, A: saturating the pyruvate binding site, and B: saturating the nicotinamide binding site (Figure 72).

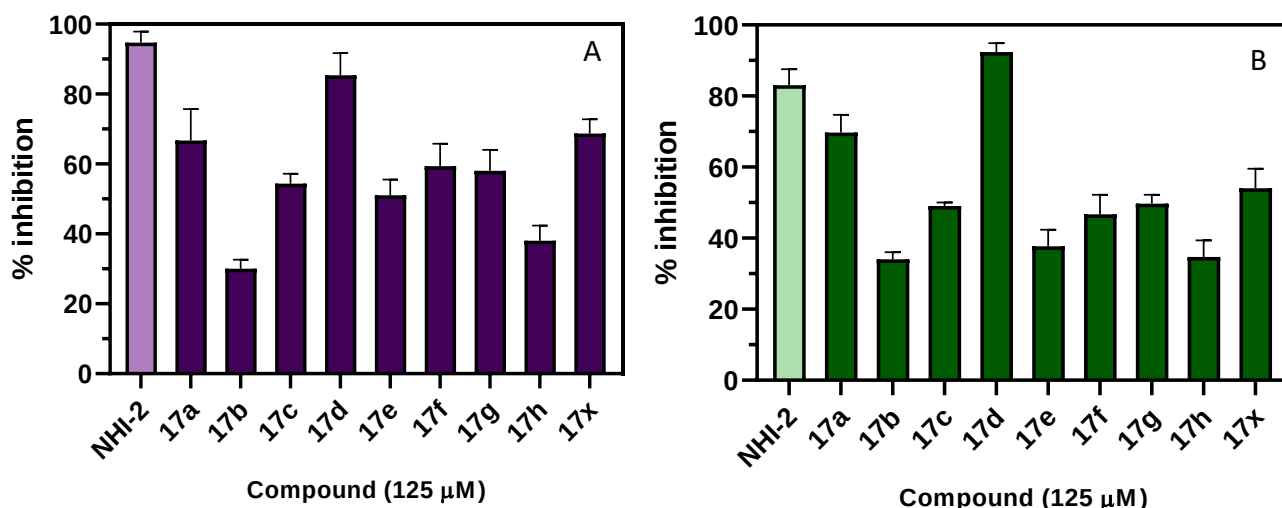


Figure 72 - Measurement of inhibition (%) of enzymatic activity of LDH-A in the presence of quinone-amino acid hybrids (17a-h and 17x) at 125 μM. Results are reported as mean ± SD (n=3).

In the NADH competition assay (Figure 72A), the cofactor was added at 90 μM, and pyruvate at 2 mM. By contrast, in the pyruvate competition assay (Figure 72B), pyruvate was added at 200 μM, and NADH at 300 μM. NHI-2 was used as positive control in both tests. These conditions have been reported previously by Granchi *et al.* and they have been used here with minor modifications. For instance, the concentration of enzyme used in the assay was optimized, so that the consumption of NADH could be observed for the total length of the experiment. In addition, the pyruvate competition assay was included, for better comparisons between active sites.

These screening experiments revealed promising results for quinone-amino acid hybrids. All screened compounds showed moderate to very good level of enzymatic inhibition, with compound **17d** at the forefront of the group in both NADH and pyruvate competition assays (92 ± 2.5% and 85 ± 6%, respectively). Compound **17d** presented very good inhibitory values, comparable to the positive control NHI-2.

Compounds **17a**, **17x** and **17g** presented remarkable inhibitory potency in both tests, with inhibitory percentages above 60% in the NADH competition assay (Figure 72A).

In these preliminary results, compounds **17c**, **17f**, and **17h** showed a modest affinity for the LDH-A binding sites, with inhibition percentages around the 50% in the NADH competition assay and closer to 40 % inhibition in the pyruvate competition assay.

Interestingly, esterification of compound **17a** caused a striking drop in the inhibitory potency of the resulting compound (**17b**), which could be appreciated in both tests. Similarly, compound **17h**, another ester, showed lower inhibitory capacity (38 ± 4% in the NADH competition assay) compared with its acid analogue, **17g**.

The screening via enzymatic assay in solution helped us to contrast the results firstly obtained in the screening with SPR, and confirmed the union of compounds to the active centre of the enzyme.

Candidates with a percentage inhibition relative to control higher than 50% were selected to further characterize their profile as LDH-A inhibitors in kinetic studies to obtain the IC_{50} and the inhibition constant (K_i) values.

Determination of the inhibition constant (K_i)

K_i - the concept

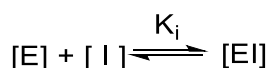
An enzymatic reaction starts when the substrate (S) reversibly binds to the free enzyme (E) to form the ES complex. This process is quantified by the dissociation constant K_D (which comprises the k_{on} and k_{off} rate constants for the formation of the ES complex). The ES complex will generate the reaction product(s) through a series of chemical modifications which are collectively defined by the rate constant k_{cat} .



Scheme 2.4- Schematic representation of the enzyme-catalysed conversion of the substrate (S) into the product(s) (P).

Copeland described the enzyme-inhibitor binding equilibria in three different ways:²⁴⁷ Inhibitors can irreversibly bind to an enzyme and render it inactive, which usually happens by the formation of a covalent bond between the inhibitor and some group on the enzyme molecule. The second binding mode possible is through tight binding, this is, the inhibitor binds so strongly that for all practical purposes they are permanently bound (the dissociation rates are very slow). These inhibitors are known as tight binding inhibitors. However, in most cases inhibitors bind reversibly to enzymes with rapid dissociation and association rates. Inhibitors that follow this third type of binding are known as classical reversible inhibitors.²⁴⁷

There are three major forms of reversible inhibitor interaction with enzymes: competitive, non-competitive and uncompetitive inhibition. The relative potency of a reversible inhibitor is measured by its binding capacity for the enzyme. The equilibrium between the binary EI complex and the free enzyme and inhibitor molecules is defined by the dissociation constant K_D , but in the case of enzyme-inhibitor interactions, the dissociation constant is often referred to as inhibitor constant and is given the special symbol, K_i .²⁴⁸



Scheme 2.5- K_i represents the dissociation constant of $[EI]$ complex.

A competitive inhibitor reversibly binds to the same site as the substrate, so its inhibition can be totally overcome by using a very high concentration of substrate. The V_{max} will not be altered, whereas the effective K_m increases.

In the case of a non-competitive inhibitor, it reversibly binds to both the enzyme-substrate complex, and the enzyme itself. This means that the effective $V_{\max}$ decreases with inhibition, but the K_m does not change.

Finally, if the inhibitor binds to the enzyme-substrate complex, but not the free enzyme, the inhibition is uncompetitive. This reduces both the effective $V_{\max}$ and the K_m .²⁴⁹

The EI complex could bind the substrate to form the ESI complex. However, the S could display a lower affinity for the EI complex than for the free enzyme. To quantify the degree to which inhibitor binding affects the affinity (K_D) of the enzyme for substrate, Copeland described the constant α .²⁴⁸ Alpha determines the mechanism of inhibition: if there is no change in substrate affinity after the formation of the EI complex, then $\alpha = 1$. This is a non-competitive inhibition. By contrast, if the formation of the EI complex prevents the binding of substrate, $\alpha \gg 1$ and the inhibition type is competitive. Finally, if the substrate's affinity for the enzyme increases upon the formation of the EI complex, then $\alpha < 1$ and the inhibition is uncompetitive.

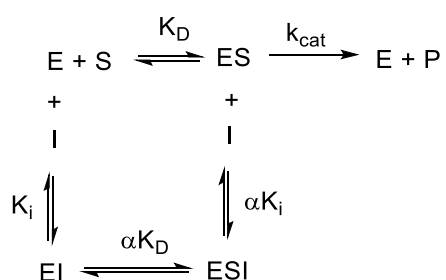


Figure 73 - Equilibrium scheme for enzyme turnover in the presence and absence of a reversible inhibitor. K_D is the dissociation constant for dissociation of the ES complex, k_{cat} expresses the overall rate of reaction after the ES complex formation. Alpha quantifies the effect of inhibitor on K_D and K_i and ultimately, the alteration substrate- enzyme and inhibitor enzyme affinity. Taken from Copeland, 2000.²⁴⁸

K_i is very advantageous for expressing the potency of an inhibitor because, unlike IC_{50} , K_i is independent from the concentration of the substrate.

Experimental obtaining of K_i

Prior to calculating the inhibition constants of each compound, the Michaelis-Menten constants (K_m) of pyruvate and NADH were obtained under steady state conditions at 37 °C (Figure 74).

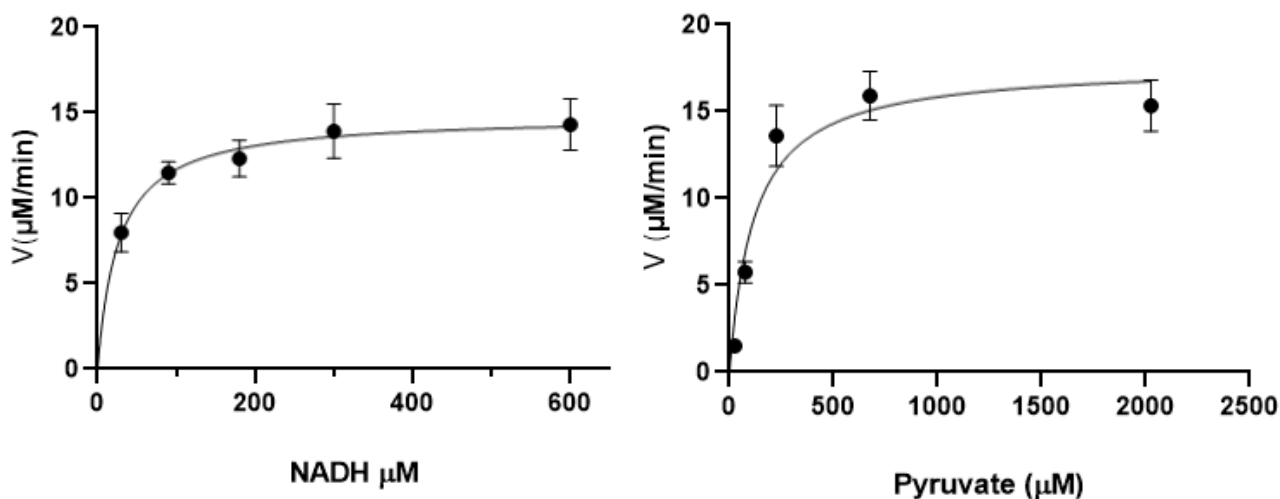


Figure 74 - Michaelis-Menten curves corresponding to NADH and pyruvate. K_m NADH= 20.7 μM ; K_m pyruvate = 118.7 μM . Data is reported as the mean of 3 independent experiments.

Under this conditions, pyruvate showed a K_m of 118.7 μM , whereas NADH presented a K_m of 20.7 μM , both in high agreement with values found in the literature (120 μM and 20 μM , respectively).²⁴⁶

We obtained the dissociation constant (K_i) of LDHA with each substrate in the presence of the quinone derivatives (Table 12). Each compound was tested at different concentrations, ranging between 2 and 80 μM . For each concentration, a scaled amount of NADH was used, while keeping the concentration of pyruvate constant, for saturating the pyruvate binding site. The same study was performed by keeping the concentration of NADH fixed to saturate its binding site, the target compounds were tested with respect to the pyruvate binding site. The alpha values were determined by mixed-model inhibition fit in GraphPad Prism software. This model includes the alpha parameter discussed above as indicator of the type of inhibition mechanism ($\alpha = 1$ non-competitive; $\alpha > 1$ competitive; $\alpha < 1$ uncompetitive).

Table 13 - K_i for LDH-A - inhibition data in the presence of quinone-amino acid hybrids **17a-h** and **17x**. Mixed-model inhibition fit (GraphPad Prism 8 software) was applied for the obtention of K_i and alpha values (α). Mean $\pm$ SD (n=3).

LDH-A K_i (μ M)					
Entry	Compound	NADH ^a	Pyruvate ^b	α NADH	α Pyruvate
1	17a	57.0 $\pm$ 2.7	49.9 $\pm$ 3.8	$\alpha > 1$	$\alpha > 1$
2	17c	130.2 $\pm$ 4.3	134.7 $\pm$ 8.8		
3	17d	5.88 $\pm$ 0.7	2.9 $\pm$ 0.35		
4	17e	153.3 $\pm$ 10.1	212.0 $\pm$ 5.1		
5	17f	83.2 $\pm$ 4.3	89.3 $\pm$ 5.1		
6	17g	81.6 $\pm$ 1.3	78.8 $\pm$ 2.4		
7	17x	31.6 $\pm$ 3.2	84.14 $\pm$ 4.1		

The smaller the K_i value, the higher the affinity of the compound for the enzyme.

^aSaturating concentration of sodium pyruvate (2 mM) and competitive increasing concentrations of NADH (30- 900 μ M). ^bCompetitive increasing concentrations of sodium pyruvate (25 μ M to 2025 μ M) and saturating concentration (300 μ M) of NADH.

According to the obtained alpha values, all the inhibitors behaved as competitive inhibitors in both binding sites of the enzyme ($\alpha > 1$). These results reinforced our previous outcomes with SPR, where the curve shape of the sensorgram indicated a reversible interaction between inhibitor and enzyme.

Compound **17d** showed the highest affinity for both nicotinamide and pyruvate sites, with K_i values of 5.88 $\pm$ 0.7 μ M and 2.9 $\pm$ 0.35 μ M, respectively. The most striking data were the K_i values of compounds **17d** and **17e**. Both entities are 1,4-anthraquinones coupled with tryptophan, that only differ on one -OH group (Figure 67). Compound **17e** has the hydroxy group in position 5' in the indole ring, whereas this group is absent in compound **17d**. The -OH substitution provokes that the affinity for both binding sites drops by $\sim$ 30-fold for the NADH site and almost $\sim$ 200-fold in the pyruvate site (Table 12, entries 3 and 4).

Compounds **17a** and **17c** are the 1,4-naphthoquinone counterparts of **17d** and **17e**. The same behaviour was observed in the pair **17a** and **17c**. **17a** lacks of the hydroxy group, and displayed higher affinity for both binding sites (57.0 $\pm$ 2.7 μ M and 49.9 $\pm$ 3.8 μ M, respectively), whereas **17c** showed K_i values of 130.2 $\pm$ 4.3 μ M and 134.7 $\pm$ 8.8 μ M (Table 12, entries 1 and 2).

The quinone derivative possessing the amino acid tyrosine, **17f**, showed moderate affinity for the LDH binding sites, with K_i values ranging 80-90 μ M (Table 12, entry 5).

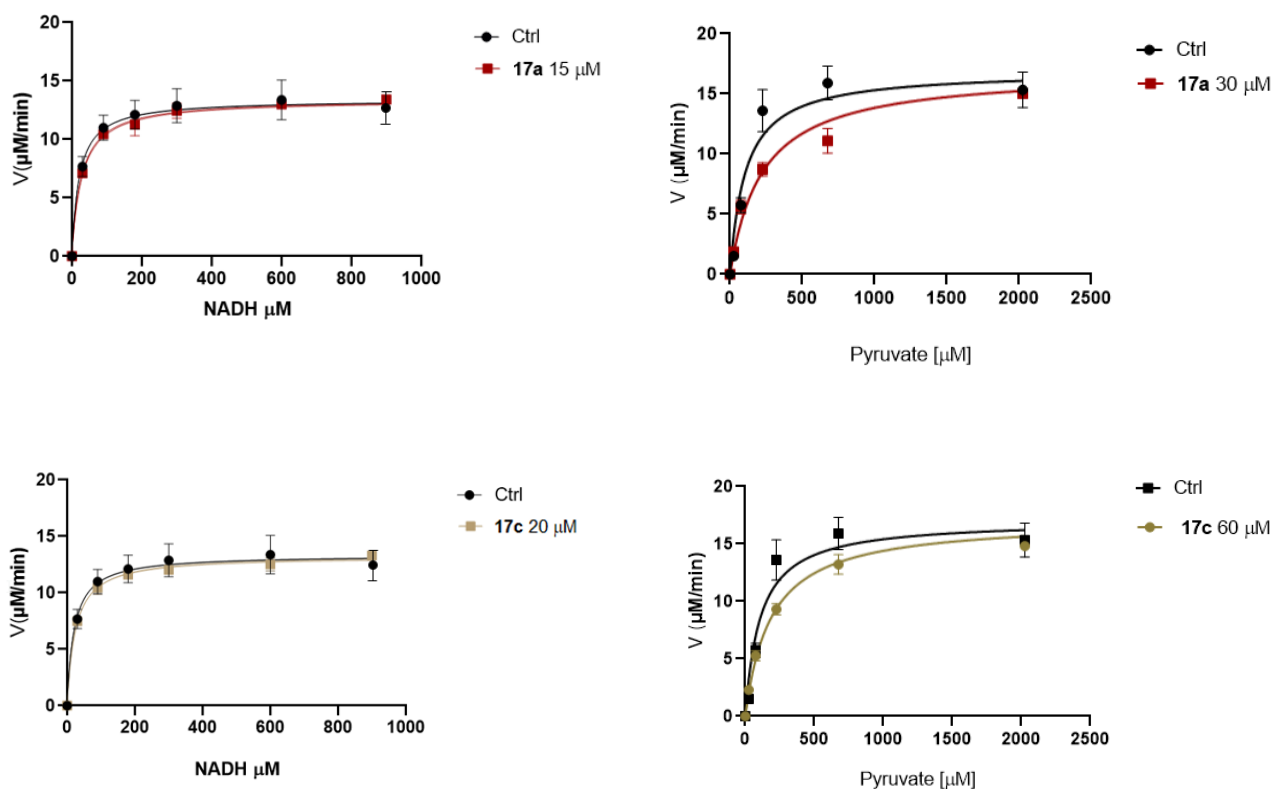
Notably, compound **17x** improved the inhibitory effect observed of compound **17g**, as it can be appreciated by comparing the kinetic constants in both in SPR and enzymatic assays (Tables 11 and 12).

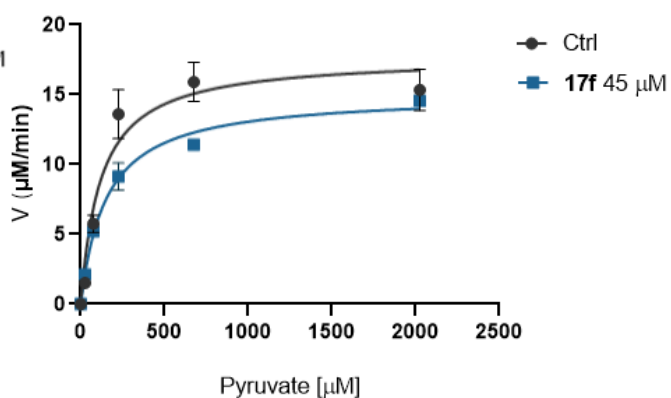
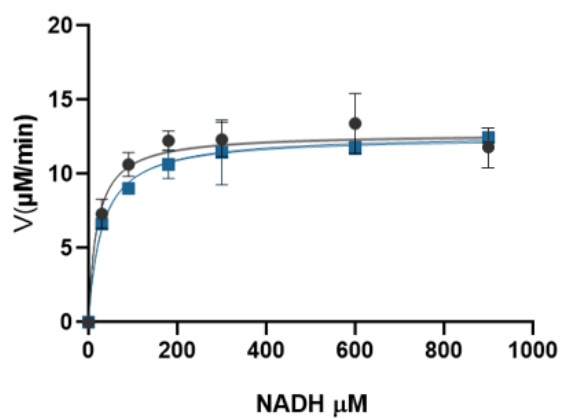
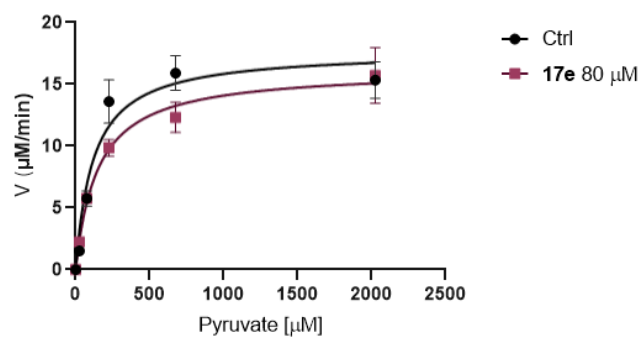
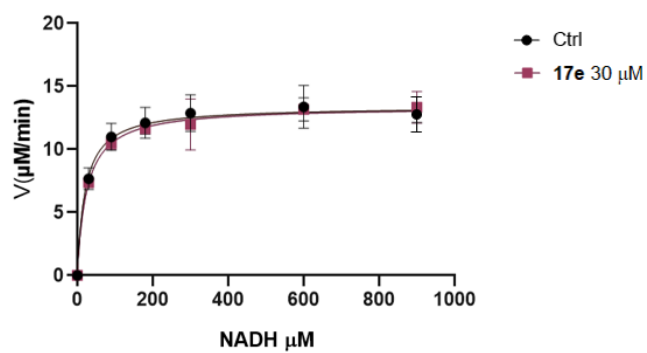
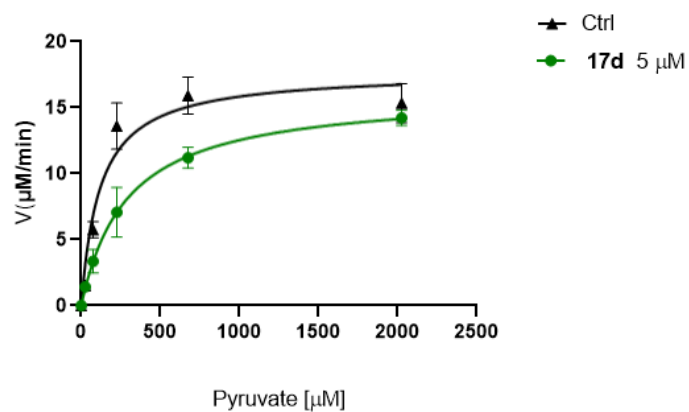
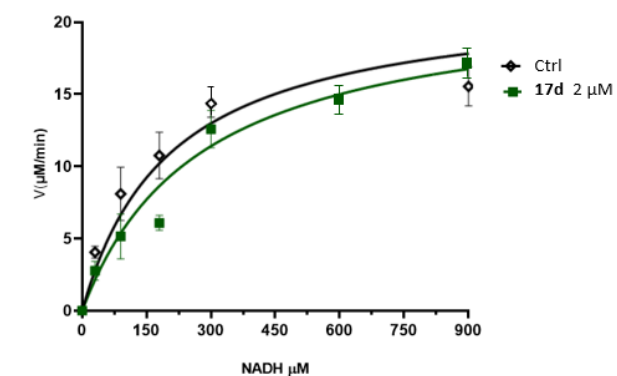
Overall, quinone-amino acid hybrids presented similar affinity for both binding sites, as it is demonstrated by their respective K_i values. Only compound **17x**, the analogue with the highest structural variability, presented a preference for NADH binding site ($K_i = 31.6 \pm 3.2 \mu\text{M}$), almost 3-fold smaller than the K_i value calculated for the pyruvate binding site ($84.1 \pm 4.1 \mu\text{M}$).

These K_i values manifest the good activity of quinone amino-acid hybrids as LDH-A inhibitors, particularly compounds **17a**, **17x** and **17d**, the latter with a K_i value of $2.9 \mu\text{M}$ in the pyruvate site and 5.8 in the nicotinamide site. These values are among the lowest K_i values found in the literature obtained by enzymatic assays as the one here performed.²⁵⁰ For example, the N-hydroxyindole-based compounds developed by Granchi and co-workers (among them our positive control NHI-2) showed K_i values ranging from 4.7 to $35.4 \mu\text{M}$.²⁴⁶

Effect of inhibitors in binding isotherms of pyruvate and NADH

Figure 75 shows to what extent an inhibitor can modify the Michaelis-Menten curve of each substrate (binding isotherms). These curves were obtained by plotting the reaction rates (V) obtained in the K_i tests versus the concentration of substrate. Increment in K_m values (with the consequent displacement of the Michaelis-Menten curve towards the right) is indicative of a competitive inhibition.





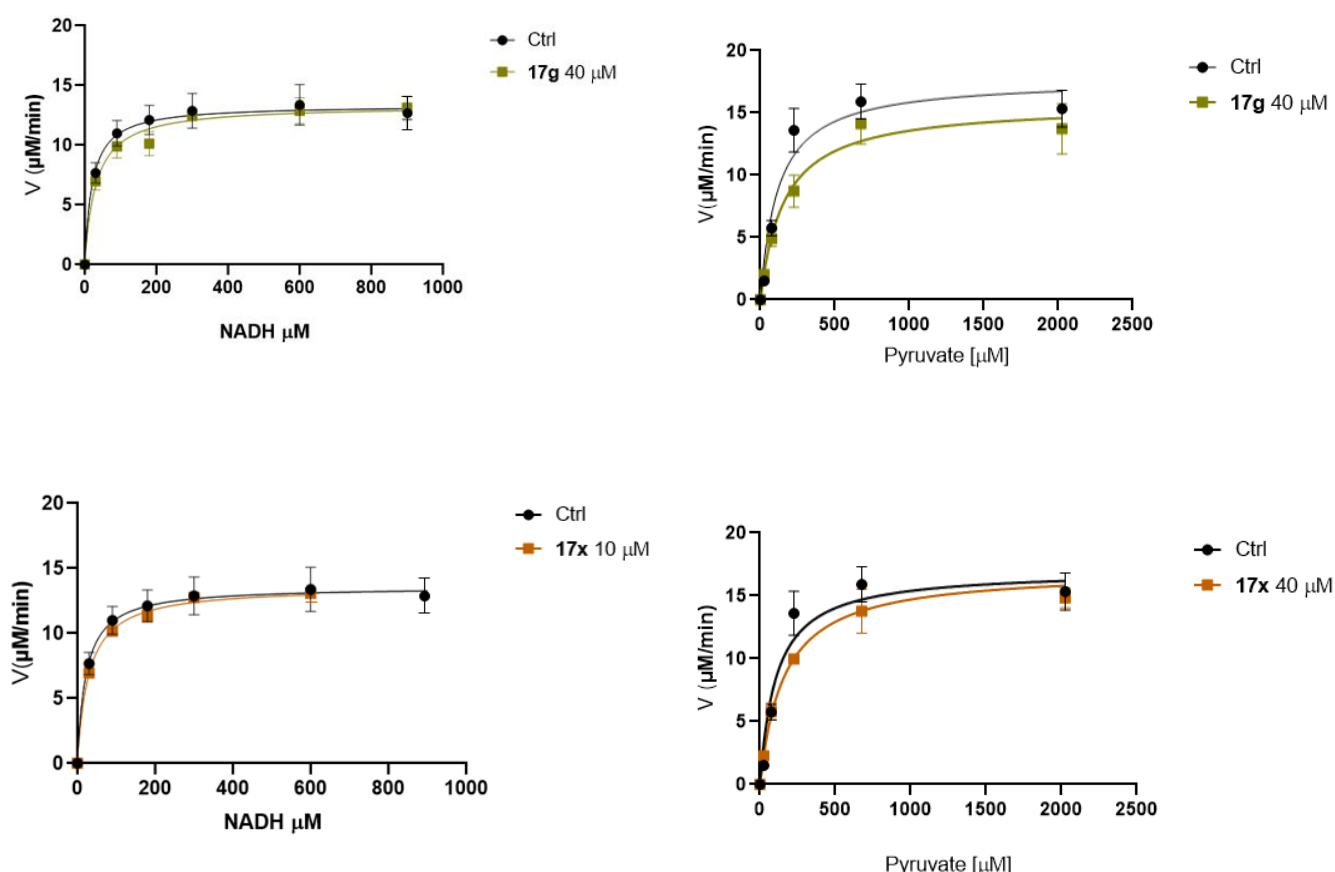


Figure 75 - Michaelis-Menten curves of LDH-A in the absence (black line) or in the presence of compounds (coloured line). Quinone derivatives were tested at a range of concentrations (2-80 μM). Curves were obtained with GraphPad Prism software.

The higher the affinity of the inhibitor for the enzyme, the larger will be the shift of the coloured curve towards the right. This phenomenon can be appreciated in some examples such as compound **17g**. Under equal concentrations, the binding isotherm of pyruvate shifts more than the isotherm of NADH. In other words, with the same quantity of **17g**, we can inhibit more efficiently the enzyme by binding the pyruvate site.

Another example is compound **17d**. As a start, we can appreciate to what extent very small quantities of compound provoke a noticeable shift in both Michaelis-Menten curves. The shift observed in the pyruvate binding isotherm is approximately 2.5 fold the shift observed in the NADH binding isotherm, which is in accordance to the K_i values obtained for this compound.

A final example is compound **17e**. A higher concentration of **17e** equal to 80 μM was needed to effectively modify the K_m of pyruvate compared with that of NADH site, where 30 μM of **17e** was sufficient. This trend is also observed in compounds **17c** and **17x**, the former with equal affinity for both binding sites and the latter with higher potency when displacing the NADH isotherm.

Different substitutions modulate the inhibitory potency

The carboxylic acid

The presence of a carboxy group is key for the inhibition activity. It is known that carboxylic acids moieties are recurrent in LDH inhibitors.^{225,231,233} Consequently, esterification of the carboxylic acid residue had detrimental effects that lead to poor results in LDH-A inhibition (compounds **1b**, **1h**). This can be explained by the presence of a carboxylic acid in the natural substrate of the enzyme: pyruvate. Indeed, oxalic acid is considered a classic competitive inhibitor of LDH. Although having K_i in the millimolar range, oxalic acid is commonly used as positive control of LDH inhibition due to its chemical structure that highly resembles pyruvate. Similarly, oxamic acid behaves as competitive inhibitor when lactate is present.²⁵¹

Furthermore, previous works demonstrated that vicinal -OH and -COOH groups are fundamental for the inhibitory activity of a several classes of LDH inhibitors²⁵² (Figure 76). For example, theazole-based compounds described by Cameron *et al.* such as 3-hydroxyisoxazole-4-carboxylic acid (HICA) and 4-hydroxy-1,2,5-thiadiazole-3-carboxylic acid (HTCA) showed IC_{50} values of 54 and 10 μ M, respectively, on LDH-A (Figure 76). In fact, both methoxy-substituted derivatives or *N*-CH₃ tautomer of HICA were inactive, as well as its ester analogues. Unfortunately, these compounds showed high affinity for both human and plasmodium falciparum-LDH enzymes.²⁵² Granchi and co-workers developed a series of *N*-hydroxyindole derivatives with a carboxylic acid in position 2- that displayed very good affinity for hLDH-A, acting as competitive inhibitor with both substrate and cofactor²⁴⁶ (Figure 76).

The inhibitory effect of the quinone-amino acid hybrids of the present work could be imputed to a similar pharmacophore, composed by the carboxylic acid and the secondary amine (Figure 76). Naphthoic acids have been previously described as LDH inhibitors,²²³ thus, naphthoquinone and anthraquinone scaffolds could also contribute to the inhibitory activity.

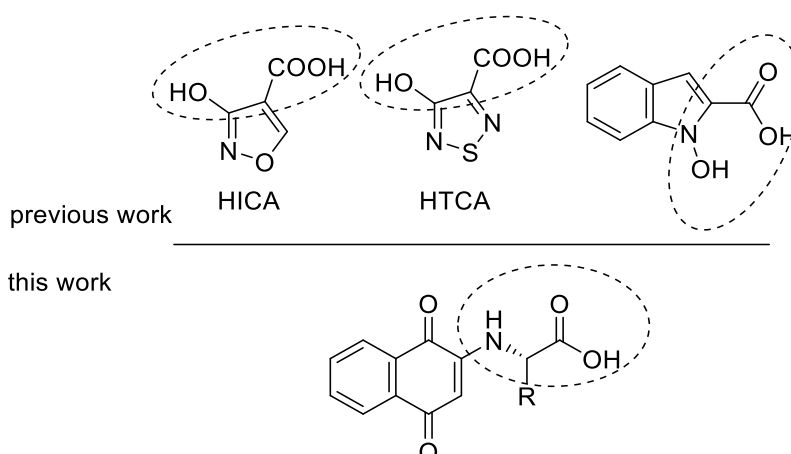


Figure 76 - Structure of LDH inhibitors bearing the OH/COOH pharmacophore and proposed pharmacophore quinone-amino acid conjugates. Key: 3-hydroxyisoxazole-4-carboxylic acid (HICA) and 4-hydroxy-1,2,5-thiadiazole-3-carboxylic acid (HTCA).

Molecular modelling with LDH-A and NHI-based compounds showed strong interactions between Arg109 and the carboxylic acid and H-bonds between OH group and His195 and Arg171 in the active site. Granchi and collaborators concluded that NHI compounds may occupy the whole substrate pocket as well as a portion of the cofactor pocket, which would explain the dual competitive behaviour of NHI derivatives observed in their enzymatic assays,²⁴⁶ and our quinone-amino acid hybrids might act in a similar way.

The hydroxy group

The next key modification is the substitution in position 5- on the indole ring of the quinone-tryptophan hybrids with a hydroxy group. This substitution was clearly detrimental in all compounds tested with this group. Compounds **17a** and **17d** showed higher inhibitory potency compared to **17c** and **17e** (Figure 77).

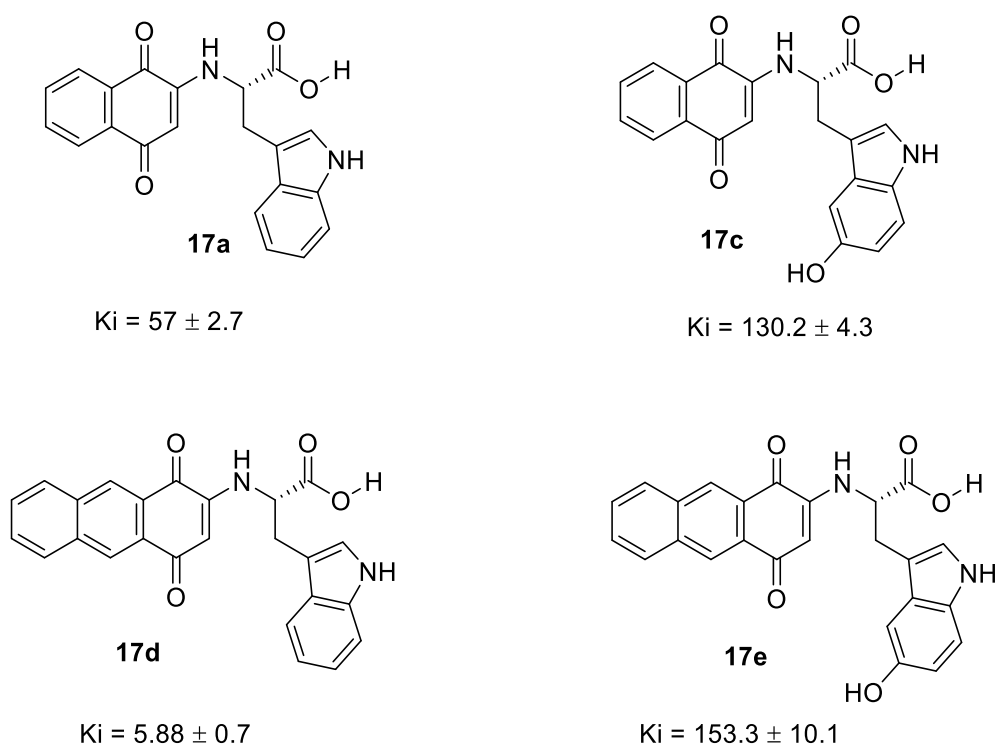


Figure 77 - Chemical structure of quinone-tryptophan conjugates. K_i values are shown for the NADH site.

A hypothetical explanation for these outcomes could lie on the ability of the hydroxyl group to form hydrogen-bonds. The formation of H-bonds in the active site of enzymes may help to adopt the most favourable position of substrate and/or cofactors for the enzymatic reaction to occur.¹⁹¹ However, this might have the reverse effect, where the hydroxy group could interact with an amino acid spatially closer to the active site, preventing the inhibitor from approaching and completely entry inside the pocket, or, once inside, obstructing the compound to adopt the correct position. These two possible situations would imply that the substrate can easily compete and displace the inhibitor from the binding site.

The quinone fragment

Another structural key feature is the anthraquinone fragment. The third ring markedly increased the inhibitory potency of compound **17d** compared to **17a** (Figure 77). The LDH pocket is a very hydrophobic area of the enzyme, in fact, within 10 Å of pyruvate, 24 out of 33 amino acids have lipophilic character.¹⁹⁵ One more possible explanation could be the total length of the molecule, being more favourable for the inhibitory activity compounds bigger in size (for a better interaction inside the pocket). Thus, the anthraquinone fragment improves the inhibitory potency of quinone-hybrids conjugates.

Bearing in mind what we have just discussed, we would expect compound **17e** to inhibit more efficiently the enzyme than **17c**. However, the opposite effect is observed: although lacking the third ring, **17c** competed more efficiently with the substrate (Figure 77). This observation implies that the introduction of a third ring in the structure combined with the substitution on position 5 increases the detrimental effect exerted by the hydroxy group. Nevertheless, the effect of replacing the anthraquinone ring by the naphthoquinone fragment is less acute than the effect observed after the substitution on position 5 in the indole ring.

The amino acid

Regarding the contribution of each amino acid to, we can conclude that *L*-tryptophan presented the best results, closely followed by *L*-histidine, and finally *L*-tyrosine.

In summary, the quinone-*L*-tryptophan subfamily presented all the above-discussed structural modifications, from which valuable insights regarding SAR are gained. Those are: the substitution of the 5' position in the indole moiety with a hydroxy group, which has a detrimental effect on inhibitory activity. Next, the esterification of the carboxylic acid afforded analogues with lower potency and finally, the type of quinone fragment, being the 1,4-anthraquinone fragment more suitable than the 1,4-naphthoquinone.

Determination of the IC₅₀

The half maximal inhibitory concentration IC₅₀ of the naphtho- and anthraquinone derivatives was obtained (Table 13). Opposite to the obtention of the dissociation constant (*K_i*), in the IC₅₀ test the concentrations of cofactor and pyruvate are fixed, and compounds are evaluated using a wide range of concentrations (0 - 1 mM). When determining the IC₅₀ in the nicotinamide site, NADH is added at competitive concentrations (90 µM), whereas pyruvate is added a saturating concentration (2 mM). Likewise, in the pyruvate competition assay, the natural substrate is added at 200 µM (competitive concentration) whilst NADH is added at saturating concentrations (300 µM).

Table 14 - Half maximal inhibitory concentration (IC₅₀) of quinone-hybrids.

Compound	LDH-A IC ₅₀ (μM)	
	NADH ^a	Pyruvate ^b
17a	253 ± 9.6	139.8 ± 4.1
17c	>500	>500
17d	38.5 ± 5.2	12.3 ± 3.0
17e	>500	>500
17f	>500	>500
17g	218.5 ± 12.6	>500
17x	174.2 ± 6.3	251.7 ± 5.5

Compounds were tested in a range of concentrations ranging 0 μM – 1 mM.

a NADH competition assay: competitive concentration of NADH (90 μM) and saturating concentration of pyruvate (2 mM). **b** Competitive concentration of pyruvate (200 μM) and saturating concentrations of NADH (300 μM). Mean ± SD (n=3).

IC₅₀ results correlated with the obtained K_i. Compound **17d** showed the lower IC₅₀ in both enzyme pockets, with 12.3 ± 3 and 38.5 ± 3.2 μM values. Compounds **17a** and **17x** displayed higher IC₅₀ values, both compounds above 100 μM in both binding sites. The remaining compounds presented IC₅₀ values superior to 500 μM.

These values were not surprising. According to the Cheng-Prusoff equation, the IC₅₀ of competitive inhibitors can be related with the K_i (Equation 2.28).

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$

Equation 2.28

Thus, values of IC₅₀ are expected to be equal or higher than the K_i values. This relationship strongly depends on the substrate concentration [S] and it is valid only for competitive inhibition.

These good results obtained by **17d** align it with those found in the literature. Ward and co-workers developed by fragment-based approach a series of malonic acid derivatives which presented IC₅₀ values on enzymatic assays on the low micromolar range.²²⁵ Our reference compound, NHI-2, displayed also very good activity (14.7 ± 2.1 μM in the pyruvate site and 10.5 ± 2.5 μM in the pyruvate) and its acid counterpart, slightly higher values (29 ± 3 and 73 ± 11 μM).²⁵³

2.3.4 K_D , K_i and IC_{50}

It worth pausing and summarise the results obtained until now. Quinone-amino acid conjugates were assessed by two techniques as potential human LDH-A inhibitors. The first one, surface plasmon resonance, provided initial knowledge of the interactions and affinity grade of quinone derivatives for LDH-A enzyme. The second one, enzymatic assays in solution, helped us to confirm the preliminary results in SPR and were used to better characterise the type of inhibition by the obtention of K_i values.

Table 15 - Summary of results obtained with quinone derivatives by SPR and enzymatic assays.

Compound	K_D (μ M)	K_i NADH (μ M)	K_i Pyruvate (μ M)	IC_{50} NADH (μ M)	IC_{50} Pyruvate (μ M)
17a	189	57.0 ± 2.7	49.9 ± 3.8	253 ± 9.6	139.8 ± 4.1
17c	-	130.2 ± 4.3	134.7 ± 8.8	>500	>500
17d	575	5.88 ± 0.7	2.9 ± 0.35	38.5 ± 5.2	12.3 ± 3.0
17e	-	153.3 ± 10.1	212.0 ± 5.1	>500	>500
17f	1100	83.2 ± 4.3	89.3 ± 5.1	>500	>500
17g	682	81.6 ± 1.3	78.8 ± 2.4	218.5 ± 12.6	>500
17x	117	31.6 ± 3.2	84.14 ± 4.1	174.2 ± 6.3	251.7 ± 5.5

In general, it is wiser to compare different LDH inhibitors tested in different laboratories based on their K_i values instead of IC_{50} values. This is due to the highly dependence of IC_{50} on the substrate concentration, according to the Cheng-Prusoff equation, as we discussed previously. Thus, the comparison we made above regarding IC_{50} values must be interpreted with caution. K_i it is a more reliable parameter since it does not possess such limitation.

Similarly, caution is advised when comparing results obtained by SPR, such as dissociation constant (K_D), with those obtained by enzymatic solution-based assays (K_i). The dissociation constant of a system is very dependent on the physical conditions that the components are tested in. Given that SPR is a surface technique, where one component has limited freedom of movement and possibly a constrained orientation, mass transport effects, etc., there are often large differences when comparing to gel-based assays (e.g. EMSAs) which are semi-constrained (caged) or solution-based assays where components are potentially free.

Hence, the different techniques employed in the present study contributed to the identification of a new series of quinone-amino acid hybrids with activity as inhibitors of human LDH-A enzyme.

2.3.5 Antiproliferative Assays

High levels of LDH-A are a hallmark of many tumours, among them GBM.^{254,255} LDH-A is considered a major molecular mediator of the Warburg effect and to play a critical role in nursing cancer's glycolytic phenotype. Remarkably, LDH-A suppression led to reduction of tumour progression.^{256,257}

In view of the good results in enzymatic assays, quinone-amino acids compounds were selected to test their cytotoxic activity in human glioblastoma cell line (U87 MG) using the SRB (Sulforhodamine B) assay.²⁵⁸ This colorimetric method was chosen as reliable alternative to the MTT assay to avoid interferences provoked by LDH inhibition (the MTT assay assesses the cell viability of cells by measuring the activity of dehydrogenase enzymes in the mitochondria).²⁵⁹ The SRB binds to protein basic amino acid residues in TCA-fixed (trichloroacetic acid) cells to quantify cellular proteins. Cells were exposed to quinone-amino acid hybrids for 48 h and the IC₅₀ for each compound was calculated (Table 15).

Table 16 - Cytotoxic effect on U87-MG cells after 48 h of treatment with quinone-amino acid hybrids, as determined by the SRB assay. Mean $\pm$ SD (n=6).

Compound	IC ₅₀ (μ M)
17a	158 $\pm$ 9
17c	256 $\pm$ 8
17d	> 500
17f	365 $\pm$ 16
17g	51 $\pm$ 10

Interestingly, only the compound **17g** showed cytotoxic activity on glioma cells, with an IC₅₀ value of 51 $\pm$ 10 μ M. Compounds **17a**, **17c** and **17f** barely showed cytotoxic activity whereas compound **17d** was inactive.

The phospholipidic bilayer in the cell membrane is impermeable to polar molecules such as glucose or amino acids. The cellular membrane naturally possesses a negative charge that ranges between -40 and -80 mV. Increased membrane charge can increase the permeability of amino acids of opposite charge,²⁶⁰ whilst repelling those with same charge. The -COOH can be easily deprotonated, thus preventing the molecules from passing through the membrane. In the case of the *L*-histidine analogue (**17g**), the imine-like N atom in the imidazole has a pKa of 6.75, and it readily gets protonated. Thus, the net charge of this molecule would be zero (-COO⁻, =NH⁺-) which could facilitate the pass through the membrane.

A potential approach to solve this problem would be masking the carboxylic groups as methyl ester derivatives. Then, subsequent cleavage of the esters into the active acid form should occur once the compounds were inside the cell.^{253,261}

The third benzene ring in compound **17d** could also prevent the permeation through the cell membrane, which would explain the differences found between **17d** and its naphthyl-analogue **17a**.

More studies are planned to assess the activity the methyl ester derivatives **17h** and **17b** as well as improve the ADME properties of this compounds, including structural modifications, and formulation of best candidates for targeted therapies-

2.4 CONCLUSIONS

A novel class of inhibitors of human LDH-A enzyme has been identified in a series of quinone-amino acids conjugates.

Results obtained in the enzymatic assays led to conclusion that this series of quinone-based molecules are competitive inhibitors of LDHA with NADH and pyruvate. Some of the tested compounds presented good K_i values. Compound **17d** showed remarkable activity as LDH-A inhibitor.

Cytotoxic activity of studied quinone-amino acid conjugates has been assessed on glioblastoma cell line. Results suggest that physicochemical properties of compounds like **17d** are not optimal for cellular permeability. By contrast, compound **17g**, which possess lower activity against LDH-A on the enzymatic assays, showed the best cytotoxic activity.

Further studies are needed to characterise the interactions with the LDHA molecule, as well as improving their ADME properties. Nevertheless, the good results on enzymatic assays encourage us to continue studying these quinone-based compounds and develop strong candidates suitable for antitumoral therapies.

2.5 MATERIALS AND METHODS

2.5.1 Materials and Equipment

Chemicals and reagents

All reagents employed as starting materials were purchased from commercial suppliers with high purity and were used without further purification. Solvents were obtained from Sigma-Aldrich, Acros and Scharlab and were employed with no additional pre-treatment. Reactions were monitored by Thin Layer Chromatography (TLC) whose spots were visualized by an UV lamp at 254 and 356 nm. In certain occasions, a solution of Vanillin or Phosphomolybdic acid, with subsequent heating, was used for better traceability of the reaction course. When needed, the purification of the synthesised compounds was performed by flash column chromatography with Silica Gel Merck-60 (230-400 mesh). All plastics for experiments and 96- well plates were obtained from Sarstedt Ltd. (Leicester, UK) or ThermoFisher Scientific. UV-VIS spectrometry assays were performed in a Multiskan Spectrophotometer (ThermoFisher Scientific).

NMR analysis

Nuclear magnetic resonance (NMR) spectra were recorded in deuterated solvents on Bruker AVANCE-300, Varian INOVA -300 Varian INOVA -400 and Varian INOVA-500 spectrometers. ^{13}C -NMR were registered with complete proton decoupling. The chemical shifts measured are reported in δ (ppm) and the residual signal of the solvent was used as the internal calibration standard. The multiplicity of the signals is reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet, ddt = doublet of doublet of triplet, br = broad signal. The coupling constant J is reported in hertz (Hz).

LC-MS analysis

HPLC-MS analyses were performed on a Waters (2695 HPLC system) apparatus, equipped with a quaternary pump and photodiode array detector (PDA), using a SunfireTM column (C18 stationary phase, 3.5 μm particle size, 4.6 $\times$ 50 mm). Mobile phase consisted of acetonitrile +0.08% formic acid (v/v) and H_2O +0.01% formic acid (v/v). The solvent gradient is specified for each compound in the in the following section. The molecular weight was determined by the nominal mass obtained from a single quadrupole mass spectrometer coupled to the HPLC system operating in positive electrospray ionization (ES^+).

UPLC-IM-HRMS analysis

The chromatographic separation was achieved on an ACQUITY UPLC® I-Class System with an FTN sample manager. An Acquity BEH C18 column (Waters) with 2.1 x 100 mm and particle size of 1.7 μm , heated at 40°C was used. The injection volume was 4 μL , and the flow rate was 0.45 mL min^{-1} . LC solvents were 5 mM ammonium formate in water +0.1% formic acid (aqueous mobile phase, A), and acetonitrile +0.1% formic acid (organic mobile phase, B). A binary gradient method was used as follows: initial equilibration at 2% B for 0.7 min; 2 – 100% B in 14.1 min; hold at 100% B for 2.9 min, reprimed initial conditions (2% B) in 0.01min, final re-conditioning at 2% B for 2.29 min. The total run time was 20 min.

The chromatographic system was interfaced with a Vion IMS QToF (Waters) operating in electrospray positive and negative mode (ESI+/-). The mass range was m/z 50 – 1000 at an acquisition frequency of 5 Hz, using the following source parameters: capillary voltage +2.0 kV and -1.7 kV, sampling cone voltage 32 and 25 V for positive and negative ion mode, respectively, source temperature 120 °C, desolvation gas temperature 650 °C, desolvation gas flow 950 L h^{-1} , cone gas flow 50 L h^{-1} . Nitrogen was used as collision gas. Two independent scans with different collision energies (CE) were alternatively acquired during the run in high definition MS^E mode (HDMS^E acquisition). A low-energy scan (CE 6 eV) was to monitor the protonated/deprotonated molecules and other potential adducts, whilst the high-energy scan (CE ramp 27–55 eV) was used to fragment the ions traveling through the collision cell. The LockSpray was used for the one-point real-time mass correction and consisted of a 50 $\text{pg } \mu\text{L}^{-1}$ solution of Leucine-Enkephalin solution in 50:50 acetonitrile:water +0.1% formic acid (LockSpray infusion rate: 15 $\mu\text{L min}^{-1}$, acquisition every 2.5 min). The TOF analyzer operated in sensitivity mode. The drift cell was set using the following parameters: IMS gas flow rate 25 mL min^{-1} , wave velocity 250 m s^{-1} , IMS pulse height 45 V. Major Mix IMS/ToF Calibration Kit (Waters, p/n 186008113) was used for mass and CCS calibration. Data acquisition and processing were performed using UNIFI software v. 1.9.4.

LDH-A Enzyme

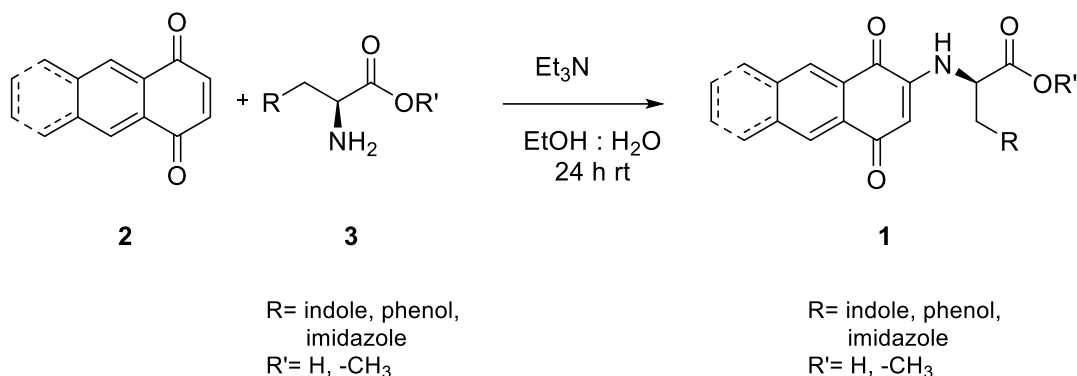
Purified human Lactate Dehydrogenase A enzyme (recombinant, expressed in *E. coli*,) was purchased at Sigma-Aldrich (Product number SAE0049, Purity >95%, Enzymatic Assay = 1014 (Activity U/ml).

Cell Culture

Cell culture reagents were obtained from Gibco and Thermo Fisher Scientific (Paisley, UK). Plastics for the enzymatic assays and cell culture were obtained from Sarstedt Ltd. (Leicester, UK) and Thermo Fisher Scientific.

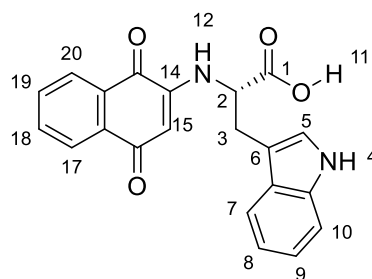
2.5.2 Synthesis and characterization of Quinone-Amino Acid Conjugates

General procedure:



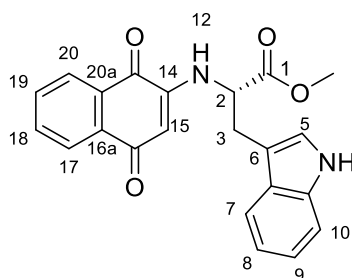
Scheme 2.6- Michael addition reaction for the functionalization of *L*-amino acids (3) with quinone-based compounds (2).

In a round bottom flask, anthracene-1,4-dione or naphthalene-1,4-dione **2** (2 eq) were added to a stirring solution containing one equivalent the corresponding *L*-amino acid **3** and Et₃N (0.7 mL/mmol) dissolved in ethanol (20 mL/mmol) and H₂O (2 mL/mmol). The reaction mixture was stirred for 24 h at room temperature, after which the solvent was removed under high vacuum. The crude reaction was then basified with saturated Na₂CO₃ and extracted three times with AcOEt. If necessary, the aqueous phase was then acidified with HCl 36% and extracted again with AcOEt. The combined organic phases were dried over anhydrous MgSO₄, filtered and evaporated under high vacuum. The crude product was purified by flash column chromatography to afford the desired quinone-amino acid conjugate derivatives **1a-i**.

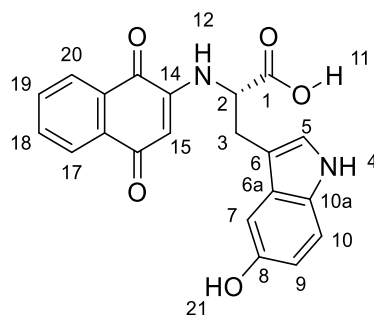


***N*-(1,4-dioxo-1,4-dihydronaphthalen-2-yl)-*L*-tryptophan, 17a.** According to the general procedure for the synthesis of quinone- amino acid hybrids, the commercially available naphthalene-1,4-dione (550 mg, 3.4 mmol), *L*-tryptophan (0.355 mg, 1.7 mmol), and Et₃N (1.19 mL, 8.5 mmol) were dissolved and allowed to react in EtOH/H₂O. The crude product was purified by chromatography (DCM: MeOH)

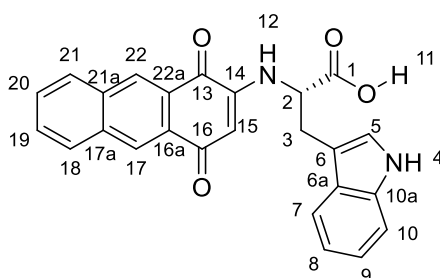
to yield a reddish solid (376 mg, 61% yield). The spectrometric data is in accordance with that found in the litterature.²⁴⁵ **¹H NMR** (500 MHz, DMSO-*d*₆) δ 10.81 (s, 1H, -OH), 7.92 (ddd, *J* = 11.7, 7.7, 1.3 Hz, 2H, **17**, **20**), 7.81 (td, *J* = 7.6, 1.3 Hz, 1H, **19** or **18**), 7.70 (td, *J* = 7.5, 1.3 Hz, 1H, **19** or **18**), 7.48 (d, *J* = 7.9 Hz, 1H, **7** or **10**), 7.28 (d, *J* = 8.2 Hz, 1H, **7** or **10**), 7.12 (dd, *J* = 12.0, 4.8 Hz, 2H, **5**, -NH), 6.99 (ddd, *J* = 8.1, 6.9, 1.1 Hz, 1H, **8** or **9**), 6.86 (ddd, *J* = 7.9, 7.0, 1.1 Hz, 1H, **8** or **9**), 5.63 (s, 1H, **15**), 4.17 – 4.14 (m, 1H), 3.31 – 3.24 (m, 2 H, **3**, **3'**). **¹³C NMR** (126 MHz, DMSO-*d*₆) δ 181.4 (C=O), 181.3 (C=O), 172.3 (C=O), 147.1 (C), 135.9 (C), 134.9 (C18 or C19), 133.1 (C), 132.2 (C18 or C19), 130.1 (C), 127.7 (C), 125.9 (C17 or C20), 125.3 (C17 or C20), 123.8 (C), 120.7 (C8 or C9), 118.27 (C7 or C10), 118.2 (C8 or C9), 111.2 (C7 or C10), 109.93 (C), 99.8 (C15), 56.6 (C2), 26.26 (C3) ppm. **HPLC-MS (ES⁺)**: CH₃CN/H₂O 30:70 to 95:5 (5 min), RT = 4.74 min, [M+H⁺] = 361.



Methyl N-(1,4-dioxo-1,4-dihydronaphthalen-2-yl)-L-tryptophanate 17b: According to the general procedure for the synthesis of quinone- amino acid hybrids, the commercially available naphthalene-1,4-dione (550 mg, 3.4 mmol), methyl *L*-tryptophanate hydrochloride (0.355 mg, 1.7 mmol), and Et₃N (1.19 mL, 8.5 mmol) were dissolved and allowed to react in EtOH/H₂O. The crude product was purified by chromatography (DCM: MeOH) to yield a brown solid (392 mg, 63% yield). **¹H NMR** (300 MHz, Acetone-*d*₆) δ 7.99 (ddd, *J* = 7.6, 3.1, 1.3 Hz, 2H, **17**, **20**), 7.79 (tt, *J* = 7.6, 1.4 Hz, 1H, **19** or **18**), 7.69 (tt, *J* = 7.5, 1.4 Hz, 1H, **19** or **18**), 7.56 (d, *J* = 8.0 Hz, 1H, **7** or **10**), 7.38 (d, *J* = 8.1 Hz, 1H, **7** or **10**), 7.28 (d, *J* = 2.5 Hz, 1H, **5**), 7.09 (ddd, *J* = 8.1, 6.9, 1.3 Hz, 1H, **8** or **9**), 7.01 (ddd, *J* = 8.1, 7.0, 1.2 Hz, 1H, **8** or **9**), 6.60 (d, *J* = 8.1 Hz, 1H, -NH), 5.77 (s, 1H, **15**), 4.61 (dt, *J* = 8.1, 5.7 Hz, 1H, **2**), 3.72 (s, 3H, -CH₃), 3.60 – 3.40 (m, 2H, **3**). **¹³C NMR** (75 MHz, Acetone) δ 181.99 (C=O), 181.29 (C=O), 171.14 (C=O), 146.95 (C), 136.7 (C), 134.6 (C18 or C19), 133.2 (C), 132.2 (C18 or C19), 130.5 (C), 127.6 (C), 125.9 (C17 or C20), 125.6 (C17 or C20), 124.0 (C5), 121.5 (C8 or C9), 118.9 (C8 or C9), 118.1 (C7 or C10), 111.50 (C7 or C10), 108.8 (C), 101.6 (C15), 55.4 (C2), 51.9 (-CH₃), 26.77 (C3). **HPLC-MS (ES⁺)**: CH₃CN/H₂O 30:70 to 95:5 (10 min), RT = 4.80 min, [M+H⁺] = 375.

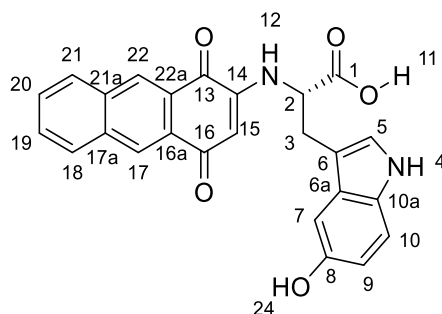


***N*-(1,4-dioxo-1,4-dihydronaphthalen-2-yl)-5'-hydroxy-*L*-tryptophane, **17c**:** According to the general procedure for the synthesis of quinone- amino acid hybrids, the commercially available naphthalene-1,4-dione (275 mg, 1.7 mmol), *L*-5-hydroxy-tryptophan (187 mg, 0.85 mmol), and Et₃N (0.6 mL, 4.3 mmol) were dissolved and allowed to react in EtOH/H₂O. The crude product was purified by chromatography (Hexane/AcOEt/MeOH; 5:2:3) to yield a brown solid (183 mg, 57% yield). ¹H NMR (300 MHz, DMSO-*d*₆) δ 10.45 (s, 1H, COOH), 8.53 (s, 1H, -OH), 7.93 (*apparent* t, *J* = 7.6 Hz, 2H), 7.81 (dd, *J* = 8.4, 6.9 Hz, 1H, **18** or **19**), 7.70 (td, *J* = 7.5, 1.4 Hz, 1H, **19** or **18**), 7.15 (d, *J* = 7.0 Hz, 1H, -NH **12**), 7.06 (d, *J* = 8.5 Hz, 1H, **10**), 6.97 (d, *J* = 2.3 Hz, 1H, **5**), 6.81 (d, *J* = 2.3 Hz, 1H, **7**), 6.52 (dd, *J* = 8.5, 2.3 Hz, 1H, **9**), 5.76 (s, 1H, **15**), 4.07 – 3.96 (m, 1H), 3.19 (qd, *J* = 14.7, 5.2 Hz, 2H, **3**, **3'**). ¹³C NMR (75 MHz, DMSO-*d*₆, 25 °C): 181.5 (C=O), 181.2 (C=O) 172.4 (CO) 150.11 (C), 146.9 (C), 134.8 (C18 or C19), 133.3 (C), 132.0 (C19 or C18), 130.4 (C), 130.2 (C), 128.5 (C), 125.9 (C17 or C20) 125.3 (C20 or C17) 124.1 (C5), 111.4 (C7), 111.0 (C9), 109.2 (C7), 102.4 (C10), 99.4 (C15), 54.9 (C2), 26.4 (C3) ppm. HPLC-MS (ES⁺): gradient CH₃CN/H₂O 20:80 to 95:5 (5 min), RT = 4.63 min, [M+H⁺] = 377.

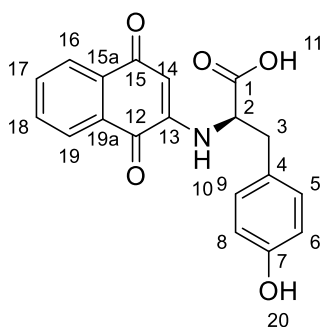


***N*-(1,4-dioxo-1,4-dihydroanthracen-2-yl)-*L*-tryptophan, **17d**:** According to the general procedure for the synthesis of quinone- amino acid hybrids, anthracene-1,4-dione (611 mg, 2.93 mmol), *L*-tryptophan (300 mg, 1.46 mmol), and Et₃N (1 mL, 7.32 mmol) were dissolved and allowed to react in EtOH/H₂O. The crude product was purified by chromatography (Hexane/AcOEt/MeOH; 5:2:2) to yield a brown solid (405 mg, 68% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.69 (s, 1H, COOH), 8.61 (s, 1H, **22** or **17**), 8.48 (s, 1H, **22** or **17**), 8.23 (d, *J* = 7.9 Hz, 1H, **18** or **21**), 8.19 (d, *J* = 7.9 Hz, 1H, **18** or **21**), 7.79 – 7.66 (m, 2H, **20**, **19**), 7.51 (s, 1H, -NH), 7.44 (d, *J* = 7.9 Hz, 1H, **10**), 7.25 (d, *J* = 8.2 Hz, 1H, **7**), 7.06 (d, *J* = 2.2 Hz, 1H, **5**), 6.94 (t, *J* = 7.1 Hz, 1H, **9**), 6.79 (t, *J* = 7.9 Hz, 1H, **8**), 5.67 (s, 1H, **15**), 3.88 – 3.79 (m, 1H, **2**), 3.28 – 3.15 (m, 2H, **3**, **3'**). ¹³C NMR (75 MHz, DMSO-*d*₆, 25 °C): 181.0 (C=O), 180.5 (C=O), 173.9 (C=O), 147.8 (C), 137.80 (C) 137.4 (C), 133.3 (C17 or C22), 132.1 (C), 130.1 (C17 or C22),

129.7 (C) 129.6 (C20 or C19) 128.6 (C) 128.4 (P), 128.0 (C21 or C18), 127.4F (C21 or C18), 126.8 (C), 126.4 (C5), 123.7 (C9 or C8), 120.5 (C9 or C8), 117.9 (C10 or C7), 112.6 (C), 111.1 (C10 or C7), 99.5 (C15), 57.3 (C2), 26.5 (C3) ppm. **HPLC-MS (ES⁺):** CH₃CN/H₂O 50:50 to 95:5 (5 min), RT = 1.93 min, [M+H⁺] = 411.

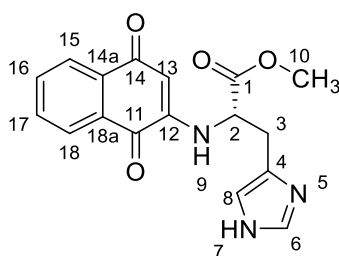


N-(1,4-dihydroanthracen-2-yl)-5'-hydroxy-L-tryptofano 17e: According to the general procedure for the synthesis of quinone- amino acid hybrids, commercially available anthracene-1,4-dione (470 mg, 2.26 mmol), L-5-hydroxy-tryptophan (250 mg, 1.13 mmol), and Et₃N (0.8 mL, 5.7 mmol) were dissolved and allowed to react in EtOH/H₂O. The crude product was purified by chromatography (Hexane/AcOEt/MeOH; 5:2:3) to yield a brown solid (254 mg, 52% yield). **¹H NMR** (300 MHz, DMSO-d₆, 25 °C): δ 8.65 (s, 1H, **22** or **17**), 8.55 (s, 1H, OH), 8.51 (s, 1H, **22** or **17**), 8.25 (d, *J* = 7.9 Hz, 1H, **21** or **18**), 8.21 (d, *J* = 7.9 Hz, 1H, **21** or **18**), 7.79 – 7.66 (m, 3H, **20**, **19**, NH), 7.10 (dd, *J* = 8.4, 4.5 Hz, 2H, **10**, NH), 7.05 (d, *J* = 2.4 Hz, 1H, **5**), 6.83 (d, *J* = 2.3 Hz, 1H, **7**), 6.56 (dd, *J* = 8.6, 2.3 Hz, 1H, **9**), 5.81 (s, 1H, **13**), 3.91-3.98 (m, 1 H, **2**) 3.24-3.34 (m, 2H, **3**) ppm. **¹³C NMR** (75 MHz, DMSO-d₆, 25 °C): 181.1 (C=O), 180.8 (C=O), 170.5 (C=O), 150.2 (C), 148.1 (C), 135.2 (C), 133.5 (C), 130.6 (C22 or C17), 130.2 (C22 or C17), 129.8 (C21 or C18), 129.8 (C) 129.7 (C), 128.8 (C21 or C18), 128.5 (C), 127.5 (C20 or C19), 126.5 (C19 or C20), 124.3 (C5), 114.5 (C) 111.5 (C7), 111.1 (C9), 109.7 (C), 102.5 (C10), 101.3 (C15), 48.7 (C2), 31.4 (C3) ppm. **HPLC-MS (ES⁺):** CH₃CN/H₂O 30:70 to 95:5 (5 min), RT = 4.43 min, [M+H⁺] = 427.

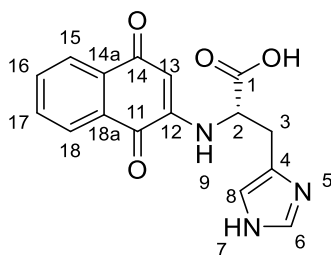


N-(1,4-dioxo-1,4-dihydronaphthalen-2-yl)-L-tyrosine, 17g. According to the general procedure for the synthesis of quinone- amino acid hybrids, naphthalene-1,4-dione (450 mg, 2.9 mmol), L-tyrosine

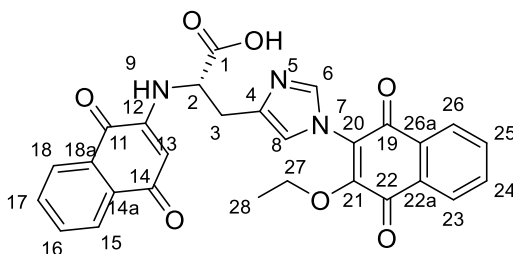
(256 mg, 1.4 mmol), and Et₃N (1 mL, 7.17 mmol) were dissolved and allowed to react in EtOH/H₂O. The crude product was purified by chromatography (Hexane/AcOEt/MeOH; 5:2:0.5) to yield a brown solid (123 mg, 26% yield). **¹H NMR** (500 MHz, Methanol-*d*₄) δ = 8.01 (d, *J* = 6.8 Hz, 2H), 7.98 (d, *J* = 7.6 Hz, 1H), 7.76 (td, *J* = 7.5, 1.3 Hz, 2H), 7.67 (td, *J* = 7.6, 1.3 Hz, 2H), 7.04 (d, *J* = 8.4 Hz, 2H), 6.68 (d, *J* = 8.4 Hz, 2H), 5.63 (s, 1H), 4.29 (m, 1H), 3.23 (dd, *J* = 14.1, 5.1 Hz, 1H), 3.09 (dd, *J* = 14.0, 6.9 Hz, 1H). **¹³C NMR** (126 MHz, cd₃od) δ 183.57 (C=O), 180.81 (C=O), 156.12 (C=O), 147.7(C), 134.5 (C17 or C18), 133.22 (C), 132.1 (C17 or C18), 130.5 (C), 130.1 (C8 and C6), 127.0 (C), 126.0 (C19 or C16), 125.4 (C19 or C16), 114.9 (C9 and C5), 100.1 (C14), 65.5 (C2), 36.0 (C3). **HPLC-MS (ES⁺)**: CH₃CN/H₂O 25:75 to 95:5 (5 min), RT = 4.07 min, [M+H⁺] = 338.



Methyl N-(1,4-dioxo-1,4-dihydronaphthalen-2-yl)-L-T , 17h According to the general procedure for the synthesis of quinone- amino acid hybrids, the commercially available naphthalene-1,4-dione (655 mg, 4.14 mmol), methyl-L-histidinate hydrochloride (425 mg, 2.07 mmol), and Et₃N (1.45 mL, 10.2 mmol) were dissolved and allowed to react in EtOH/H₂O for 24h. The crude product was purified by chromatography using DCM/MeOH under gradient conditions to yield a brown solid (52 mg, 38% yield). **¹H NMR** (500 MHz, Chloroform-*d*) δ 8.02 (m, 2H, **15**, **18**), 7.76 – 7.66 (m, 2H, **6** and **16** or **17**), 7.60 (ddd, *J* = 8.8, 5.2, 2.0 Hz, 1H, **16** or **17**), 7.00 (d, *J* = 7.5 Hz, 1H, -NH, **12**), 6.88 (s, 1H, **8**), 5.67 (s, 1H, **13**), 4.44 – 4.32 (m, 1H, **2**), 3.74 (s, 3H, **10**), 3.32 – 3.18 (m, 2H, **3**, **3'**). **¹³C NMR** (126 MHz, Chloroform) δ 183.5 (C=O), 181.5 (C=O), 170.9 (C=O), 147.3 (C), 135.5 (C6), 134.8 (C16 or C17), 134.2 (C), 133.4 (C), 132.3 (C17 or C16), 130.6 (C), 126.5 (C15 or C18), 126.2 (C15 or C18), 115.2 (C8), 102.1 (C13), 55.1 (C2), 52.9 (C10), 29.6 (C3) ppm. **HPLC-MS (ES⁺)**: CH₃CN/H₂O 20:80 to 95:5 (5 min), RT = 4.51 min, [M+H⁺] = 326.



***N*-(1,4-dioxo-1,4-dihydronaphthalen-2-yl)-*L*-histidine, 17g.** According to the general procedure for the synthesis of quinone- amino acid hybrids, the commercially available naphthalene-1,4-dione (202 mg, 1.28 mmol), *L*-histidine (100 mg, 0.64 mmol), and Et₃N (0.95 mL, 6.54 mmol) were dissolved and allowed to react in EtOH/H₂O 24 h. The crude product was purified by chromatography using Hexane/AcOEt/MeOH [5:3:1] to yield a brown solid (90 mg, 45% yield) **¹H NMR** (300 MHz, DMSO-d₆, 25 °C): δ = 9.78 (br, 1 H, COOH) 7.99 (d, 1 H, *J* = 7.3 Hz, **15**) 7.91-7.96 (m, 1 H, **18**) 7.84-7.86 (m, 1 H, **6**) 7.71-7.76 (m, 2 H, **16**, **17**), 7.54 (t, 1 H, *J* = 7.5 Hz, NH), 6.94 (s, 1 H, **8**), 6.88 (d, *J* = 8.4 Hz, 1 H, NH), 5.67 (s, 1 H, **13**), 3.63-3.66 (m, 1 H, **2**), 3.12-3.18 (m, 2 H, **3**, **3'**) ppm. **¹³C NMR** (75 MHz, DMSO-d₆, 25 °C): 181.8 (C=O), 181.1 (C=O), 170.0 (C=O), 147.6 (C), 147.6 (C), 135.8 (C6), 134.9 (C17 or C16), 133.7 (C), 132.4 (C), 130.4 (C17 or C16), 125.9 (C), 125.3 (C15 or C18), 125.2 (C15 or C18), 112.9 (C8), 101.0 (C13), 54.7 (C2), 54.7 (C2), 21.0 (C3) ppm. **HPLC-MS (ES⁺)**: CH₃CN/H₂O 25:75 to 95:5 (5 min), RT = 3.59 min, [M+H⁺] = 312.



***N*-(1,4-dioxo-1,4-dihydronaphthalen-2-yl)-*N'*-(3-ethoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-*L*-histidine, 17x.** Naphthalene-1,4-dione (510 mg, 3.2 mmol), *L*-histidine (250 mg, 1.6 mmol), and Et₃N (1.12 mL, 7.9 mmol) were dissolved and allowed to react in EtOH/H₂O for 72 h. The crude product was purified twice by chromatography using Hexane/AcOEt/MeOH under gradient conditions to yield product 1x (82 mg, 10% yield). **¹H NMR** (400 MHz, DMSO-d₆) δ 8.02 (dd, *J* = 7.7, 1.4 Hz, 1H), 7.95 (ddd, *J* = 7.8, 4.8, 1.3 Hz, 2H), 7.89 – 7.82 (m, 3H), 7.75 (ddd, *J* = 14.8, 7.5, 1.4 Hz, 2H), 7.59 (td, *J* = 7.5, 1.4 Hz, 1H), 7.10 (s, 1H), 5.70 (s, 1H, **13**), 4.57 (s, 1H), 4.15 (q, *J* = 7.1, 2H), 3.21 (s, 2H), 1.19 (t, *J* = 7.1 Hz, 3H). **HPLC-MS (ES⁺)**: CH₃CN/H₂O 20:80 to 95:5 (10 min), RT = 5.82 min, [M+H⁺] = 512

2.5.3 Enzymatic assays

The oxidation of NADH was observed at 340nm, which is a direct measure of the consumption of pyruvate and its transformation into lactate. The reaction rate of LDH-A was determined by a decrease in absorbance of NADH at 340 nm in a UV-VIS spectrophotometer. All the assays here described were carried out by measuring the optical values every 15 seconds for 3 min at 37 °C.

In order to avoid loss of activity, commercial LDH-A solution was slowly thaw on ice and aliquoted in plastic eppendorf tubes (30 µL/eppendorf). Aliquots were kept at -18 °C and thaw immediately prior to use. Enzyme stocks were kept on ice for the whole length of the assay. The NADH stock solution was freshly prepare before each assay and kept on ice covered in foil. By contrast, pyruvate stocks solutions are stable and can be prepared in advance and kept in the fridge when not used. Buffer for assays was kept warm (40 °C) in a water bath. In every assay, calculations were made for a total volume of 200 µL/well.

Sodium phosphate buffer (100 mM, pH 7.4) was used to prepare all the stock solutions of reagents.

Enzyme stock preparation:

Commercial LDH-A enzyme possessed an activity of 1014 U/mL. A first diluted stock of LDH-A at 6 U/mL was freshly prepared by diluting 6 µL of enzyme solution at 1014 U/mL in 994 µL of buffer 0.1M pH 7.4. From this stock at 6 U/mL, a second 10-fold diluted stock (0.6 U/mL) was prepared (100 µL in 1 mL of buffer, total volume). 20 µL of this second stock were added to reaction wells, diluted 1 to 10 in reaction media, affording the desired 0.06 U/mL.

Determination of Michaelis-Menten constants:

Michaelis-Menten constants (K_m) for both substrates of LDH-A (NADH and Pyruvate) were calculated in saturating conditions as described elsewhere with minimal modifications:²⁴⁶

- NADH K_m determination: in a 96-well plate, a range of NADH concentrations (12 µM- 900 µM) were combined with 2 mM of sodium pyruvate (pyruvate-saturated) and 0.06 units/mL of LDH-A.
- Pyruvate K_m determination: in a 96-well plate, a range of pyruvate concentrations (25-2025 µM) were combined with 300 µM of sodium pyruvate (NADH-saturated) and 0.06 units/mL of LDH-A.

The reaction velocity V [concentration (µM)/min] of LDH-A was obtained by applying two equations. First, the decrease of absorbance of NADH per minute was calculated according to equation 2.29:

$$\Delta Absorbance/min = \frac{[OD_{t=0} - OD_{t=3}]}{3}$$

Equation 2.29

The optical density (OD) value at time = 3 minutes was subtracted to the OD value at time zero and divided by 3 to obtain the variation of absorbance of NADH per minute ($\Delta Absorbance/min$).

The Lambert-Beer law (Equation 2.30) directly correlates the concentration and the absorbance of a particular substance in the solution, thus allowing to calculate the concentration of that substance by measuring the absorbance.

$$A = C \times l \times \varepsilon \quad \text{Equation 2.30}$$

where A = absorbance; C = molar concentration and l = path length (cm). The molar extinction coefficient (ε , M^{-1}) of NADH at 340 nm is 6.2 mM^{-1} .

Lambert-Beer equation can be rewritten as

$$C = A / l \times \varepsilon \quad \text{Equation 2.31}$$

The Absorbance/min value obtained in equation 2.29 is then included in the Lambert-Beer law equation (Equation 2.31) to calculate the reaction velocity, V , which is expressed as $\mu\text{M}/\text{min}$:

$$V = C/\text{min} = \frac{A (\Delta\text{Absorbance} / \text{min})}{l \times \varepsilon} \quad \text{Equation 2.32}$$

Data analysis was performed by nonlinear regression analysis with GraphPad Prism 8.4 software (GraphPad – USA).

Opposite to experiments in cuvettes, where light pass through the cuvette horizontally, experiments performed in 96-well plates the direction of the incidental light is from top to bottom. Therefore, the path length will depend on the volume added in each well. All the assays carried out in this study had 200 μL as final volume. The path length was determined experimentally accordingly to 200 μL total volume.

Determination of the path length in 96-well microtiter

Materials: Thioflavin T (ThT), cuvette (1 cm path length) and 96-well plate with flat bottom.

ThT is a coloured substance whose molar extinction coefficient ε at 412 nm is known (31600 M^{-1}). ThT was weighted and dissolved in deionized water at 1 mM (MW = 318.86 g/mol). This stock solution was filtered and diluted further to 0.05 mM (50 μM). Absorbance of ThT (expressed as optical density, OD) was measured at 412 nm in a spectrophotometer.

- Determination of the absorbance of ThT in the cuvette (A_1):

2 mL of ThT solution at 50 μM were introduced into the cuvette and measured at the above-mentioned wavelength. The average absorbance (A_1) of three replicates was obtained:

$$A_1 = 1.093$$

- Determination of the absorbance of ThT in the 96-well plate (A_2)

200 μL of ThT solution at 50 μM were dispensed in the microtiter and the OD values were acquired at 412 nm. The OD average value, A_2 , of three replicates was calculated:

$$A_2 = 0.523$$

We have two Lambert-Beer equations, one for the cuvette test (equation 2.33) and one with the microplate test (equation 2.34):

$$A_1 = \epsilon \times C_1 \times l_1$$

Equation 2.33

$$A_2 = \epsilon \times C_2 \times l_2$$

Equation 2.34

ϵ depends only on the employed wavelength. Here we have used the same value (412 nm), therefore, ϵ is constant. C is the molar concentration of ThT. In both test the concentration was the same, thus, $C_1 = C_2$.

Only the path length is different. The length of the cuvette (l_1) is standard (1 cm), whereas the path length of 200 μL in the microtiter is our unknown value.

If C_1 is equal to C_2 , then:

$$\frac{A_1}{\epsilon \times l_1} = \frac{A_2}{\epsilon \times l_2}$$

Equation 2.35

ϵ is constant, so we can remove it from the equation and get the equation for l_2 :

$$l_2 = \frac{A_2 \times l_1}{A_1} = \frac{0.523 \times 1}{1.093} = 0.48 \text{ cm}$$

Equation 2.36

According with equation 2.36, the path length (l) for a microtiter well plate filled up with 200 μL is 0.48 cm. This value has been used on Equation 2.32 for rate reaction determination in this study.

Obtention of UV-visible spectrum of quinone-amino acid conjugates

The UV-visible spectrum of compounds was obtained, so any possible interference of quinone-amino acid hybrids with NADH absorbance.

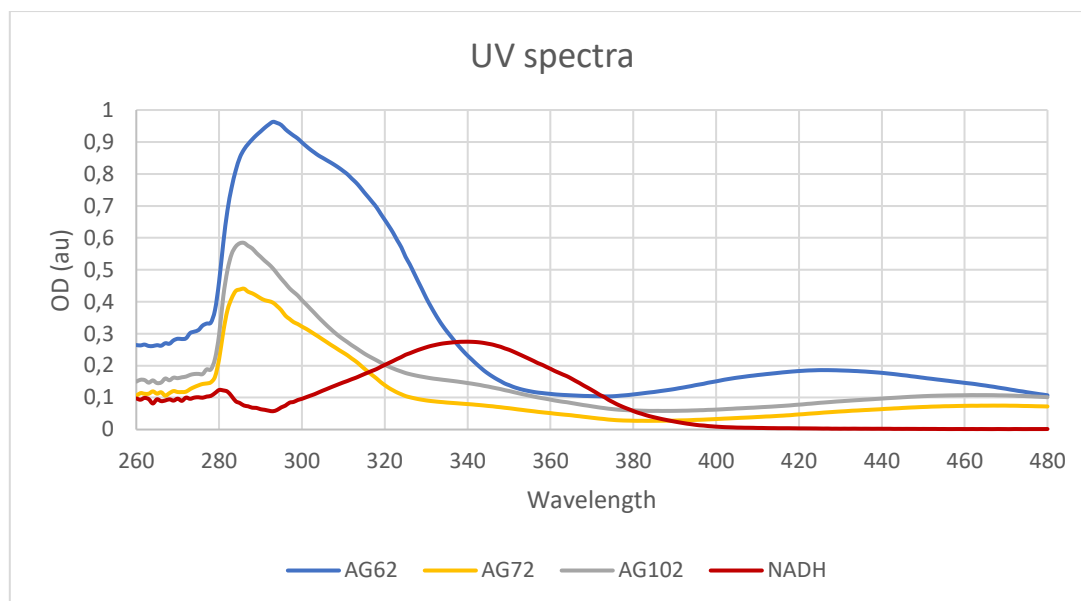


Figure 78 - UV-visible absorbance spectra of some quinone-amino acid hybrids and NADH at 100 μM . OD values were acquired from 200 to 600 nm, although only the important window of values is shown.

The maximum absorbance of NADH was found at 340 nm. Compound **17d** presents a blue shift due to the presence of a third ring in its structure, the anthracene core. In all assays, background absorbance corresponding to quinone derivatives was calculated and subtracted.

Screening of quinone - amino acids hybrids as LDH-A inhibitors.

Inhibitory properties of compounds were initially screened in an enzymatic assay against human LDH-A. 0.06 units of enzyme were combined with the cofactor NADH (300 μM), the substrate pyruvate (200 μM) and the candidate inhibitors at 125 μM dissolved in 0.1 M of sodium phosphate.

Enzyme stocks were prepared as specified at the beginning of this section 2.5.3. Stocks of compounds were prepared in pure DMSO at 50 mM. 10-fold more concentrated diluted stock solutions of compounds (1.25 mM, 2.5% DMSO v/v) were prepared by diluting 2 μL of 50 mM stock in 78 μL of 0.1 M phosphate buffer. Then, 20 μL of 1.25 mM stock were added to reaction wells, diluted 1 to 10 in reaction media, affording the desired 125 μM concentration of inhibitor. The final percentage of DMSO per well was 0.25% (v/v), except for compound **17b** and the reference molecule, NHI-2, which were evaluated at 2% of DMSO, due to their low solubility at lower DMSO percentages.

Enzyme inhibition (%) was determined by measuring the reduction of NADH absorbance at 340 nm (Equation 2.37):

$$\% \text{ enzyme inhibition} = 100\% - \left[\frac{V(\mu\text{M}/\text{min})_{\text{inhibitor}}}{V(\mu\text{M}/\text{min})_{\text{control}}} \times 100 \right]$$

Equation 2.37

The percentage of enzyme inhibition was calculated by subtracting the remaining enzyme activity (enzyme activity in the presence of inhibitor) to the maximum enzyme activity (100%). The remaining enzyme activity was determined by dividing the enzyme reaction rate in the presence of the inhibitor by the reaction rate of control and multiplying it by 100.

Determination of the half maximal inhibitory concentration (IC₅₀)

These assays were conducted in 96-well plates containing a solution with reagents dissolved in 0.1 M sodium phosphate buffer at pH 7.4. Six different concentrations (in triplicate for each concentration) of compound were used to generate a concentration – response curve. As previously done in the screening test, DMSO stock of compounds were used for preparing stock dilutions. The final percentage of DMSO on each test did not exceeded 2%.

In the NADH competition assay, compounds were tested in the presence of 90 μ M of NADH and 2 mM of pyruvate (saturating conditions). NADH and pyruvate stock solutions were freshly prepared 10-fold more concentrated (i.e. 900 μ M and 20 mM, respectively). Both NADH and pyruvate were weighted separately and diluted with sodium phosphate buffer 0.1 M, pH 7.4.

In the pyruvate competition assay, compounds were tested in the presence of 200 μ M of pyruvate and 300 μ M of NADH (saturating conditions). Similarly, NADH and pyruvate stock solution were 10-fold more concentrated. Both were weighted separately and diluted with sodium phosphate buffer 0.1 M, pH 7.4.

Pyruvate and NADH stock solutions (20 μ L) were dispensed dissolved in the buffer. Compound solution (20 μ L) was mixed with enzyme (20 μ L from stock 0.6 U/mL) and incubated for 1 minute separately from substrate and cofactor. Once the incubation period finished, the mixture containing both inhibitor and enzyme (*Mix I+E*) was added to the corresponding well (total volume = 200 μ L). The amount of consumed NADH was monitored every 15 seconds for 3 minutes. Volumes used in the assay are collected in Table 16:

Table 17 - Volumes used in IC₅₀ and Ki calculations.

Reagent		Volume /well (μ L)
NADH		20
Pyruvate		20
Mix <i>I+E</i>	LHD	20
	Inhibitor	20
Buffer		120
<i>Total volume</i>		200

The order of addition of reagents was kept constant:

Pyruvate – NADH – buffer – mix *E+I* – Start

IC₅₀ values were generated using the curve-fitting tool for nonlinear regression, modality [*Inhibitor*] vs. *normalized response -- Variable slope*, with GraphPad Prism 8.4 software.

Determination of the inhibition constant (K_i)

In the kinetics experiments, the progression of the reaction in the presence of the inhibitor was evaluated. Two values of K_i for LDH-A were calculated, one under pyruvate-saturating concentrations and another under NADH saturating-concentrations.

Compound stocks were prepared at 50 mM in pure DMSO. Diluted stock solutions were prepared by diluting with buffer sodium phosphate 0.1 M pH 7.4. The final percentage of DMSO on each test only exceeded the 0.5% in the case of compounds **17b** and NHI-2 (which was 2%). Negative controls were prepared with the same percentage of DMSO as sample tests.

Calculation of K_i – Pyruvate-saturating concentrations

0.06 units of enzyme were mixed externally with the compound at concentrations ranging from 0.2 μM to 80 μM and incubated for 1 min (concentrations were selected depending on the potency of the inhibitor shown in the previous screening assay). After the incubation period was finished, the mix was added to a reaction mixture containing scalar concentrations of NADH (30-900 μM) and 2 mM of pyruvate (saturating concentration). All tests were performed in triplicates.

Calculation of K_i – NADH-saturating concentrations

0.06 units of enzyme were mixed externally with the compound (0.2-80 μM) and incubated for 1 min. Then, the mix was added to a reaction mixture containing scalar concentrations of pyruvate (25-2025 μM) and 300 μM of NADH (saturating concentration). All tests were performed in triplicates.

Volumes of reagents for both assays are indicated in Table 16. Experimental data was analysed with GraphPad Prism software by non-linear regression using the mixed-model inhibition fit.²⁴⁸

2.5.4 Surface Plasmon Resonance (SPR) Studies

Surface plasmon resonance experiments were carried out on a Biacore X100 instrument at 25 °C. Human LDH-A was immobilised on a CM5 sensor chip by standard amine coupling reactions. Buffer acetate (pH 5.0) was used to immobilize 9700 resonant units (RUs) of enzyme in the second channel, whereas channel 1 was block as reference. One RU represents approximately 1pg protein/mm³ of chip surface.^{180,181} Chip was inactivated by a consecutive injection of ethanolamine 1.0 M pH 8.5.

HBS-P buffer (HEPES 10mM, NaCl 150 mM, EDTA 3mM, 0.05% tween) was used as running buffer. Stock solutions of compounds at 10 mM in pure DMSO were used to prepare a 200 μM diluted stock solution. 4 μL of 10 mM stock solution were dissolved in 196 μL of running buffer, to obtain the desired 200 μM diluted stock solution at 2% DMSO (v/v). This stock solution was used to prepare serial dilutions (2-100 μM) diluting with running buffer enriched with 2% (v/v) DMSO.

The flow rate was 30 μL /min. As contact and dissociation times, 50 sec were selected, total time of experiments 300 sec. After each injection, an extra wash with 50% DMSO (v/v) in water was completed. No chip regeneration was needed.

2.5.5 Cell Culture and Cytotoxicity Tests

Human glioblastoma cell line, U87 MG, was obtained from the European Collection of Cell culture (ECACC, No. 89081402, Lot 11K009, P21 to P28) and cultured in growth medium DMEM supplemented with 10% (v/v) of heat-inactivated FBS, 1% (v/v) of non-essential amino acids and 1% Penicillin-Streptomycin.

For all assays, U87 MG cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C, and the medium refreshed every 2-3 days. When cells reached ~80% of confluency, the cells were washed with PBS (~5 mL) and incubated with 2-3 mL of TrypLE Enzyme 0.25% at 37°C for 3 minutes. Then, growth medium (3 mL) was added and cells suspension was centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded, and the pellet re-suspended in 1 mL of complete medium. Cell number was obtained using the Trypan Blue Exclusion Assay²⁶² and the haematocytometer.

The Sulforhodamine B assay

The Sulforhodamine B (SRB) assay measures cell density by determining the cellular protein content. This assay was chosen for the evaluation of the antiproliferative properties of LDH inhibitors in tumoral cells. The SRB assay was performed as describe previously in the literature ^{258,263} with some modifications:

1. Cell preparation:

U87MG cell line was seeded in a sterile 96-well plate at cell density 12,500 cells/well. Medium: DMEN (Dulbecco's Modified Eagle Medium) enriched with 10% fetal bovine serum (FBS), 1% NEAA and 1% Penicillin-Streptomycin.

2. Treatment solution preparation:

Compound of choice was dissolved in 100% (v/v) DMSO. Serial dilutions were prepared from stock using 5% (v/v) DMSO in sterile buffer phosphate (PBS). Final percentage per well of DMSO was 0.5%.

Vehicle-control dilution is also prepared (10 μL of 5%DMSO/PBS + 190 μL fresh medium).

Procedure:

Day -1: Cell seeding at 1.25×10^4 cell per well and allow attachment overnight.

Day 0: Media was removed and replaced by 200 μL /well of freshly prepared treatment solutions or vehicle control dilution. Plates were incubated at 37°C, 5% CO_2 for 48 h.

Day 2: Cell fixation with trichloroacetic acid (TCA). Without removing the supernatant, 100 µL of cold TCA 10% (wt/v) in H₂O were added to each well. Plates were incubated at 4 °C for 1h.

Next, plates were washed four times with slow-running tap water via plastic tubing connected directly to a faucet, and the excess of water was removed using paper towels. Plates were allowed to air-dry at room temperature (20–25 °C).

Day 3: 100 µL of 0.057% (wt/vol) Sulforhodamine B* solution were added to each well and left at room temperature for 1h, after which plates were quickly rinsed four times with 1% (vol/vol) acetic acid to remove unbound dye. Plates were allowed to dry at room temperature. *SBR solution is prepared in 1% v/v AcOH in deionized water.

Day 4: Optical density (OD) measurement and analysis of results. 200 µL of 10 mM Tris base solution (pH 10.5) were added to each well and plates were placed on a gyratory shaker for 5 minutes to solubilize the protein-bound dye.

UV absorbance was measured at 570 nm and 690 nm (the latter for background subtraction). *Cell growth (%)* and *cell growth inhibition (%)* were obtained by applying equations 2.38 and 2.39:

$$\% \text{ cell growth} = \frac{\text{Mean OD sample}_{510 \text{ nm}} - \text{Mean OD sample}_{690 \text{ nm}}}{\text{Mean OD control}_{510 \text{ nm}} - \text{Mean OD control}_{690 \text{ nm}}} \times 100$$

Equation 2.38

$$\% \text{ cell growth inhibition} = 100 - \% \text{ cell growth}$$

Equation 2.39

CHAPTER 3

Preparation and evaluation of IIPS04- loaded TPGnRH nanofibers

3.1 INTRODUCTION

3.1.1 Gliomas and their classification

Gliomas are the most frequent primary tumours in the central nervous system accounting for 27% of all brain tumours.²⁶⁴ Gliomas have been divided in different subtype of tumours according to the WHO classification of 2007 based on the origin of the cell type. Hence, there are four main categories: diffuse astrocytoma (with glioblastoma as the most common and aggressive representative), oligodendroglial phenotype (oligoastrocytomas), oligoastrocytic and ependymal tumours. A fifth group, named mixed neuronal-glial tumours, encompasses gliomas with mixed characteristics of different subtypes of glial tumours. From a histologically point of view, gliomas are very similar to normal glial cell (oligodendrocytes or astrocytes), however the origin of these cancerous cells remains unclear.²⁶⁵ Until 2016, the World Health Organization (WHO) classified tumours of the central nervous system based mainly on the histology of the tumour.²⁶⁶ Then, a malignancy grade (WHO grade I to IV) was assigned, depending on the presence/absence of marked mitotic activity, necrosis and microvascular proliferation.^{266,267}

Important changes in the criteria for classifying a tumour have been introduced, giving more relevance to molecular types of glioma. The new classification incorporates diagnostic and prognostic markers such as mutation in the isocitrate dehydrogenase (IDH) enzyme, chromosome 1p/19q deletion, and histone mutations. This way, there are 2 main molecular categories: IDH wildtype and IDH-mutant and few subcategories (chromosome deletion and others).

With the new 2016 WHO Classification, astrocytoma and glioblastoma are classified into IDH mutant and IDH wild types. Oligodendroma belong to the IDH mutant category, and are 1p/19q co-deleted. Diffuse gliomas are classified by the histone H3 K27M mutations. A “not otherwise specified” (NOS) category was introduced for cases where molecular testing could not be performed, or the results were not conclusive.²⁶⁸

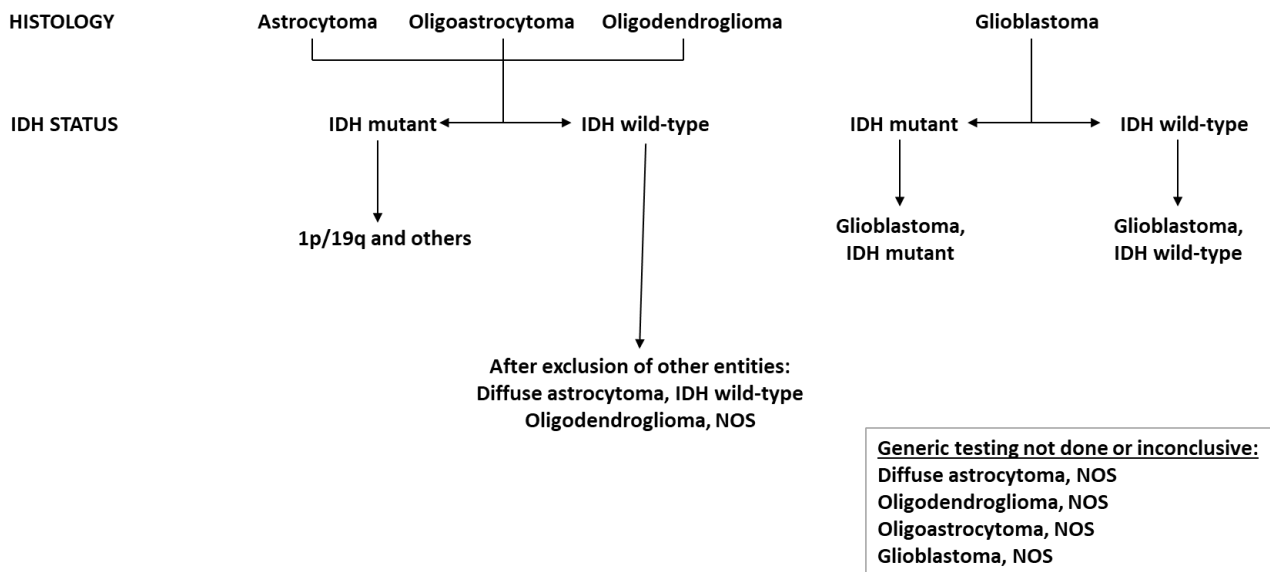


Figure 79 - Schematic algorithm for classification of gliomas. Not only the histology but also molecular genetic features are nowadays considered for classifying gliomas. IDH: isocitrate dehydrogenase; NOS: not otherwise specified.

3.1.2 Glioblastoma Multiforme

Glioblastoma multiforme (GBM) or simply glioblastoma, is the most prominent (with an incidence of 4.13/100.000 individuals per year) and malignant tumour astrocytoma subtype in adults.^{264,269} The incidence of GBM is higher in individuals above age 65, and more predominant in male over female.

The median overall survival of patients with primary glioblastoma is 4.7 months.²⁷⁰ Treatment with surgical resection, radiation therapy and TMZ (the STUPP protocol²⁷¹) increases the survival of patients with an assigned WHO grade IV to 12-14 months.²⁷²

GBMs are divided into GBM *IDH wild-type* (IDH^{wt}, 90% of the cases) which closely corresponds with the clinically defined as primary (*de novo*) tumour, and *IDH mutant* 1 and 2 (10% of the cases) which presents the characteristics of secondary GBM.²⁷³ If IDH status is unavailable, the diagnosis of GBM is NOS. In general, both GBM has poorly differentiated neoplastic astrocytes, with mitotic activity, microvascular proliferation, nuclear atypia and necrotic tissue. IDH1 and IDH2 mutations correlates with better outcome and increased overall survival (31 months),²⁷⁴ which is notably longer than the survival 14-month survival in patients with IDH^{wt} glioblastoma.²⁷⁵ Furthermore, patients with anaplastic astrocytoma carrying IDH mutations showed improved outcome compared to the wild type: the average survival was 65 months for patients with mutations and 20 months for those without them.²⁷⁴

Treatment of GBM

The Stupp protocol is the standard treatment of glioblastoma since its publication in 2005. It comprises radiotherapy and chemotherapy with Temozolomide after surgery.²⁷¹

Surgery

Although almost all GBM tumours recur after surgery, resection of the tumour is a key factor for increased survival.²⁷⁶ The aim of the surgery is to obtain diagnosis and relieve clinical symptoms by removal of the tumour.

Finding a balance between aggressive removal of malignant cells and minimizing the risk of inducing neurological damage is critical in order to determine the extent of resection (EOR) that provides maximal survival and functional guarantees. If the lesion is located near or involving critical areas, this is, those that if removed will generate linguistic or sensorial deficits, a subtotal resection or tissue biopsy will be performed.²⁷⁷ This situation invariably might leave residual disease (even with microscopic size) that can favour the reactivation of the tumour and further progression. For this reason, surgery is always supported by co-adjuvant therapies for tackling GBM.

Radiotherapy

Radiation therapy is the application of high energy X-rays to reduce a tumour. Post-operative radiation has been in use since the early 70's. The standard dose is 60 gray (Gy) in 1.8 to 2.0 Gy fractions. Surgery combined with these fraction sizes of radiotherapy have proven to be effective and increase survival of patients in clinical trials (14 weeks survival increment among patients with only resection surgery compared to 29.5 weeks of those that also received dosed radiotherapy).²⁷⁸

There are some cases in which re-irradiation is applied for recurrent gliomas, however, there is not enough evidence to support it, and is used mostly as palliative therapy.²⁷⁶

Chemotherapy

Temozolomide (TMZ) is currently the first-line treatment of primary GBM. It is one compound of a series of imidazotetrazinones²⁷⁹ that acts as an alkylating agent.

The molecular structure of TMZ is advantageous because of the presence of three nitrogen atoms that confer unique physicochemical properties, with similar antitumor activity and less toxic than its parent compound, mitozolomide²⁸⁰ (Figure 80).

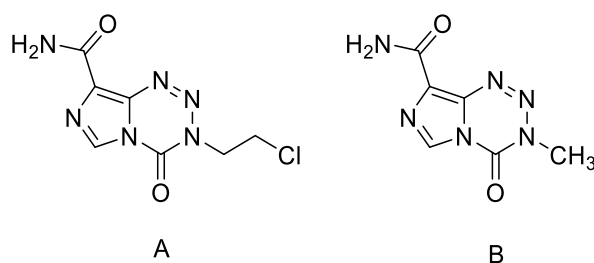


Figure 80 - Chemical structures of Mitozolomide (A) and Temozolomide (B).

TMZ is an orally bioavailable (96-100%) prodrug,²⁸¹ which doesn't need hepatic activation to convert into its active metabolite. It is readily absorbed in the intestine and widely distributed in tissues, among them the brain (brain/plasma area under the curve ratio of 17.8% and peak concentration of $0.6 \pm 0.3 \mu\text{g mL}^{-1}$ after 2.0 ± 0.8 hours of a single dose of 150 mg m^{-2}).²⁸² TMZ possesses an imidazotetrazinone core with a carboxamide moiety (Figure 80B) and due to its small size (194 Da) and lipophilicity, it can penetrate the blood-brain barrier.²⁸³ Temozolomide is stable at acidic pH, whereas at physiologic pH ($\text{pH} > 7$) the tetrazinone ring opens by the nucleophilic attack of H_2O on the highly electropositive carbon C^4 , yielding CO_2 and the active antitumor metabolite: the monomethyl triazene 5-(3-methyltriazene-1-yl)-imidazole-4-carboxamide, MTIC. MTIC breaks down further, when the secondary amine receives a proton, releasing the methyldiazonium ion, $^+\text{N}_2\text{CH}_3$, which easily transfers the methyl group to nucleophiles (NuH) such as the DNA bases²⁸⁴ (Figure 81).

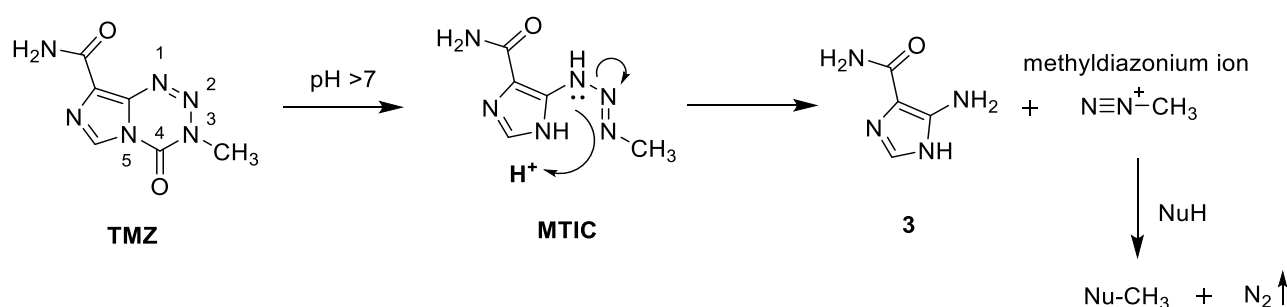


Figure 81- Scheme of TMZ pH-dependent conversion into its active form. Adapted reference²⁸⁵.

Among the lesions produced by TMZ on DNA, the most common are N-methylation on the N^7 position of guanine and O-methylation on the O^3 position of adenine bases.^{283,284,286} The cytotoxic mechanism of TMZ is related to the inability of DNA mismatch repair (DNA MMR) system to find a complementary base for the methylated guanine. Methylation of DNA results in disruption of the DNA replication and accumulation of long-lived nicks in the DNA, which persist into the next cell cycle, where they ultimately obstruct replication in daughter cells, stopping the cell cycle at the G_2/M phase, followed by apoptosis of the cell due to detention of growth process.

TMZ showed antitumoral activity in high-grade glioma^{287,288} and increased considerably the effectiveness of radiotherapy when combined with TMZ, as well as a better survival rate compared to radiotherapy alone.²⁷¹ However, GBM cells can become resistant to TMZ due to the action of O^6 -alkylguanine alkyltransferase (AGT, also known as MGMT) enzyme, which specifically removes methyl groups at the O^6 position of guanine. The high expression of AGT in GBM (~40 % of all GBM) clearly limits the efficacy of alkylating agents such as TMZ.

It has been observed that patients bearing the IDH-mutations respond better to Temozolomide and have a better prognosis than patients carrying the histone H3-mutation (a very common mutation among patients diagnosed with diffuse midline gliomas, DMGs).²⁸⁹ IDH mutations are known to be gain-of-function mutations and are associated with a high diffuse DNA hypermethylation and MGMT promoter methylated.^{290,291} As a result, the MGMT transcription is stopped and glioma cells are more sensitive to Temozolomide. By contrast, histone H3-mutations is associated with the opposite effect,

this is, a hypomethylation of DNA and therefore, lower levels of MGMT promoter methylation.^{292,293} The higher expression of the MGMT enzyme make of H3-mutation positive- patients unreactive to TMZ.

If GBM becomes recurrent, the treatment regime that would be effective is not so well demonstrated. It usually includes the combination of TMZ with other alkylating agents such as procarbazine, lomustine or carboplatin, or alkaloid agents (irinotecan or vinblastine).²⁹⁴ Newer approaches/therapies are Gliadel® implants, containing carmustine (3.85% w/w), a mustard gas-related drug. The implant contains the co-polymer of poly[bis(p-carboxyphenoxymethyl)propane to sebacic acid in 20:80 molar ratio.²⁹⁵ When hydrated, the polymer releases carmustine in the tumour. Carmustine can partially cross the BBB, however it is limited by its short half-life (13-20 min)²⁹⁶ and significant immunosuppression. Although clinical trials have shown an increase in the survival of patients treated with this implant of carmustine, the important side-effects (brain oedema and intracranial infections among others) limits the translation of Gliadel® as a first-line treatment of GMB.^{295,297,298}

A recombinant humanised monoclonal immunoglobulin G1 antibody, bevacizumab (Avastin®, Hoffman-La Roche Ltd.) is currently in clinical trials for its use in primary tumours.²⁹⁹⁻³⁰¹ Bevacizumab selectively binds endothelial growth factor (VEGF), which plays a key role in angiogenesis and regulation of the permeability of the BBB. Due to Bevacizumab VEGF pathway is blocked, and it cannot bind to its receptors (VEGFR-1 and VEGFR-2). This situation restores the abnormal tumour vasculature, reducing the vascular permeability and blood supply around the malignant tissue.³⁰² Up to date, the European Medicines Agency have not yet approved the use of Bevacizumab as co-adjuvant agent for the treatment of recurrent glioblastoma due to the scarcity of robust trial data demonstrating benefit. However, some European countries do administrate it in patients with recurrent glioblastoma, due to the lack of definitive treatments.

Similarly, this antibody has been evaluated for its use in newly diagnosed glioblastomas, although it did not significantly improve the patient's survival either.³⁰³ The most widely applied regimen for bevacizumab monotherapy is 10 mg/kg intravenously every 2 weeks until disease progression or inadmissible toxicity.³⁰⁴ In 2015, the cost of bevacizumab was \$66.6 per 10 mg,³⁰⁵ which means that an average patient of 70 kg would cost to the administration ~ \$4,660 every fifteen days.

3.1.3 New approaches for the treatment of glioblastoma: Endocrine targeted therapy

Gonadotropin-releasing hormone (GnRH) is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) firstly described in 1971. GnRH is synthesised in the septal-preoptic-hypothalamic region of the brain and released in a pulsatile way by neurons.³⁰⁶ GnRH plays a crucial role in the control of reproductive functions by stimulating the synthesis/release of luteinizing hormone (LH) and the follicle-stimulating hormone (FSH).³⁰⁷

Once secreted by the hypothalamic neurons into the portal system, GnRH reaches the anterior pituitary where it interacts with the Gonadotropin-releasing hormone receptor (GnRH-R), to stimulate gonadotropin synthesis and release. GnRH-R is a member of the G protein-coupled receptor (GPCR) family, which contains seven transmembrane domains. It also possess a short carboxyl-terminal cytoplasmic tail, a region implicated in the coupling to G proteins and believed to be important for receptor internalization³⁰⁶ GnRH-R is coupled to a G_{αq/11} protein to activate phospholipase C, which transmits downstream signalling to diacylglycerol and inositol 1,4,5-triphosphate (IP3). Next, intracellular Ca²⁺ is released by IP3 and DAG activates the intracellular protein kinase C (PKC) pathway.³⁰⁶ Phospholipase A₂ and phospholipase D are also activated by GnRH and both activate PKC. Phospholipase A₂ yields long-chain unsaturated fatty acids such as arachidonic acids, whereas phospholipase D acts on the membrane phospholipid phosphatidylcholine, converting it into phosphatidic acid. This fatty acid can be further metabolized into DAG, thus prolonging the PKC activation.³⁰⁸ PKC is a major mediator of the activation of the MAPK cascades. GnRH-R intracellular signalling has been found to activate all four MAPK cascades: ERK1/2, c-Jun N-terminal kinase (JNK), p38MAPK, and big MAPK1 (also known as ERK5). MAPK cascade is the link for the transmission of signals from the cell surface to the nucleus, leading to transcription factor (e.g. Elk1 and c-Jun), phosphorylation and ultimately triggering gonadotropin synthesis and release. Both GnRH agonist and antagonist, the former if a sustained dose is present, can cause desensitization of the GnRH-R, which translates into antiproliferative effect.

Recent research has indicated that GnRH-R are overexpressed in cancer tissues, either related (i.e. breast, ovarian, prostate and endometrial cancers) or unrelated (i.e. glioblastoma, lung or melanoma).^{309,310} Western blot and mRNA protein expression have confirmed the overexpression of GnRH in U87MG glioblastoma cells that if treated with micromolar concentration of GnRH agonists can result in a concentration dependent antiproliferative effect.³⁰⁹ This could be mainly explained by three mechanisms: crosstalk with growth factor receptor, upregulate JNK inducing apoptosis, and downregulate cAMP reducing cell proliferation (as shown in figure 82) and the latter has been demonstrated by GnRH agonists in U87MG cells.³⁰⁹ However, GnRH peptides have not been translated into treatments for GBM due to their short half-life or their inability to across the BBB.³¹¹

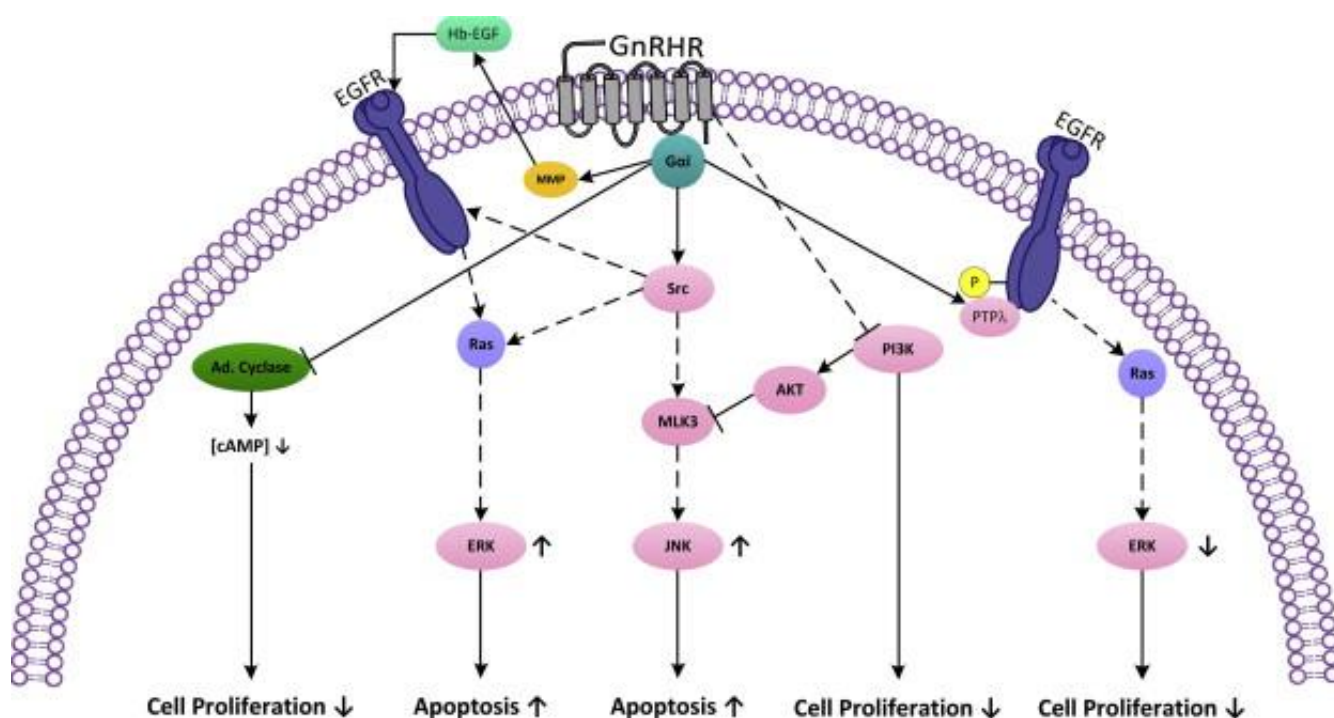


Figure 82 - Signalling pathways involved after the activation of the GnRH receptor and their effects on glioblastoma cells. Key: dashed arrows: indirect activation. Solid arrows: direct activation. Solid line with the transversal line: direct blocking.

Tyrosyl⁵ palmitoyl GnRH (TPGnRH) nanofibers as a targeted endocrine GBM therapy

Chemically-modified neuropeptides by lipidation has proven to enhance their amphiphilicity and, in some cases, allow their self-assembly into supramolecular structures (micelles, vesicle, nanotubes, and nanofibers).^{309,312} Peptide nanofibers have been shown to be able to enhance permeability across the BBB and result in therapeutic levels of the peptide *in vivo*.^{312,313}

Dr Lalatsa's group has developed a lipidized analogue of GnRH by grafting a palmitic tail via an ester bond at position Tyr⁵ (Figure 83). TPGnRH has been shown to be able to bind to GnRH-R and maintain agonistic activity and to have a strong dose-dependent antiproliferative effect in GBM U87MG cells (IC₅₀ 10.31 μM), while being able to elicit a G₂/M phase arrest and trigger apoptosis.³¹⁴ TPGnRH self-assembles into long axial nanofibers that are able to be loaded with high concentrations of cytotoxic drugs (namely paclitaxel (~2mg/mL), lomustine, doxorubicin) and target them in GBM cells. TPGnRH nanofibers and paclitaxel loaded nanofibers are able to cross across an *in vitro* 2D human BBB model prepared by human brain endothelial cells (hCMEC) and human astrocytes (SC-1800), while being able to increase paclitaxel's permeability by 30-fold. Peptide nanofibers are stable upon dilution and possess an excellent biological half-life (Table 17).

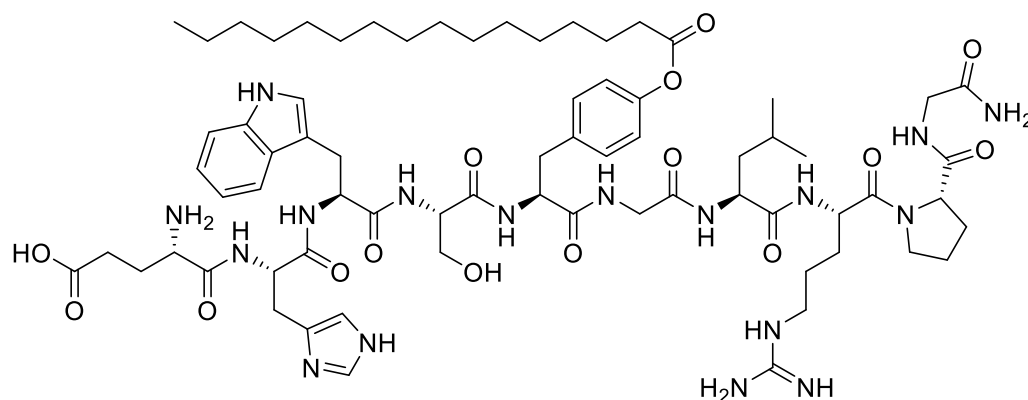


Figure 83 - Molecular structure of Tyrosyl⁵ palmitoyl GnRH (TPGnRH) peptide.

Table 18 - The half-life (minutes) of Glu-GnRH and TPGnRH in 50% plasma or brain or liver homogenate.

Homogenate	Glu-GnRH (minutes)	TPGnRH (minutes)
Plasma 50%	17.66 ± 4.22	445.14 ± 4.95
Brain 50%	3.59 ± 0.08	1951.40 ± 8.01
Liver 50%	0.67 ± 0.03	4.94 ± 2.61*
U87MG	82.46 ± 20.05	196.47 ± 84.90**
* half-life alpha phase ~60%		
** half-life beta phase		

TPGnRH nanofibers counteracted FSK-induced cAMP intracellular accumulation, implying that GnRH-R was coupled to a $G_{\alpha i}$ protein as previously described for other GnRH D-analogues in GBM cells.³⁰⁹ TPGnRH at 35 μ M neutralizes cAMP accumulation in a concentration – dependent manner, affording 13-fold decrease in the cAMP levels.³¹⁴ It is thought that $G_{\alpha i}$ protein could be the major mediator of alteration of proliferation or apoptosis signals.³¹⁰ TPGnRH nanofibers have demonstrated its ability to elicit an antiproliferative effect by activating the GnRH-Rs coupled to the $G_{\alpha i}$ protein and the cAMP -dependent signalling cascade in the GBM cells. Additionally, TPGnRH nanofibers were able to generate G_2/M phase arrest followed by apoptosis on U87MG cells. This is similar to other studies of stable GnRH analogues such as triptorelin (a D-analogue of GnRH) causes antiproliferation on HEK293 cells transfected with functional GnRH receptor by altering the MAPK pathway, causing G_2/M phase arrest and apoptosis.³¹⁵ GnRH activation in the U87-MG cells might be also associated with similar alterations, thus, MAPK pathway alterations could be another mechanism for TPGnRH to produce antiproliferative effects.

At low concentrations of TPGnRH (up to 7 μ M), apoptotic effects may be also involved, as there is no alteration in the cell cycle³¹⁶ but there is an increase of apoptotic cells. In the study with the GnRH

analogue Triptorelin, alterations in some genes involved in apoptosis (caspase-8, cFLAR, Fas-associated protein) were observed after 72 to 96 hours of treatment.³¹⁵ In GBM cells, TPGnRH might act similarly, and trigger apoptosis either by a direct apoptotic pathway or by an alteration of cells cycle regulators. More studies are needed in this behalf to fully elucidate the mechanism of action of TPGnRH.

3.1.4 microRNA as treatment of cancers

Protein synthesis is a highly controlled and conserved process on cells. The information that provides the instructions for the synthesis of any protein in our body is contained in the DNA. When a specific protein should be synthesised, a copy of its DNA-sequence is copied as messenger RNA (mRNA). This mRNA travels out of the nucleus to the cytoplasm and reaches the ribosome. Inside the ribosome, information encoded in the sequence of bases in the mRNA is translated by transfer RNA (tRNA) molecules, that carry specific amino acids at one end and bind to the mRNA by the other end.³¹⁷ The ribosome links every new amino acid to the growing peptide chain (Figure 84).

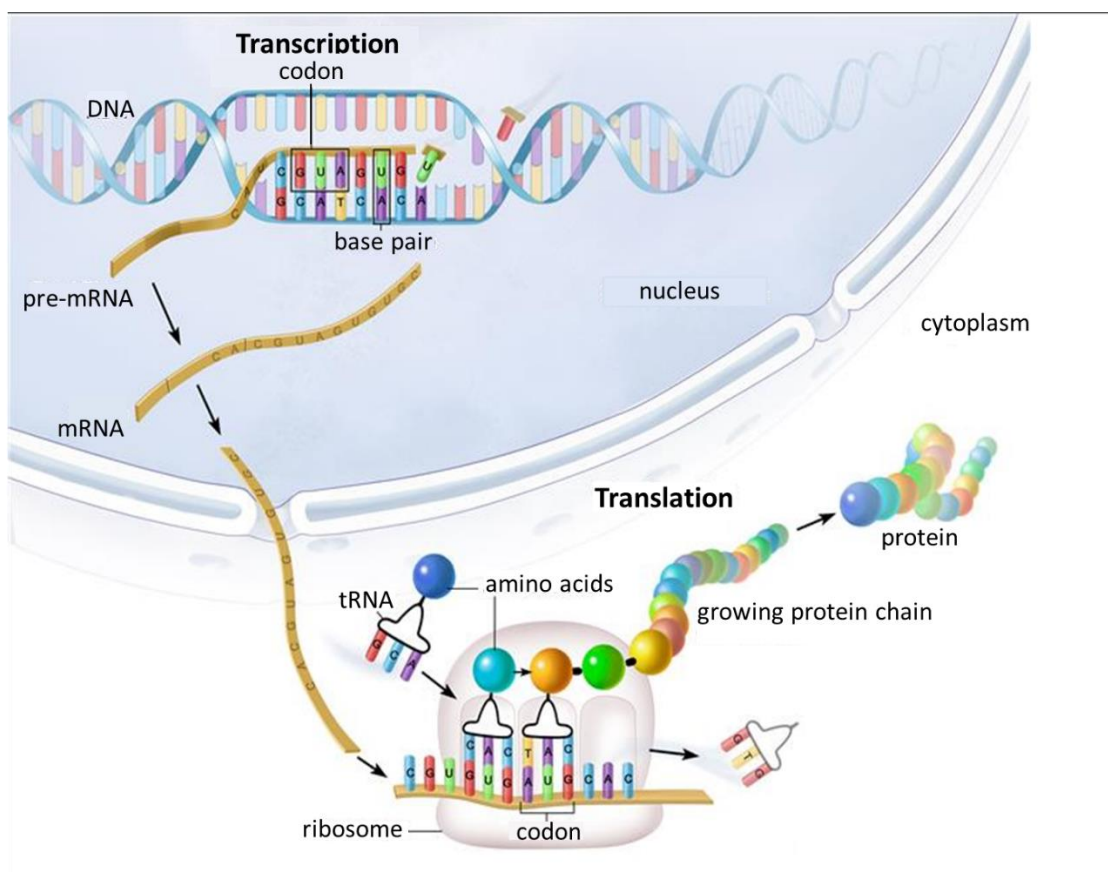


Figure 84 - Transcription and translation processes for peptide biosynthesis on cells. Adapted from <https://www.cancer.gov/>.

At the end of the last century, Fire and co-workers discovered the presence of double-stranded RNA in *Caenorhabditis elegans* that induced potent and specific gene silencing, a phenomenon termed RNA interference (RNAi).³¹⁸ Gene silencing is a well-conserved process that functions in several species, including mammals. The inhibitory capability of RNA in mammalian cells is carried out by single-stranded RNAs or microRNA (miRNA).

MicroRNA (miRNA) are small non-coding transcripts of 19 to 25 nucleotides in length without protein-coding ability, but able to regulate protein expression at the post-transcriptional level. miRNA negatively regulate synthesis of proteins by base-pairing to complementary or partially complementary sites on target mRNAs.³¹⁹ Up to 60% of the human transcriptome is under the negative regulation of miRNAs activity. Thus, miRNAs constitute one of the most important and abundant classes of gene-regulatory systems in animals.³²⁰

There are ~ 2,000 known human miRNAs but the knowledge about their specific target mRNA is limited. However, a clear function of this extensive regulatory network is the control over processes that regulate normal cell proliferation, differentiation and apoptosis, as well as determination of the final phenotype of other human diseases, such as cancer, thus defining carcinogenesis and metastatic potential.³²¹

Despite their global dysregulation, most miRNAs (oncomiRNAs) are repressed in cancer tissue relative to normal tissue, indicative of a loss of differentiation of tumour cells.³²⁰ However, several miRNAs are upregulated, some of them playing key oncogenic roles.

3.1.5 microRNA-21

We will focus our attention into microRNA-21 (or miR-21). miR-21 is abundantly expressed in mammalian cells, whose upregulation is associated with numerous types of cancer.^{322,323}

Mature miR-21 is a 22-nucleotide long molecule whose sequence is 5'-UAGCUUAUCAGACUGAUGUUGA. In humans, miR-21 gene is located on chromosome 17q23.2. For most miRNAs, among them miR-21, the transcription process starts on the nucleus by RNA polymerase II, which transcribes the miR-21 genes and generates long primary transcripts (pri-miR-21). Subsequently, RNase III enzyme (Drosha) trims pri-miR-21 to release pre-miR-21 hairpin, which is transferred to the cytoplasm by exportin 5.^{324,325} In the cytoplasm, the pre-miR-21 is further processed by a protein complex containing dicer protein (Dicer) and the transactivation response RNA binding protein (TRBP) that further cut pre-miR21 to form double-stranded miRNA fragment, which finally unwinds into mature single-stranded miRNA (Figure 85).

Like other mature miRNAs, mature miR-21 incorporates into the RNA-induced silencing complex (RISC). RISC tethers, by means of the miRNA fragment, to total or partially complementary motifs in target mRNAs. This will provoke post-transcriptional gene silencing of the target mRNA and (or) promotion of its degradation.³²⁶

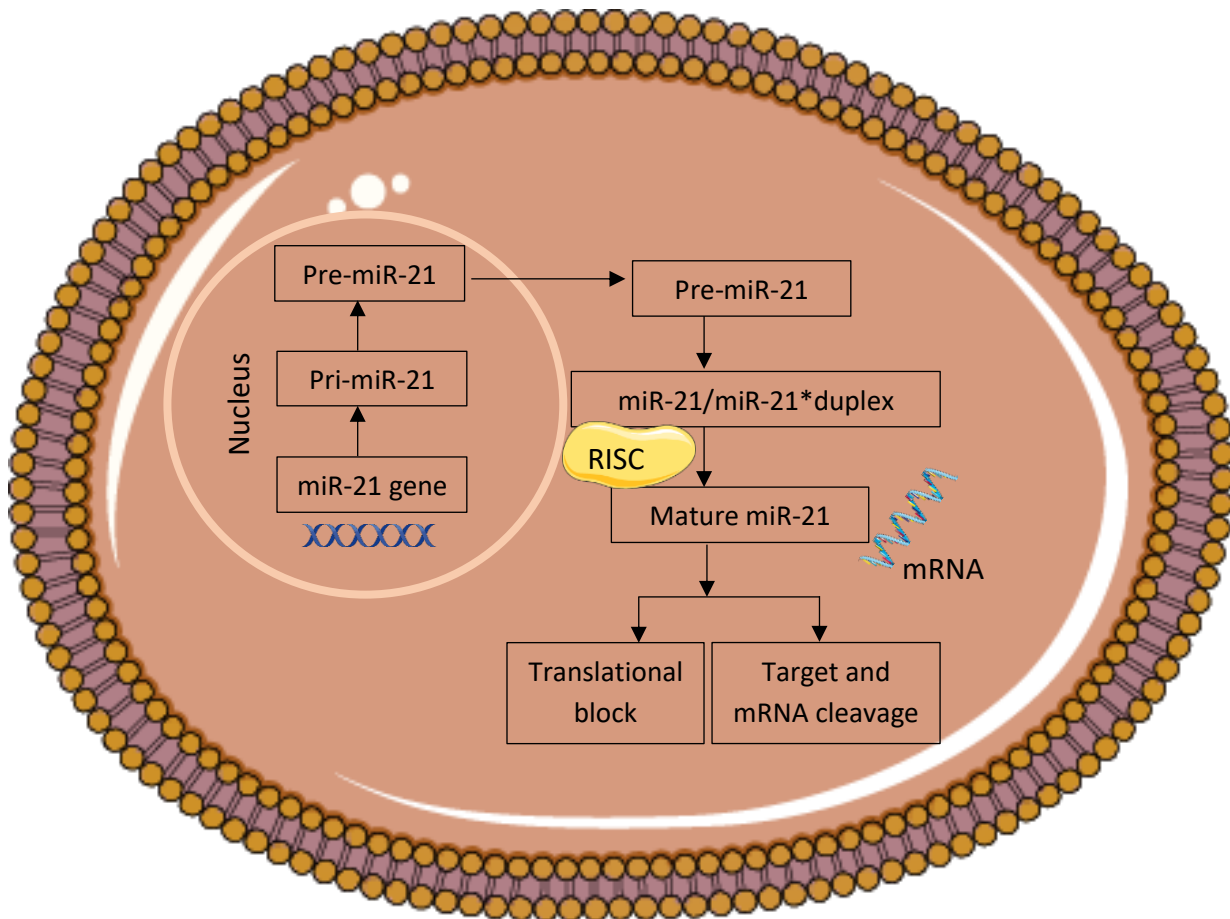


Figure 85 - Biosynthesis of miR-21. The course followed by miR-21 transcript is indicated with arrows. Mature miR-21, coupled with RNA-induced silencing complex (RISC), binds target mRNA in the cytoplasm and provokes translational block and (or) mRNA cleavage. Picture adapted from 326 and prepared with Servier Medical Art (<https://smart.servier.com/>).

Role of miRNA-21 in GBM

It has been demonstrated that miR-21 overexpression functions as an oncogene, this is, it can cause the growth of cancer cells.³²⁷ In a study that profiled 540 clinical samples of cancer patients, miR-21 was found to be the only one that consistently was upregulated.³²⁸ miR-21 is also expressed in hematopoietic cells, including B/T cells, monocytes, dendritic cells and macrophages. For these reasons, miR-21 is considered a biomarker and target for cancer treatments.³²⁹

Chan and colleagues confirmed the overexpression miR-21 in gliomas by Northern blot in different mammalian tissues and cells.³¹⁹ Its expression was increased 5- to 100-fold in human GBM tissue compared to non-neoplastic brain, and strongly elevated in all high-graded glioma samples tested.³³⁰ Similarly, miR-21 expression was remarkable elevated in six model cell lines, commonly used as models for human glioblastoma (LM299, U373, A172, U87, LN308 and LN428). Interestingly, miR-21 expression was present in other primary brain tumours such as oligoastrocytoma, oligodendroglioma and anaplastic astrocytoma, but such high levels of miR-21 were only found in glioblastoma.

The phenotypic effect of miR-21 inhibition has been also studied. To investigate the effect of miR-21 inhibition, glioblastoma cells (U87, A172, LN229 and LN208 cell lines) were transfected in parallel with 2'-O-methyl-oligonucleotide or with antisense oligonucleotides containing LNA (locked nucleic acid) complementary to miR-21. They both are molecules that can be engineered with a very high affinity for miRNA *in vitro* and *in vivo*, and when bound to a miRNA, prevent miRNA interaction with its mRNA target.³¹⁹ Both techniques, 2'-O-methyl and LNA-oligonucleotides, showed similar effectiveness and specificity, with a significant drop in cell number, whereas unrelated 2'-O-methyl- or LNA/DNA-oligonucleotides (non-targeting miR21) did not affect cells. The metabolic activity was clearly reduced after the suppression of miR-21, but not with the control or with the suppression of two other miRNAs (miR-124a and miR125b) which are abundant in normal and tumour brain.

The decrease in cell number was related with a marked enhance in cell apoptosis. Enzymatic activities of caspases-3 and caspase-7 (key executioners of apoptosis) increased 3-fold by 48 hours post-transfection with the antisense-modified oligonucleotides, 2'-O-methyl-miR-21 or LNA/DNA-miR-21. This means that the aberrant overexpression of miR-21 in glioblastoma cells down-regulate the translation of mRNAs coding for apoptosis-related genes, and therefore, miR-21 acts as an antiapoptotic factor in GMB.

Targeted molecular pathways by miR-21 in GBM

miR-21 targets multiple genes including associated with glioma cell apoptosis, migration and invasiveness. Figure 86 gather key downstream pathways that are altered by miR-21 upregulation in multiple cancers.

Cells possess survival signalling pathways whose expression is upregulated by the effect of miR-21. An example of these survival pathways is the PI3K/AKT. PI3K catalyses phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3), which recruits AKT and phosphoinositide dependent kinase (PDK) to allow PDK to phosphate and activate AKT. Activated AKT increases cell proliferation and decreases cell apoptosis. Phosphatase and tensin homolog (PTEN) is a tumour suppressor mRNA and protein that negative regulate of PI3K/ AKT pathway, by turning PIP3 into PIP2. miR-21 directly target mRNA of PTEN, with subsequence increase of AKT activation.³³¹ In a very recent study, it has been observed that PTEN loss of function in GBM directly upregulates oncogene expression of the histone-chaperone (DAXX) and histone variant (H3.3) complex, which are involved in tumorigenesis.³³²

Other known mRNA targets of miR-21 are the programmed cell death protein 4 (PDCD4), Maspin, matrix metalloproteinases inhibitors RECK and TIMP3, Bax and tumour suppressor gene tropomyosin 1 (TPM1).³³³

PDCD4 is a potent tumour suppressor and one of the principal targets of miR21. It can down regulate tumour promotion and progression³³⁴ and inhibits invasion and intravasation.³³⁵ PDCD4 inhibits eIF4A activity to suppress translation of p53 and Sin1. Inhibition of Sin1 translation attenuates cell proliferation, invasion and metastasis. PDCD4 may directly bind with mRNAs of c-Myb transcription

factor, anti-apoptotic protein Bcl-xL and X chromosome-linked inhibitor of apoptosis (XIAP) to inhibit their translations, leading to suppression of proliferation and anti-apoptosis. Moreover, knockdown of Pdcd4 also causes a decrease in expression of several epithelial markers including E-cadherin, α -catenin and γ -catenin as well as an increase in expression of mesenchymal markers like β -catenin, N-cadherin and fibronectin, suggesting that PDCD 4 knockdown induces epithelial to mesenchymal transition (EMT) and ultimately, increases cell migration and invasiveness potential.³³⁶

RECK is a membrane-anchored inhibitor of metalloproteinases (MMPs). Reduced expression or inactivation of RECK is critical for the angiogenesis, invasiveness and metastasis of diverse cancers like glioblastoma.³³⁰ Tissue inhibitor of metalloproteinases 3, TIMP3, acts in similarly to RECK, not only by inhibition of MMPs but also by participation in other cellular processes, such as apoptosis. Krichevsky and co-workers demonstrated that TIMP3 activates caspases and induce apoptosis on human glioma cells.³³⁷ MMP levels and activities are significantly elevated in human gliomas, which contributes to glioma cell invasion of the surrounding normal tissues, metastasis, and angiogenesis through cell surface degradation.³³⁸

Bax is a key component for cellular induced apoptosis through mitochondrial stress. Bcl-2 is an antiapoptotic protein and constitute one of the major obstacles for chemotherapy in many cancer cells. Under proper stimuli, Bax forms toxic oligomers which drill the mitochondrial membrane, releasing pro-apoptotic proteins such as Cytochrome C and apoptosis-inducing factors. Leakage of pro-apoptotic mediators results in activation of caspase pathways that ultimately precipitate cell death.³³⁹ miR-21 protects glioma cells by reducing the Bax/Bcl-2 ratio and lessening the activity of caspase 3.³⁴⁰

Tropomyosin 1 (TPM1) another suppressor gene whereas Maspin (mammary serine protease inhibitor) is a well-known tumour suppressor. Both mRNAs are targeted by miR21. TPM1 is involved in cell migration and invasion; Maspin regulates cell growth, invasiveness and metastasis.³⁴¹

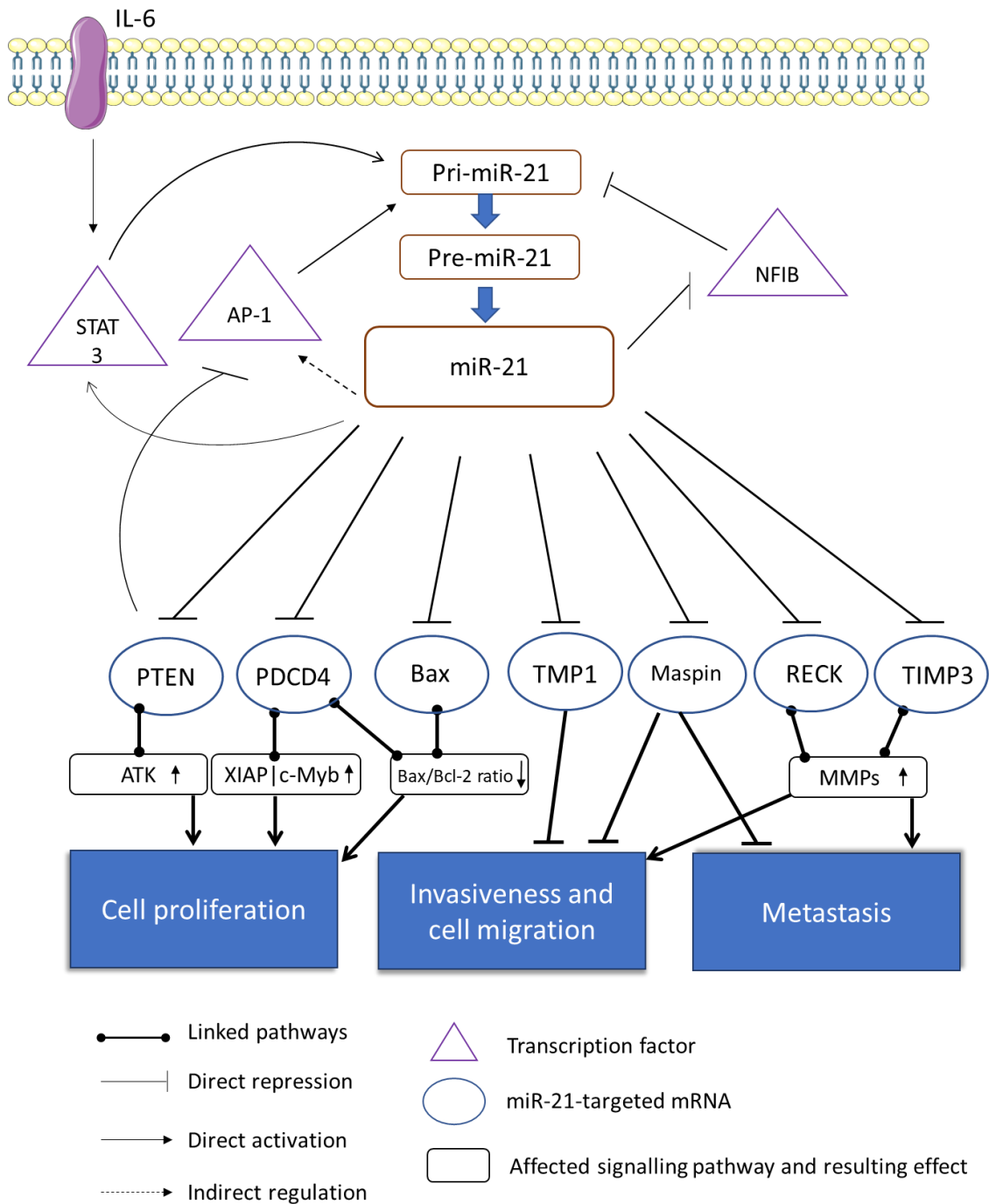


Figure 86 - Scheme of miR-21 signalling pathways for carcinogens and feedback regulation. As a result of the direct repression on targeted mRNA, many signalling pathways involved in cell proliferation, invasiveness and metastasis are altered and initiates carcinogenesis phenomena in the cell. Key: miR, microRNA; pri-mRNA, primary mRNA; pre-mRNA, precursor mRNA; AP-1, activated protein-1; NFIB, nuclear factor I/B; Maspin, mammary serine protease inhibitor; PDCD4, programmed cell death protein 4; TPM1, tropomyosin 1; RECK, reversion-induced cysteine-rich protein with Kazal motifs; TIMP3, tissue inhibitor of metalloproteinases 3; MMP, matrix metalloproteinase; PTEN, phosphatase and tensin homologue; c-Myb transcription factor; XIAP, X

chromosome-linked inhibitor of apoptosis; STAT3, signal transducer and activator of transcription 3. Picture made using Servier Medical Art (<https://smart.servier.com/>).

How does miR-21 maintains its expression over the time?

Sustained miR-21 expression might involve miR- 21 itself and its direct target Nuclear Factor I/B (NFIB). NFIB is a transcriptional repressor that suppresses basal expression of the miR-21 gene. In stimulated or cancer cells, NFIB can be displaced from the miR-21 promoter (e.g. by AP-1), which may lead to elevation of miR-21 levels. Then miR-21 would bind to NFIB mRNA inducing down-regulation of NFIB production and therefore further upregulation of miR-21 expression (Figure 86).

Another related mechanism of continuous miR-21 expression might implicates its transcriptional inducer AP-1 and PDCD4 regulator. miR-21 represses expression of PDCD4, a protein that blocks the transactivation of AP-1 by interfering with c-Jun phosphorylation and activation. Therefore, miR-21, in turn, is capable of inducing AP-1 activity and AP-1-dependent transcription by two, likely independent, mechanisms to maintain its expression.

The Signal Transducer and Activator of Transcription 3 (STAT3) is a transcriptional factor which is constitutively activated in tumoral tissues. STAT3 promotes tumour growth, angiogenesis, tumour invasion and metastasis. Many cytokines, growth factors, miRNAs (miR-21) and G-protein coupled receptors induce STAT3 phosphorylation. Ganguly *et al.*³⁴² demonstrated that Glioma Initiating Cells (GICs) are strongly dependent on STAT3 factor. Simultaneously, STAT3 is one of the factors that induce miR-21 transcription in various cancers, such as myeloma and GBM.³³⁷ The STAT3-mediated IL-6-miR21 autocrine feedback stabilises miR-21 levels in glioma cells (Figure 86).

3.1.6 Cancer stem cells and miRNAs

Cancer stem cells (CSCs) present self-renewal capacity, limitless proliferative and metastasis potential, making of them critical drivers of tumour progression, recurrence and malignancy.³³³

Epithelial-to-mesenchymal transition (EMT), as defined by Li and co-workers, is a well-recognized integral component of invasion and migration processes, which is characterized by loss of cell adhesion, changing in the composition of the cytoskeleton and acquisition of migration ability and invasive traits.³⁴³ It is hypothesized that cells that undergoes EMT acquire cancer stem cell characteristics.

It has been reported that CSCs are also formed in glioblastoma (GSCs or GICs), making them more resistance to radiation, chemotherapy with TMZ and biological treatment.³⁴⁴

miR-21 can also support CSCs development in GBM. The mechanism by which miR-21 boots GSCs formation is by the inhibition of FASLG protein expression, an driver of apoptosis that belongs to the Tumoral Necrosis Factor α (TFN- α) family.³⁴⁵

miR-205

miRNA-205 belong to the miRNA-200 family and plays a key role in glioblastoma. Although its expression can vary from one type of tumour to another (being upregulated or downregulated), it behaves as tumour suppressor. In glioblastoma, miR-205 is downregulated, situation that facilitates tumour initiation and proliferation. miR-205 directly targets the vascular endothelial growth factor A, provoking apoptosis and cell-cycle arrest and reduce viability and invasive properties of glioma cells.³⁴⁶

Another target of miR-200 family is Zinc finger E-box binding homeobox 1 (ZEB1), a transcriptional factor that enhances tumour invasion and metastasis by inducing EMT on carcinoma cells.³⁴⁷ It has been observed that glioma cells overexpress ZEB1, due to the low levels of miR-205. EMT is then undertaken for many cancerous cells gaining CSCs profiles.

3.1.7 11PS04, TMZ and other strategies for cancer stem cells

In view of the key role played by miRNAs in cancer, and specifically that of miR-21 and miR-205 in GBM, it is obvious that novel antitumoral therapies are oriented towards these targets. Malignant gliomas are treated with a combination of surgery, radiation, and chemotherapy (TMZ). Sadly, in many occasions this is not enough for total health recovery due to tumour recurrence. CSCs in glioblastoma are recurrent even though TMZ significantly reduces its formation. Therefore, there is need of new therapies that target miRNAs and restore their physiological levels as well as completely avoid the formation of CSCs.

The most common approach for repressing miRNA expression is by treating cells with *anti*-miRNA oligonucleotides, both for studying the signalling pathways affected and for the treatment of glioblastoma. A second option is the use of antisense peptide nucleic acids (PNAs), which bind complementary RNA with high binding affinity.

A very recent study employed an anti-miR-21 oligonucleotide and a PNAs to inhibit miR-21 expression.³⁴⁸ In order to overcome the rapid clearance of these oligonucleotides and help them to across the BBB, the miR-21 inhibitors were loaded in two nanoparticles (NP) formulations: the first formulation used the cationic polymer poly(amine-co-ester) (PACE) modified with apolipoprotein E (ApoE) in the NP surface, and was employed to load the anti-miR-21). The second formulation was a poly(lactic acid) based NP with different chemical modification on the surface. Convection-enhanced delivery (CED) of NPs formulations facilitated the intracranial distribution of the active nucleotides, which did not provide a survival benefit in the animal models when dosed alone but it did it when combined with TMZ.

A different strategy is by using chemotherapeutics to modify the expression and/or the signalling pathways altered by oncomiRNAs.

Candance and co-workers developed gamma-secretase inhibitors that enhanced the effect of TMZ on neurosphere (CSCs that mimics those from GBM patients in glioblastoma) by targeting the Notch pathway, which has demonstrated to *be able to* enhance glioma cell survival.³⁴⁹ Their gamma-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-5-phenylglycine t-butyl ester) partially succeeded in combined treatment with TMZ. Pre-treatment and co-treatment of glioma cells with DAPT before addition of TMZ reduced the efficacy of TMZ whereas post-treatment did reduce the neurosphere formation.³⁵⁰

Our group has developed a novel molecule with a pyrano-oxazolone core named 11PS04 ((3aR,7aS)-2-(3-propoxyphenyl)-7,7a-dihydro-3aH-pyrano[3,4-d]oxazol-6(4H)-one) with a demonstrated capacity to fight oncogenicity in vitro and in vivo by targeting both miRNAs 21 and 205.³⁵¹

The antitumor profile of 11PS04 was validated by real-time PCR, which confirmed the inhibition of miR-21 as well as the enhancement of miR-205 expression in a dose-dependent manner in U87MG cells and MCF-7 cell lines.

After 24 h hours cell exposition to 11PS04, the expression of miR-21 and miR-205 targeted mRNAs were measured: PDCD4 increased significantly compared to control ($p < 0.005$) whereas ZEB1 decreased ($p < 0.005$). E-Cadherin, a downregulated protein under the expression of ZEB1 was also measured to evaluate the restoration of miR-205 levels. miR-21 was reduced in both cell lines as well as miR-205 expression was found significantly higher compared to control (Figure 87).

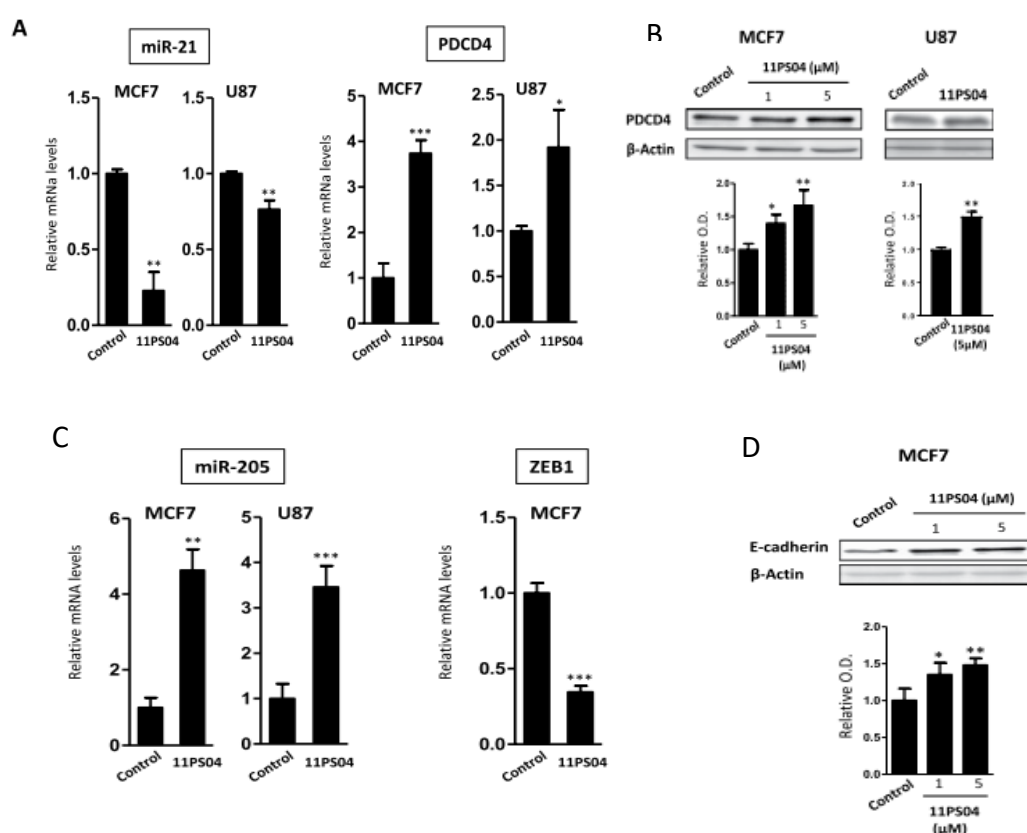


Figure 87 - 11PS04 regulates the expression of known targets of miR-21 and miR-205 in MCF-7 and U87 cells. Regulation of proteins that are downstream targets of miR-21 and miR-205: PDCD4 and E-

Cadherin, respectively. Exposure to 11PS04 (5 μ M, 24h exposure time) led to the transcriptional regulation of miR-21 (A) PDCD4 (B) miR- 205 and ZEB1. Transcription was compared with the endogenous control: ACTB ribosomal RNA. The accumulation of the miR-21 and miR-205 target proteins in extracts of treated MCF-7 and U87 cells was measured in Western blots, PDCD4 (C) and E-Cadherin (D) respectively (mean $\pm$ SEM; n = 3; *P < 0.05; **P < 0.01; ***P < 0.005 (Student's t-test). Taken from reference 351.

Furthermore, 11PS04 restricted the potential of CSCs to form oncospheres. The frequency of sphere formation (f) was assessed *in vitro* by using an extreme limited dilution analysis (ELDA) which can indicate the number of tumour cells capable of forming a single sphere. 5 μ M of 11PS04 significantly suppressed the formation of stem cells both in breast cells and glioma cells relative to controls. For instance, MCF-7 control cells presented a f of one nanosphere out of eight cells ($f = 1/8$). Same cells after treatment with 11PS04 showed a f value of 1/75.5. Similarly, U87 cells reduced the sphere formation from 1/3.6 to 1/38.

It is known that miR-21 is involved in resistance to solid cancer cells to multiple drugs, among them TMZ.³²¹ 11PS04 proved its ability to sensitise U87 MG cell line to TMZ effect, both against glioma cells and glioma stem cells (GSCs) (Figure 88).

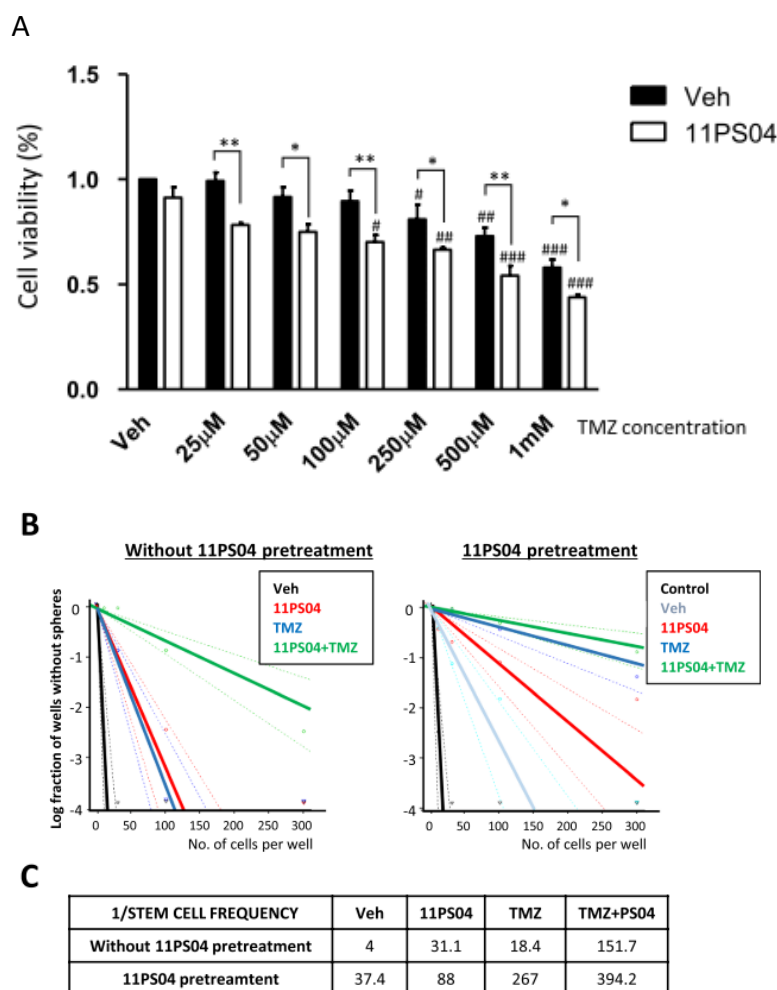


Figure 88 - (A) Cell viability of U737 MG cells after 72 h of treatment with Temozolomide (TMZ). Cells were pre-treated for 48 h with 11PS04. MTT test ($n = 3$): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to the vehicle pre-treated; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ relative to vehicle cells. (B) Sphere frequencies from U737 single-cell suspensions are plotted relative to the number of cells seeded per well, in the presence of 11PS04 (5 μ M), TMZ (100 μ M) or 11PS04 (5 μ M) plus TMZ (100 μ M) during the formation of the spheres. Solid lines represent the frequency estimation and the dashed lines the 95% confidence intervals. The right panel shows the frequency of spheres produced by U737 cells pre-treated with 11PS04 (5 μ M) or the vehicle alone, and then exposed to 11PS04 (5 μ M), TMZ (100 μ M) or 11PS04 (5 μ M) plus TMZ (100 μ M) during the formation of the spheres. The controls are the cells pre-treated and treated during sphere formation with the vehicle alone. (C) The frequency of initiating cancer stem cells was calculated using the ELDA platform. Taken from reference 351.

Therefore, 11PS04 is an miR-21 inhibitor and enhance TMZ effect via PDCD4 pathway as well as restores miR-205 levels. Both effects on miR expression influence the behaviour of CSC and their participation in oncogenesis, tumour progression, metastasis and recurrence.

3.2 HYPOTHESIS, AIM AND OBJETIVES

Large amounts of evidence point to miRNAs as key players the regulation of many proteins which are essential for cell-cycle control, stress response, differentiation, migration and metabolism. miRNAs are found in cells with physiological activity, but a significant dysregulation of their functionalities (then become into oncogenic miRNAs) trigger the activation of multiple signalling pathways implicated in multiple diseases, among them cancer. Thus, they are not only biomarkers for the diagnosis of a specific disease but also targets of antitumoral treatments.

Our group has developed 11PS04, a new chemical entity with the ability of modify and restore miR-21 and miR-205 to normal levels, and therefore increasing the susceptibility of GSCs to Temozolomide (Temodar®) as well as potentiating the antitumoral effect of Temozolomide over glioblastoma cells.

The success of many therapies sometimes relays on their optimal pharmacokinetic properties. In fact, being able to across the BBB is basic for any drug that aspires to treat a brain disease. TPGnRH has emerged as targeted nanomedicine for GBM, not only because of its antiproliferative activity on glioma cells but also due to its ability to cross the BBB barrier carrying chemotherapeutics such as Paclitaxel (Taxol®).

We aim:

1. to load TPGnRH nanofibers with 11PS04 and TMZ in clinically relevant concentrations
2. to assess 11PS04-loaded nanofibers efficacy in GBM cells.
3. to evaluate the effectiveness of combined therapy with 11PS04-loaded nanofibers with TMZ in GBM cells.

3.3 RESULTS AND DISCUSSION

3.3.1 11PS04 and 11PS04 – TMZ loaded TPGnRH Nanofibers Characterization

Particle size and zeta potential measurements. Quantification of loading using HPLC

Three formulations were prepared (F1-3) and characterised for drug loading, particle size and zeta potential (Table 18). F1 contained 11PS04 loaded into TPGnRH (molar ratio 1:8); F2 was prepared with 11PS04-TMZ loaded nanofibers (molar ratio 1:4:8) and F3 was made with 11PS04-loaded nanofibers (molar ratio 1:3). Drug loading was determined using HPLC-UV.

Table 19 – Average particle size (nm), peaks (% by volume), Z potential and drug loading of 11PS04-loaded and 11PS04-TMZ-loadend TPGnRH formulations. Peak (%) noted by volume. Mean $\pm$ SD (n=3).

	Average particle size (nm)	Peak 1 (nm) %	Peak 2 (nm) %	Peak 3 (nm) %	PDI	Z potential (mV)	Drug loading (% w/w)
F1	1311 $\pm$ 140.1	196 $\pm$ 13.49 (3.3%)	1677.3 $\pm$ 22.85 (51.5%)	2620 $\pm$ 137.1 8 (45.2%)	0.656 $\pm$ 0.073	10.2 $\pm$ 2.33	2.0 $\pm$ 0.4
F2	580.7 $\pm$ 59.07	700.9 $\pm$ 518.11 (62.1%)	3698.5 $\pm$ 239.7 (34.7%)	-	0.404 $\pm$ 0.06	13.0 $\pm$ 1.79	11PS04 2.2 $\pm$ 0.07 TMZ 5.4 $\pm$ 0.7
F3	434.1 $\pm$ 11.73	136.9 $\pm$ 22.2 (35.7)	916.3 $\pm$ 317.9 (67.1)	164.5 $\pm$ 32.6 (2.8%)	0.445 $\pm$ 0.017	10.1 $\pm$ 0.23	5.7 $\pm$ 0.23

Key: PDI: polydispersity index; F1: Formulation 1 - 11PS04 loaded into nanofibers, molar ratio 1:8; F2: Formulation 2 - 11PS04-TMZ loaded nanofibers; F3: Formulation 3 – 11PS04-loaded nanofibers, molar ratio 1:3.

All formulations were cationic as expected for TPGnRH nanofibers that are protonated at physiological pH. Polydispersity values were relatively high, due to the long axial nature of the particles. Formulation 2 contained 2.2 $\pm$ 2.9% w/w for 11PS04 (90.9 $\pm$ 2.9 % encapsulation efficiency), while TMZ demonstrated a final concentration of 5.4 $\pm$ 0.7 % w/w (89.3 $\pm$ 1.5% encapsulation efficiency). Formulation 1 and 3 presented drug loading values of 2.0 $\pm$ 0.4 and 5.7 $\pm$ 0.23 % w/w, respectively. Peaks were well separated using HPLC and retention times for TMZ, 11PS04 and TPGnRH were 3.29, 8.60 and 14.81 respectively (Figure 89).

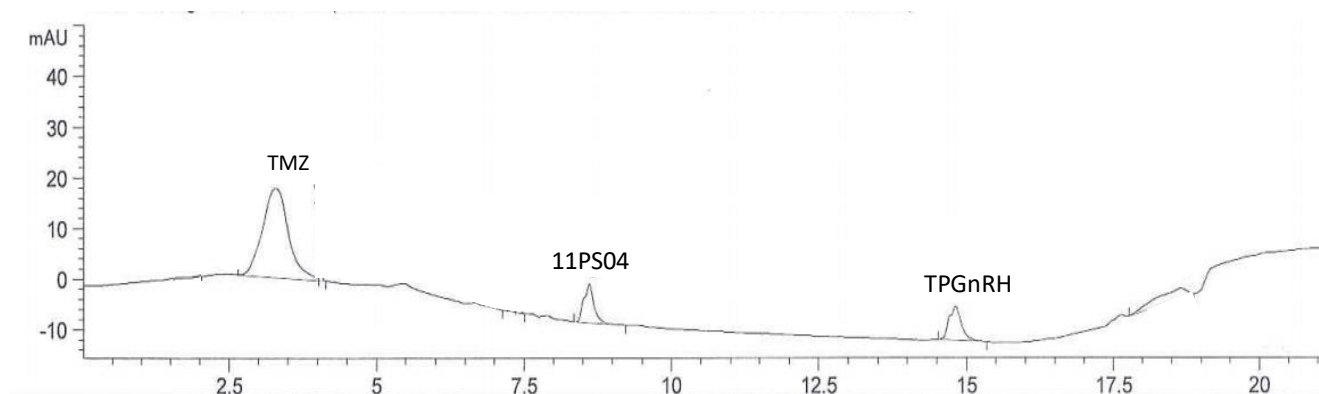


Figure 89 - HPLC chromatogram at 254 nm of TMZ and 11PS04-loaded TPGnRH nanofibers showing the peaks of TMZ, 11PS04 and TPGnRH at 3.294, 8.607 and 14.819 minutes respectively.

Transmission electron microscopy (TEM) and Atomic Force Microscopy (AFM)

TEM studies of 11PS04 and TMZ-loaded TPGnRH formulations confirmed the long-axial morphology of the nanofibers (Figure 90). Nanofibers showed ribbon-like appearance and had an average width of 27.4 ± 7.0 nm. 11PS04 loaded-TPGnRH nanofibers (Formulation 1) forms thin fibers with a diameter of 10.3 ± 1.9 nm ($n=10$) and an average length of 587 ± 100 nm ($n=10$). Formulation 1 was also imaged using AFM (Figure 91) and the measured diameter was 17 ± 2 nm ($n=10$) (which is higher possibly due to lower quality of AFM images and probe selected).

Formulation 2 (containing 11PS04 and TMZ loaded into nanofibers) showed fibers with an average diameter of 8.52 ± 2.0 nm ($n=10$) and a length of 847 ± 244 nm ($n=10$). These fibers were different from those formed on formulation 1 (P value < 0.05). If forming bundles, the diameter of fibers in Formulation 2 was 31.23 ± 8.5 nm. Loaded fibers of TPGnRH with 11PS04 in Formulation 3 appeared mostly forming bundles with diameters of 23.0 ± 2.72 nm ($n=10$) and an average length of 726 ± 79 nm ($n=10$) (Figure 90).

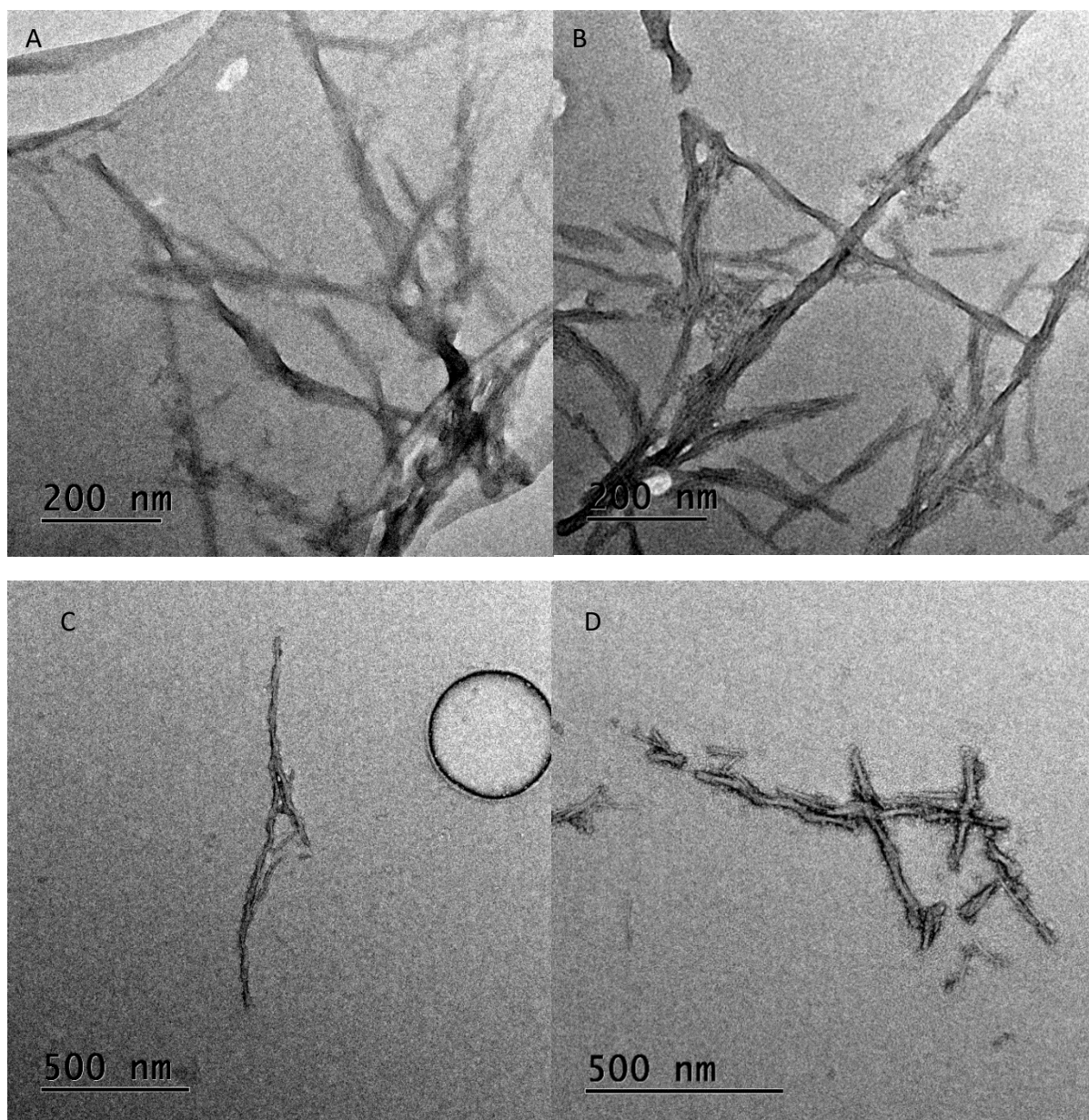


Figure 90 - Negatively stained TEM images of the 11PS04-TMZ-loaded TPGnRH (**A,B**) and 11PS04-loaded TPGnRH corresponding to F1 and F3 (**C,D**). Formulations were diluted (1:100 v/v) in 0.01 M of PBS (pH 7.4) and negatively stained with 2% uranyl acetate prior to their visualization in TEM.

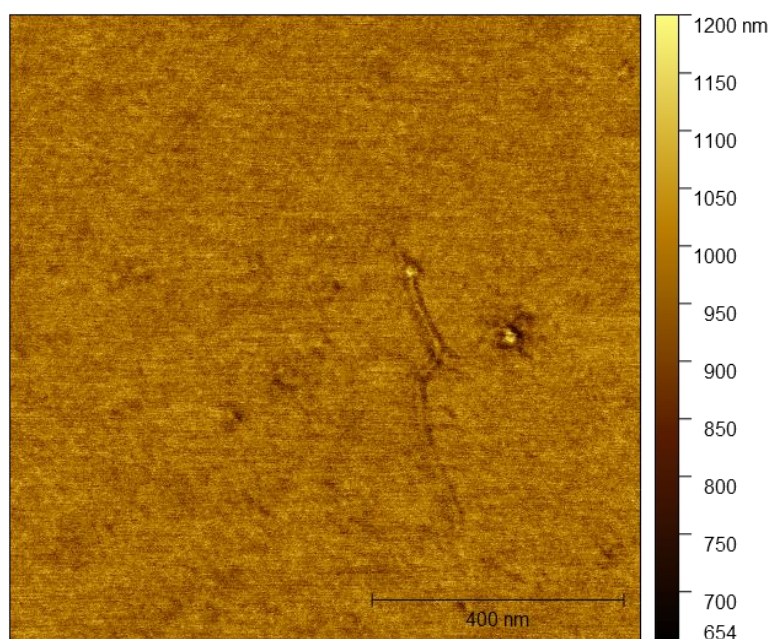


Figure 91 - AFM image of 11PS04-loaded TPGnRH nanofibers (Formulation 1). AFM images were analysed) using Gwyddion software.

AFM imaging revealed a fiber whose size was $0.45\ \mu\text{m}$ and its diameter was $17 \pm 2\ \text{nm}$ ($n=10$), which is in accordance with the sizes observed by TEM

3.3.2 Antitumoral Activity of 11PS04, Temozolomide and TPGnRH

11PS04 and Temozolomide

We evaluated the effect of different concentrations of TMZ combined with 5 μ M of 11PS04 on U87MG for a total of four days. This amount of 11PS04 was chosen as it has previously proven to be able of modify the expression of miR-21 and miR-205.³⁵¹ In this assay, the dose of 11PS04 was added on day 2 and on day 4 (refer to Figure 104 for more details). The effect of TMZ alone on U87 MG cell line was also evaluated after its addition on the 4th day.

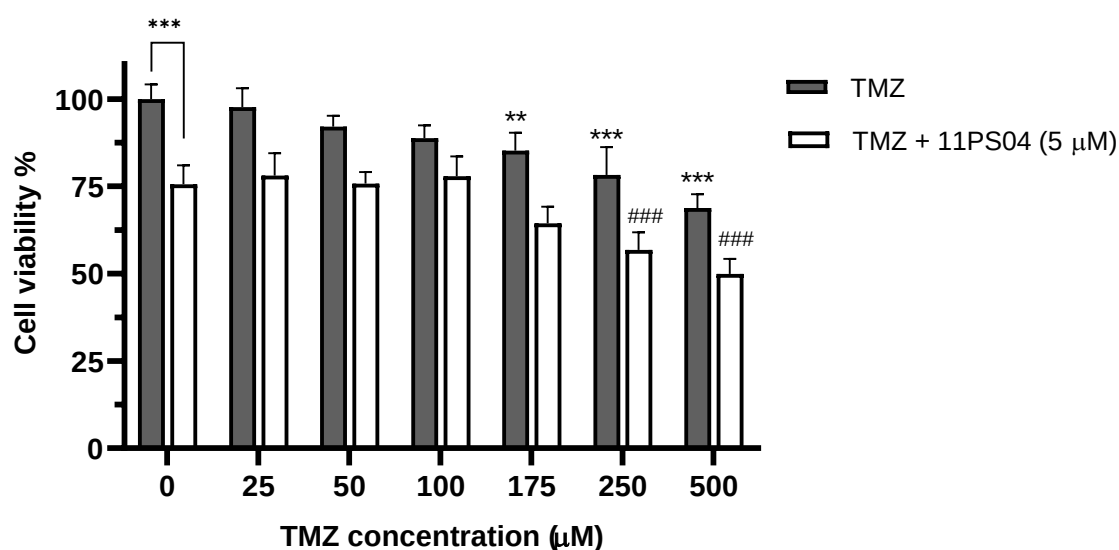


Figure 92 - Cell viability of U87 MG cells treated with Temozolomide without (black bars) or with 11PS04 (white bars). U87 MG cells were seeded at 1400 cells/cm² in complete medium and allowed to attach and grow for 3 days. Cells were treated with 11PS04 every two days for four days. Treatment with TMZ was added on the 4th day alone or in combination with 11PS04. Cell metabolic activity was measured at the end of the assay by the MTT test. Statistical comparisons among black bars: P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to non-treated cells (0 μ M of TMZ); comparisons among white bars: P values # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ relative to treated cells with 5 μ M of 11PS04 and 0 μ M of TMZ. Two-way ANOVA with Dunnett's post hoc test. Mean $\pm$ SD (n=3).

The treatment with only TMZ did not produce significant cytotoxic effect on cells at low concentrations of the drug (<175 μ M). Above 175 μ M, TMZ started to alter the cell viability of U87 MG cells (15% effective). These results correlate with those obtained by Aguado et al.,³⁵¹ showed in Figure 88A; however, higher concentrations of TMZ (250 μ M and 500 μ M) were more effective when measuring the cell viability after 48 h than after 72 h. This effect has been previously reported in the literature²⁸⁶ and supports the necessity of frequent renewal of TMZ doses. Essentially, this particular type of cells present the ability to auto-recover after administration of an active agent such as TMZ

if being maintained in culture for a longer period of time without repeating the treatment (i.e. treatment lasted for two days instead of three), which is the case of the experiment showed in figure 88A. The antitumoral effect of therapeutic TMZ concentrations is potentiated after 48 h of treatment, when cells are sensitised previously with 11PSO4. Prolongation of treatment of U87MG with 11PSO4 (5 μ M) over 4 days reduced viability to $75.6 \pm 5\%$ ($p < 0.05$) (Figure 92). An additive effect was only observed when TMZ was added at concentrations above 250 μ M. The combination of 11PSO4 and TMZ reduced the cell viability by approximately $43 \pm 5\%$ when TMZ was dosed at 250 μ M, and by $50 \pm 4\%$ when added at 500 μ M.

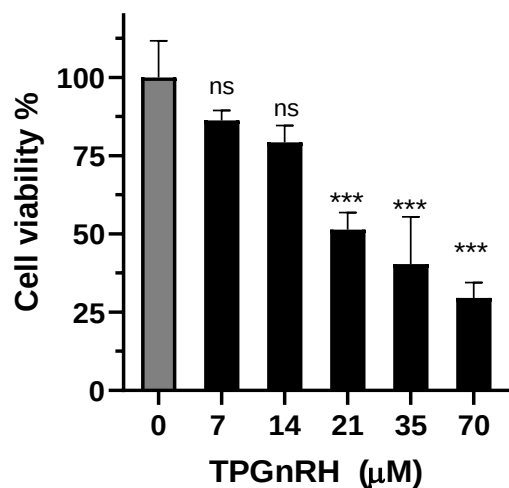
The Triple-Treatment

The effect of combining TPGnRH in a range of concentrations (7-70 μ M) with 11PSO4 (5 μ M) and TMZ at two levels, 100 and 250 μ M, was assessed and cells were treated every two days for a total of 6 days (Figure 93). Treatments were performed every two days, for a total duration of the treatment equal to six days. TPGnRH reduced cell proliferation after 48 h of treatment at higher concentrations than 21 μ M (Figure 93A) in a concentration dependent manner and the effect is potentiated with increasing duration of treatment. Addition of 11PSO4 to TPGnRH nanofibers did not elicit additive antiproliferative effects, but effects were driven by TPGnRH concentration. Cells treated with both TPGnRH nanofibers and TMZ did not show additive effects at low TMZ doses of 100 μ M but did show a significant effect when higher TMZ concentrations were added. (Figure 93D). However, when cells were pre-treated with 11PSO4 and also TPGnRH and TMZ, even low TMZ doses (100 μ M) resulted in an added additive effect that were significant (Figure 93C). At higher TPGnRH concentrations (35 and 70 μ M), addition of TMZ did not improve antiproliferative effect as efficacy was driven by TPGnRH efficacy primarily. The triple-treatment can allow for lower doses of TMZ and TPGnRH when combined (Table 19), which can result in lower systemic side-effects (Figure 93). Addition of TMZ reduced TPGnRH IC₅₀ fourfold and when cells were pre-treated with 11PSO4 before TMZ was added. TPGnRH was only needed in nanomolar concentration to elicit 50% reduction in antiproliferation (Table 19).

After two days of treatment with only 11PSO4 (Figure 93B), the cell viability was 85%, which is in accordance with the results obtained by Aguado *et al.*³⁵² However, by contrast to what expected, the effect of TPGnRH on cells did not increase when 11PSO4 was added (Figure 93B). The cell viability percentage was practically the same in the presence and absence of 11PSO4.

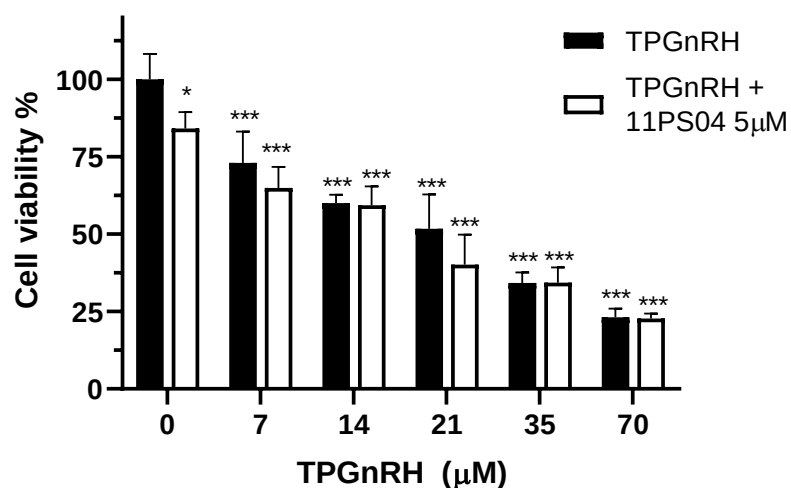
MTT test results after 2 days of treatment

A

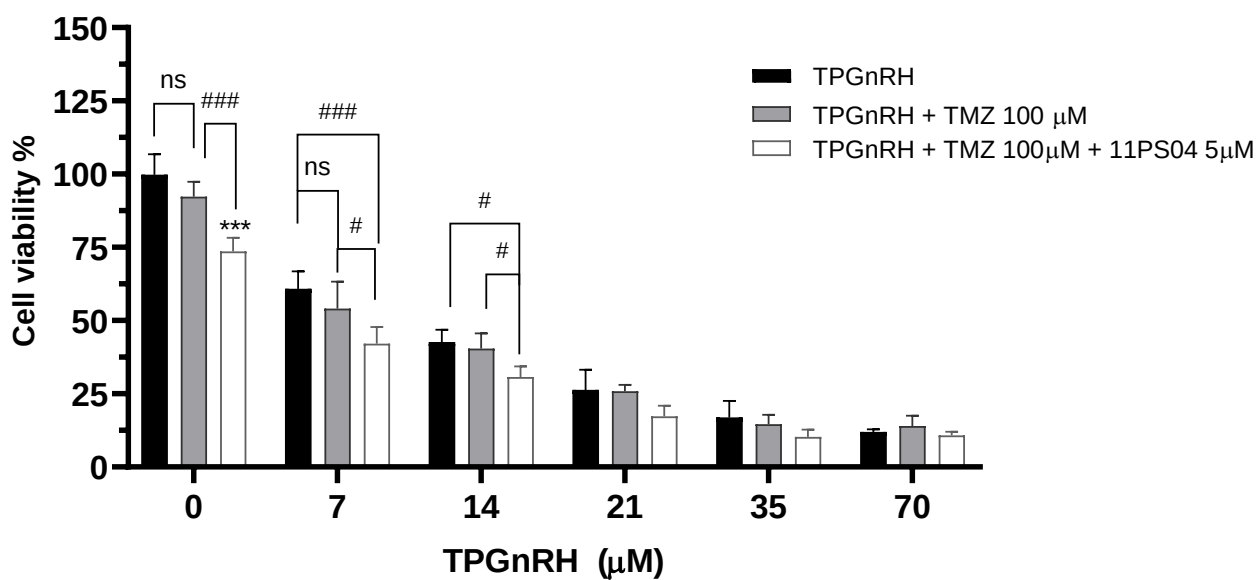


MTT test results after 4 days of treatment

B



C

MTT test results after 6 days of treatment (TMZ = 100 μM)

D

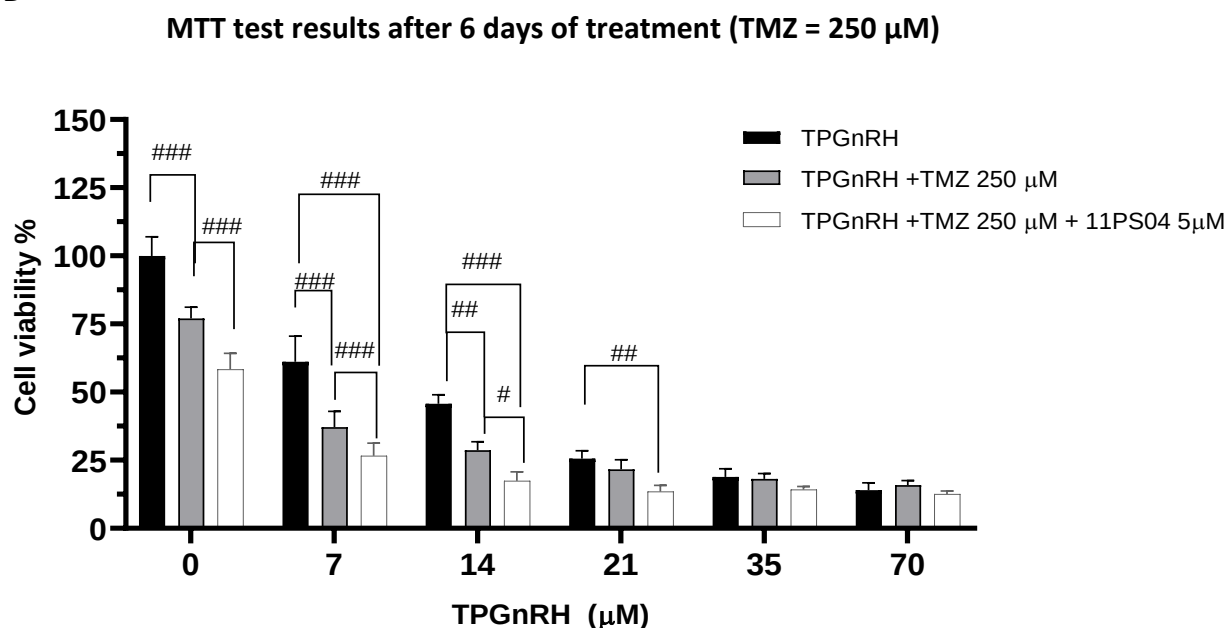


Figure 93 - Cell viability of U-87 MG cells during the triple-treatment. U87-MG cells were seeded at 1400 cells/cm² and allowed to attach and grow for 3 days. Cells were treated with TPGnRH (7-70 μ M) every two days for 6 days in every test performed. Treatment with only TPGnRH is represented by black bars, treatment with TMZ and TPGnRH in grey colour, and the triple-treatment with 11PS04, TMZ and TPGnRH in white colour. Cell metabolic activity was evaluated by the MTT assay at day 2 (TPGnRH alone, A), at day 4 (TPGnRH with or without 11PS04, B), and day 6 (TPGnRH with or without 11PS04 and two different doses of TMZ (TMZ at 100 μ M (C) and 250 μ M (D)). P values * p < 0.05, ** p < 0.01, *** p < 0.001 comparing treated cells with untreated cells. P values # p < 0.05, ## p < 0.01, ### p < 0.001 indicated for multiple comparisons between groups. Two-Way ANOVA with Dunnett's post-hoc and Tukey's Post-hoc. Mean $\pm$ SD (n=3). See protocol 2 on materials and methods for more details.

The next table shows how the IC₅₀ of TPGnRH is modified and reduced by the presence of TMZ and 11PS04 after 6 days of treatment:

Table 20 - Half maximal inhibitory concentration (IC₅₀) of TPGnRH alone, TPGnRH with 250 μ M of TMZ and TPGnRH with TMZ (250 μ M) and 11PS04 (5 μ M) after 6 days of treatment.

Treatment	IC ₅₀ (μ M)
TPGnRH	10.39 $\pm$ 0.8
TPGnRH + TMZ 250 μ M	2.56 $\pm$ 0.68
TPGnRH + TMZ 250 μ M + 11PS04 5 μ M	0.64 $\pm$ 0.3

TMZ lowered the IC₅₀ of TPGnRH fourfold (10.4 μ M to 2.6 μ M), whereas 11PS04 with TMZ contributed to further reduce the IC₅₀ in nanomolar concentrations. As it can be appreciated, the use of the three

compounds in combination clearly improved the effectiveness of the treatment, thus allowing to reduce the amount of peptide dosed as the final use.

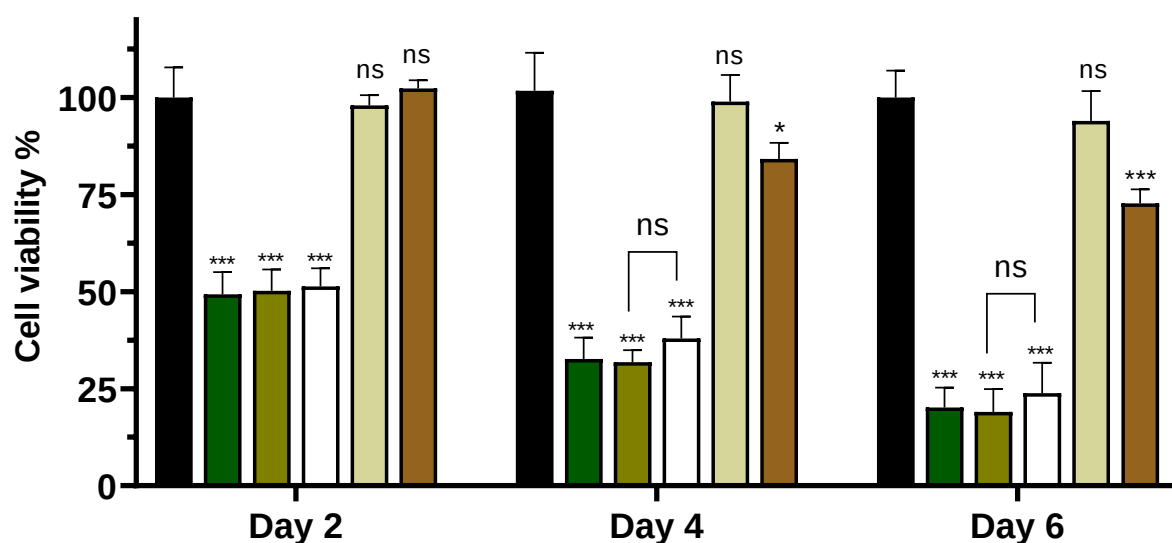
3.3.3 ANTITUMOURAL ACTIVITY OF LOADED TPGnRH NANOFIBERS

11PS04 – TMZ loaded nanofibers

Previous studies have demonstrated the ability to load high concentrations of paclitaxel in TPGnRH nanofibers when the latter are used in excess ideally at equal or above 1:2 molar ratio and preferably 1:4 molar ratio.³¹⁴ However, the amount of TMZ (~250 μ M) needed to load to elicit an antiproliferative effect are 6584-fold higher compared to paclitaxel that is active in nanomolar doses (~1-10 nM), which makes loading difficult without utilising high doses of TPGnRH. As doses of TPGnRH greater than 40 μ M are resulting in near complete inhibition of U87MG cells, we decided to set a TPGnRH concentration of 40 μ M for these studies and use the lowest needed molar ratio between TPGnRH and TMZ of 2:1 respectively. On other hand, as 11PS04 effective dose is in the micromolar range (5 μ M), we formulated these nanofibers in higher molar ratio of 11PS04 to TPGnRH of 1 to 8 respectively. Combined formulations were prepared in isopropanol by thin film method and resuspended in acetate buffer (pH 4.5). Formulations were freshly diluted in saline phosphate prior their use, (see protocol 3 in Materials and methods section for more details).

Treating U87MG cells with 40 μ M TPGnRH nanofibers concentration resulted in 50% inhibition of cells by day 2. No further enhancement of antiproliferative effects was demonstrated after another 2 days of treatment with TPGnRH nanofibers (Figure 94, Treatment A) and the treatment with 11PS04 loaded TPGnRH nanofibers (Treatment C) or added 11PS04 and TPGnRH nanofibers (Treatment B) ($p>0.05$, Two-way ANOVA). However, pre-treatment with 11PS04 and TMZ resulted in enhanced antiproliferative activity. At day 6, the remaining cell viability following the three treatments (A, B and C) was very similar and not statistically different, with values of $20 \pm 5\%$, $19 \pm 5\%$ and $23 \pm 7\%$, respectively. This suggests that the formulation containing TMZ and 11PS04 worked as the three components did when individually added (treatment B). Previous studies indicated that TMZ at 20 or 25 μ M were not able to elicit antiproliferative effects but did show some antiproliferative effects when combined with 11PS04 ($82 \pm 2\%$, Figure 10.A, $p<0.01$, Two-way ANOVA). Inclusion of 11PS04 and TMZ into nanofibers demonstrated similar efficacy to solubilised doses of each drug added separately. This indicates release of TMZ and 11PS04 from the nanofibers.

Benefit of loading these agents in TPGnRH remains permeability across the blood-brain barrier and targeting of these agents specifically to GBM cells as the peptide nanofibers can bind the GnRH receptor overexpressed in GBM cells. Loading of TMZ at therapeutic concentrations was difficult but this can be overcome in clinical practise by oral administration of TMZ as currently used. TMZ could be given orally in a range of doses in combination with 11PS04-loaded TPGnRH doses, allowing for a wider array of possibilities for tailoring the treatment regime to the patient's needs.

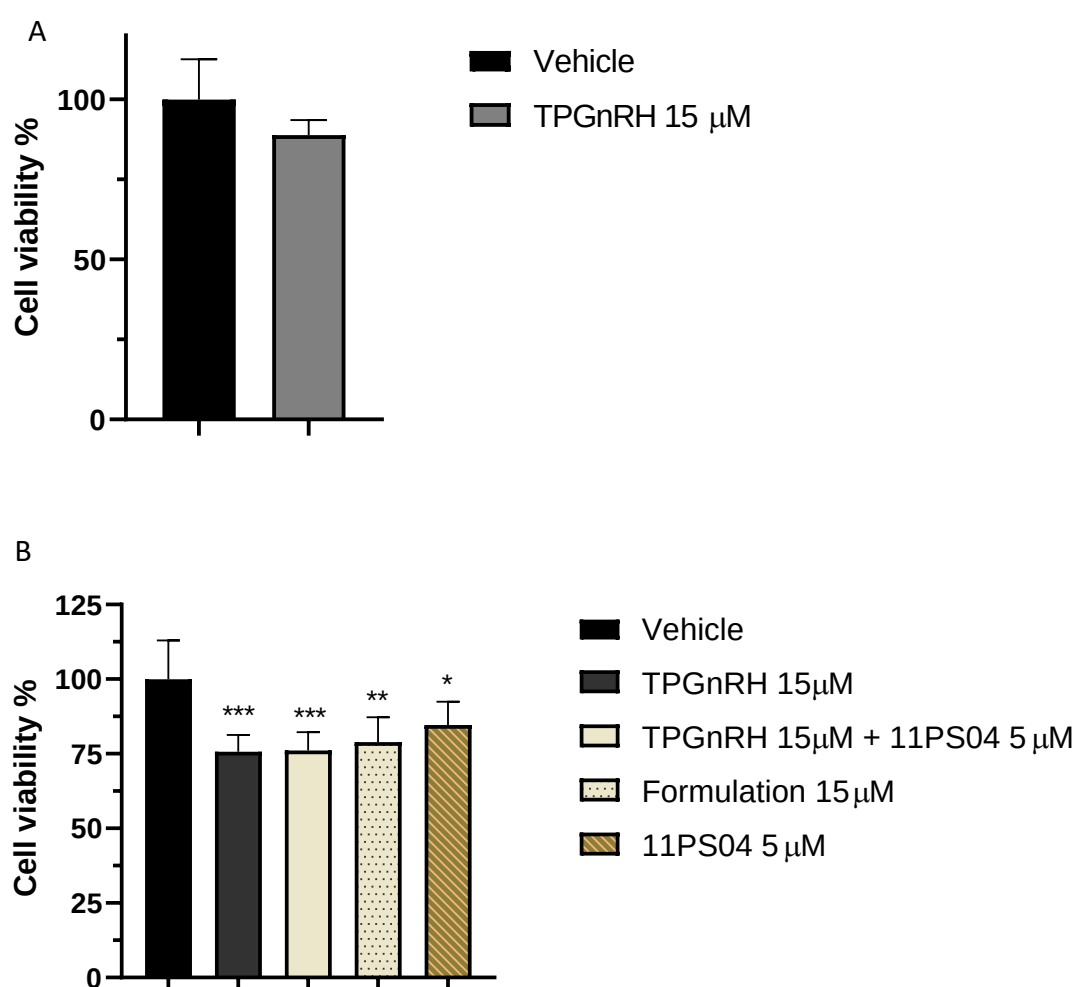


- Control- day 0, day 2 and day 4: Vehicle (DMSO 0.5% v/v in PBS)
- Treatment A- (day 0: TPGnRH 40 μ M; day 2: TPGnRH 40 μ M; day 4: TPGnRH 40 μ M)
- Treatment B- (day 0: TPGnRH 40 μ M; day 2: TPGnRH 40 μ M + 5 μ M 11PS04; day 4: TPGnRH 40 μ M + 11PS04 5 μ M + TMZ 20 μ M)
- Treatment C - (day 0: TPGnRH 40 μ M; day 2: Formulation 1; day 4: Formulation 2)
- Treatment D- (day 0: vehicle; day 2: vehicle; day 4: 20 μ M TMZ)
- Treatment E (day 0: vehicle; day 2: 11PS04 5 μ M; day 4: 20 μ M TMZ + 5 μ M 11PS04)

Figure 94 - Cell viability (%) of U87MG cells after treatment with 11PS04 and TMZ-loaded nanofibers as determined by the MTT assay. U87-MG cells were seeded at 1400 cells/cm² and allowed to attach and grow for 3 days. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing treated cells with untreated cells, and multiple comparisons between groups were done. Two-Way ANOVA with Dunnett's post-hoc and Tukey's Post-hoc. Mean $\pm$ SD (n=4). Key Formulation 1 included 11PS04-loaded nanofibers; formulation 2 contained 11PS04-TMZ-loaded nanofibers. Treatment A corresponds to TPGnRH dosed alone. Treatment B corresponds to TPGnRH, 11PS04 and TMZ externally added. Treatment C corresponds to TPGnRH, 11PS04 and TMZ formulated. Treatment D corresponds to TMZ alone. Treatment E corresponds to 11PS04 and TMZ.

Combining 11PS04 loaded nanofibers with TMZ

As TMZ loading was difficult in clinical relevant concentrations, we chose to also study the effect of 11PS04 loaded TPGnRH nanofibers with externally added TMZ in a range of concentrations. In this case, the molar *ratio 11PS04 to peptide* was 1:3 (F3), which allowed us to test TPGnRH at 15 μ M and 11PS04 at 5 μ M. Drug loading percentage was 5.7 ± 0.23 % (w/w) and encapsulation efficiency was 84.2 ± 3.4 %. Particle size (Table 18) and morphology (Figure 90) were similar as with 11PS04-TMZ loaded nanofibers tested above (formulation 2, $p > 0.05$) but smaller than 11PS04 loaded nanofibers (formulation 1, $p < 0.05$) (One-way ANOVA, Tukey's post hoc test). The antiproliferative effects of F3 are shown in Figure 95 as per protocol 4 in Figure 107.



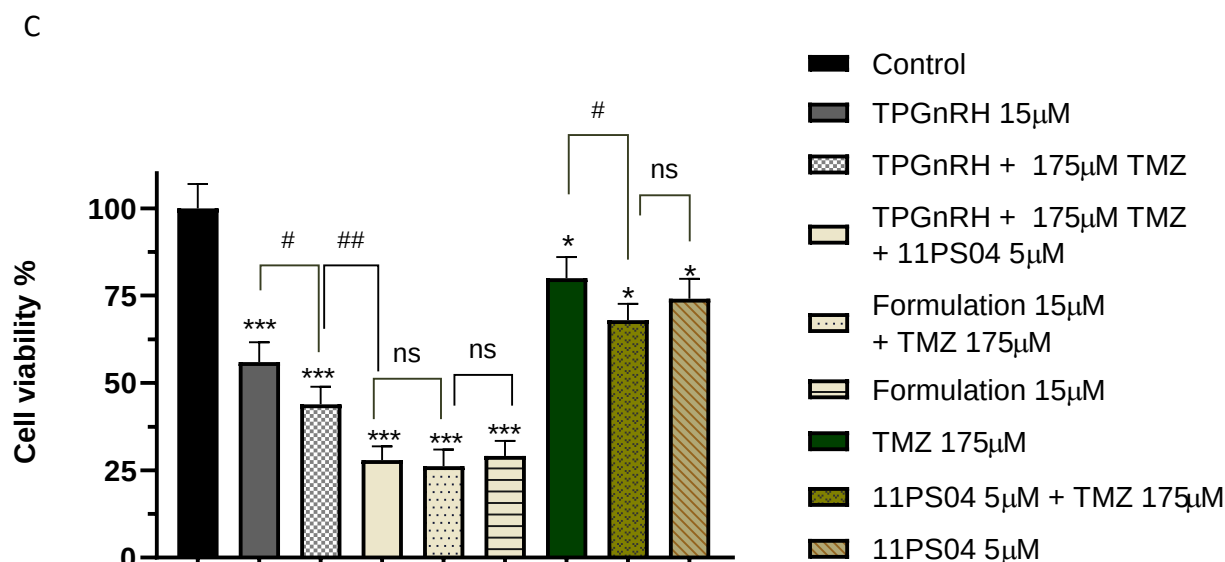


Figure 95 – Evaluation of cell viability (%) of U87 MG cells by MTT test, after the addition of 11PS04 loaded into TPGnRH nanofibers formulation, with TMZ externally added. U87-MG cells were seeded at 1400 cells/cm² and allowed to attach and grow for 3 days and received treatments every 2 days for 6 days. Results are shown for day 2 (A), day 4 (B) and day 6 (C). P values *ns*: no significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001 are relative to untreated cells (vehicle), One-Way ANOVA with Dunnett's post hoc test. One-Way ANOVA with Tukey's post hoc test was run for multiple comparisons between groups, #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001. Mean ± SD (n=3).

TPGnRH nanofibers (15 µM) did not reduce U87MG proliferation after 2 days. After 4 days of treatment and addition of F3 for a further 2 days resulted in similar antiproliferative effects to TPGnRH and 11PS04 added separately (*p* > 0.05, One-Way ANOVA with Tukey's post hoc test). These further confirms that TPGnRH and 11PS04 do not elicit additive effects after 2 days in co-treatment. Combining TMZ (175 µM) with F3 resulted in a reduction of cell viability to 26 ± 5%, which was statistically insignificant to effects produced by addition of the three drugs separately (27 ± 4 %) (Figure 95C). Thus, 11PS04 was successfully loaded into TPGnRH nanofibers and improved the effectiveness of the peptide when the latter was used at a low concentration of 15 µM. The triple-treatment showed improved antiproliferative effects, when compared to cells treated only with TPGnRH nanofibers and with the co-treatment: TMZ + nanofibers (55 ± 5 % and 43 ± 5%, respectively).

At the tested concentration, TMZ has shown to be cytotoxic when added alone, but no additive effect was observed in combination with 11PS04. This would imply that the effect observed by the three compounds is essentially due to an additive effect between TPGnRH and 11PS04. This finding was confirmed by the evaluation of the F3, added without TMZ (stripped bar). The results from F3 supported the concentration-dependant behaviour of TMZ previously observed (Figure 93C), where 100 µM of TMZ was not active, and only the combined effect of TPGnRH and 11PS04 was observed. TMZ was used at 175 µM which is the minimum concentration demonstrating cytotoxic effects in

U87 MG cells. Thus, we hoped to have shown that utilising smaller doses of each active anticancer compound would be able to elicit a similar antiproliferative effect to that obtained by high doses of the peptide alone by acting by different mechanistic pathways.

Doubling the dose of 11PS04

11PS04, when incubated for 48 h with cells, is capable of sensitizing cells without producing high cytotoxicity.³⁵¹ When 11PS04 was added for 4 days at 5 μ M, 11PS04 showed an antiproliferative effect reducing viability by 28 ± 3 % which is additive to the effect produced by the nanofibers. As the next step in this investigation, we wanted to explore to what extent the concentration of 11PS04 would affect the cytotoxic effectiveness of TPGnRH nanofibers. (See Figure 108 for more details of addition protocol followed). Thus, we also assessed the effect of 11PS04 at 10 μ M when combined with 15 μ M of TPGnRH nanofibers (both components were individually added) (Figure 96). Higher concentrations of 11PS04 (10 μ M) resulted in higher antiproliferation (cell viability of 58 ± 9 %) which was similar to that of combined 11PS04 and TPGnRH (61 ± 7 %). Once again, TPGnRH and 11PS04 did not show additive effect when added together.

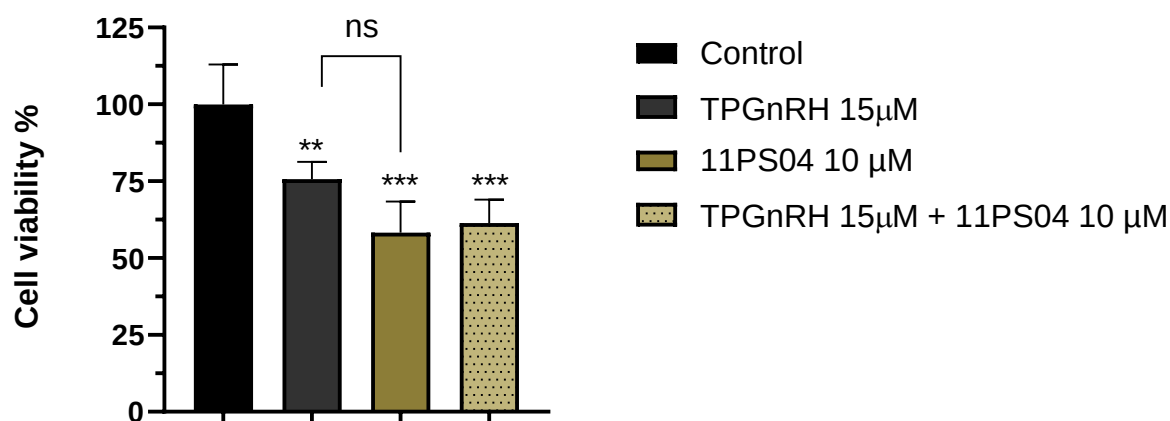


Figure 96 - Cell viability of U87MG cells at day 4 after their exposure to 10 μ M of 11PS04 in the presence and absence of TPGnRH nanofibers. U87-MG cells were seeded at 1400 cells/cm² and allowed to attach and grow for 3 days. Cells were pre-treated with TPGnRH (15 μ M) for two days prior to adding 11PS04 (Protocol 5, Figure 108). P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are relative to untreated cells (vehicle), One-Way ANOVA with Dunnett's post hoc test. Mean $\pm$ SD (n=3).

However, when treatment was continued for a further 2 days, an additive effect was evident as also previously observed (Figure 95C). These results confirm that the length of exposure plays an important role in the effect of co-treatments composed by 11PS04 together with TPGnRH.

Interestingly, the cell survival after the treatment with 15 μ M TPGnRH and 10 μ M 11PS04 was lower than with 5 μ M of 11PS04, reaching values of only 7 ± 2 %, which lies well below the values produced

by TPGnRH fibers (15 μ M) or 11PS04 alone (10 μ M) ($p < 0.001$, One-way ANOVA with Dunnett's post hoc test). Further studies are needed to fully understand and describe the mechanism behind these results.

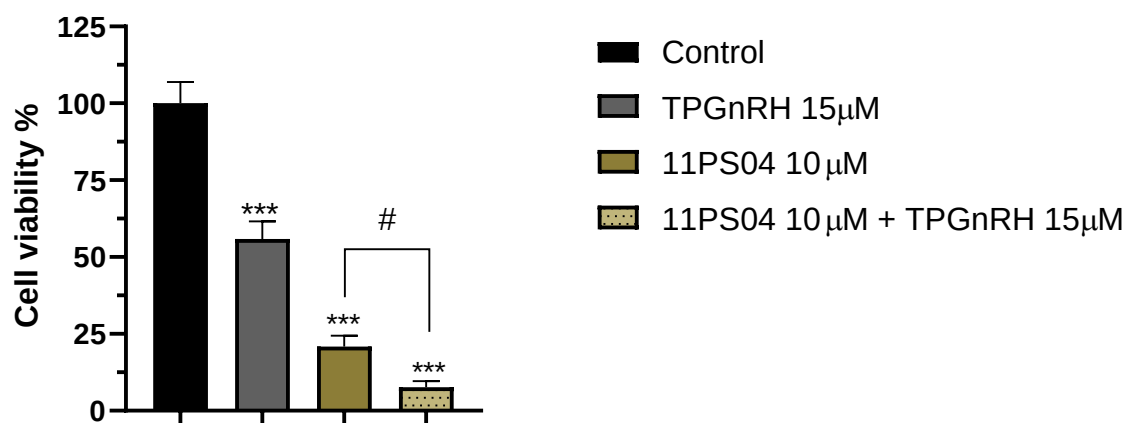


Figure 97 - Cell viability of U87MG (%) on the 6th day. P values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are relative to untreated cells (vehicle), One-Way ANOVA with Dunnett's. Mean $\pm$ SD ($n=3$). Tukey's post hoc test for multiple comparisons between groups (# $p < 0.05$). Mean $\pm$ SD ($n=3$).

3.3.4 Discussion

The current standard of care of GBM is surgical resection, followed by radiation and chemotherapy, usually with TMZ. However, these therapeutic interventions provide only a modest improvement in survival and result in nearly universal recurrence. TMZ is an alkylating agent, whose active metabolite (MTIC) crosses the BBB and penetrates into cancer cells, where it is further metabolised releasing the highly reactive methyldiazonium ion ($^+N_2CH_3$, Figure 3.3), which transfers the methyl group to nucleophilic residues such as the amine groups present in DNA bases. However, tumoral cells have developed a mechanism to repair the methylated bases: the enzyme O⁶-methylguanine methyltransferase (MGMT).

As our understanding of GBM continues to advance, it has become increasingly clear that treatments need to address the complexity and heterogeneity of the disease. There are many studies that indicate that dysregulation of miRNAs, referred to as oncomiRs, show differential expression levels in cancer and are able to affect cellular transformation, carcinogenesis and metastasis, acting either as oncogenes or tumour suppressors. GBM cells overexpress miRNA-21 (miR-21), a non-coding RNA fragment able to silence the expression of proteins involved in the control of cell-cycle regulation, proliferation, migration and invasiveness.³¹⁹ Furthermore, another miRNA belonging to the miR-200 family has its expression altered in GBM. This miRNA is miR-205 (miR-205), which is downregulated in GBM cells. miR205 is key to maintain under control the expression of ZEB1 protein and, by extension, the activation of the epithelial to mesenchymal transition (EMT) program on cancerous cells. ZEB1 and EMT facilitates cells to enter in a metastasis-initiating cell state and favour the creation of cancer stem cells.

11PS04 has demonstrated its ability to enhance the susceptibility to TMZ in GBM cells and glioma stem cells.³⁵¹ In our studies, we saw a very similar trend to that shown by Aguado and co-workers (Figure 88A). TMZ did not show significant effect when dosed alone at concentrations below 100 μ M (Figure 92 and 93C). At 175 μ M, TMZ presented low antiproliferative effects that were potentiated only at concentrations higher than 250 μ M (Figure 92). Cell viability reduction was higher after 48h than 72 h of treatment with TMZ (Figure 88A vs Figure 92), which has been previously observed,²⁸⁶ due to the effect of the alkyltransferase MGMT enzyme. After three days of TMZ treatment, cells have had more time to express higher amounts of the enzyme that counteract the alkylating activity of the dosed TMZ. However, many tumours with low levels of MGMT are chemoresistant to alkylating agents.³⁵³ TMZ induces apoptosis on cells by increasing the activity of caspase 3. However, due to the characteristic overexpression of miR-21 in glioma cells, caspase 3 activity is downregulated,^{340,354} thus, protecting tumoral cells against TMZ.

In recent years, the role of GnRH receptor has gained interest as potential target for hormonal therapy because of its overexpression in cancer tissues, such as GBM. TPGnRH, a stable lipidized analogue of the GnRH neuropeptide has been shown to cross the BBB, be stable to plasma, brain and liver enzymes, while able to bind and activate the GNRH-R exerting antiproliferative effects. TPGnRH causes cell cycle arrest at G₂/M phase and apoptosis, while being able to reduce the intracellular cAMP levels, which can trigger additional mechanism for cellular apoptosis. TPGnRH is able to self-

assemble in peptide nanofibers that can entrap antitumoral agents that are brain impermeable or labile in certain physiological media and enable their delivery across the BBB and to cells overexpressing the GnRH-R. Thus, TPGnRH can be utilised as carrier for labile drugs such as TMZ that is unstable under basic conditions or 11PS04 that possesses a lactone ring that can open under both strong basic and acidic conditions before reaching the target cell. Therefore, entrapping 11PS04 and TMZ in TPGnRH nanofibers can result in their enhanced chemical stability as well as elicit a treatment protocol that is effective and ideally targeted to only GBM cells.

Prior to evaluating the three compounds together, the effect of combining 4 days of 11PS04 and 2 days of TMZ treatment was assessed. The antiproliferative effect of TMZ was enhanced in the presence of 5 μ M of 11PS04 (Figure 92) especially at therapeutic TMZ concentrations (250 and 500 μ M). We have previously shown that 11PS04 dosed at 5 μ M is able to inhibit miR-21 expression and restores the synthesis of PDCD 4 protein (Figure 87), that result in downregulation of the MAPK/ERK cascade and JUNK/c-Jun/AP-1 pathway (Figure 86). Taken all together, G₂/M phase arrest can be elicited as well as potential cell apoptosis. Thus, 11PS04 can be employed not only as sensitizer (previous work of Aguado *et al.*, Figure 88A) but also as an adjuvant to TMZ therapy to reduce the dose and length of exposure to TMZ needed (Figure 92) as some antiproliferative effects were visible after 4 days of treatment at 5 μ M.

Combining 11PS04 and TMZ with TPGnRH can potentially have many advantages. Loading 11PS04 into TPGnRH nanofibers can enable its permeability across an intact BBB and ensure its targeting to GBM cells reducing potential side effects. Ideally co-formulating with an alkylating agent such as TMZ or doxorubicin could elicit cell sensitisation and reduction in alkylating agent needed to elicit antiproliferative effects required.

The results obtained in this chapter will be discussed by type of treatment or combination treatment employed. This way, we will be focus on TPGnRH alone, then TPGnRH with TMZ followed by TPGnRH with 11PS04. Finally, treatments comprising the three components together (TPGnRH + TMZ + 11PS04) will be examined.

TPGnRH

In order to figure out which concentration of nanofibers would be optimal for combining it with TMZ and 11PS04, TPGnRH was assessed in a set of concentrations that ranged from 7 μ M to 70 μ M (the triple-treatment assay, Figure 93).

The morphological characteristics and secondary structure of TPGnRH in aqueous dispersion has been previously elucidated by Dr. Lalatsa's group. TPGnRH self-assembles in nanofibers depending in micromolar concentrations and when formulated in a concentration above the critical aggregate concentration for formation of nanofibers (> 189 μ M) there is a hysteresis upon dilution as nanofiber structure is maintained.³⁵⁵ Nanofibers possess a poly(proline)-helix type II (PPII) structure as demonstrated by circular dichroism and XRD studies.³⁵⁵ TPGnRH has enhanced stability in plasma, brain, liver and U87MG cell homogenates which allow dosing every two days. When U87MG cells

were treated with three doses every two days, the IC_{50} was in the micromolar range under normoxia ($10.31 \pm 3.46 \mu\text{M}$) and hypoxia ($16.56 \pm 5.92 \mu\text{M}$)³¹⁴ and nanofibers were able to elicit G2/M phase arrest. Additionally, TPGnRH nanofibers can result in early and late apoptosis and reduce forskolin induced cAMP levels in a concentration dependent manner, which indicates that antiproliferation results from activation of G_{ai} protein inhibiting the cAMP dependent pathway. AFM studies have also indicated the specificity of binding of TPGnRH to cells overexpressing the GnRH receptor. Other mechanisms might explain the apoptotic effect of TPGnRH at low concentrations of TPGnRH, when no alteration of the cell cycle is observed, but there is cell death. Some genes involved in apoptosis, such as cFLAR, caspase-8 or FAS protein, showed activity after 72 to 96 h after the treatment with a GnRH-analogue.³¹⁵ It is possible that TPGnRH might trigger the same signalling pathways, although more transcriptomic studies are needed to confirm this theory.

TPGnRH showed significant antiproliferative effects even after only two days of treatment in a concentration-dependent manner (Figure 93A). The antiproliferative effect is cumulative and further doses reduce concentration necessary for reducing cell viability. Results obtained are in accordance to previous studies produced in our group.

TPGnRH and TMZ

In order to understand if an additive effect exists between TMZ and TPGnRH, the combination of these two components have been tested in two modalities: 1) TMZ 100 μM + TPGnRH at increasing concentrations (5 points, range: 7 – 70 μM); 2) TMZ 250 μM + TPGnRH at increasing concentrations as before.

The alkylating agent showed a lack of effect when tested alone at 100 μM and when combined with any of the TPGnRH concentrations (Figure 93C). However, when TMZ was tested at 250 μM , it improved the effect of TPGnRH on glioblastoma cells especially when low doses of TPGnRH are used. TMZ can induce apoptosis by activating caspase-3, therefore, the additive effect observed could be attributed to the combined effect of caspases 3 and 8 (hypothesised for TPGnRH at low concentrations), but further studies are needed to confirm this. However, when 175 μM of TMZ were tested with TPGnRH (Figure 95C) an improved TPGnRH antiproliferative effect was possible. This means that 175 μM of TMZ could be the minimum dose needed for showing an additive effect combined with the nanofibers for *in vitro* treatment of U87 MG. However, we were unable to load TMZ in concentrations that showed additive efficacy (250 μM). Formulation 2 loaded with 20 μM of TMZ lacked significant effects on cells which was expected.

TPGnRH + 11PS04

11PS04 is a pyran-oxazolone that can modify the expression of two key miRNAs in glioblastoma: miR-21 and miR-205. Doses of 5 μM effectively reduce miR-21 levels when U87MG cells were exposed for 24 h. In the case of miR-205, qPCR showed restoration of “normal” levels after the cell exposure to 11PS04 for the same period of time (Figure 87). Inhibition of miR-21 expression can have multiple

effects on the post-transcriptional level. The main targeted mRNAs of miR-21 are shown in Figure 86. Previous transcriptional studies have been done regarding the expression of tumour suppressor protein PDCD 4 after subjecting glioblastoma cells to 11PS04 (Figure 87).³⁵¹ The inhibition of miR-21 caused by 11PS04 did increase the levels of PDCD 4 protein. Therefore, the phenotypic response due to this change in PDCD 4 expression cell was expected to be visible. For example, inhibition of Bcl-2 and XIAP expression by PDCD 4, producing anti-apoptotic and antiproliferative effect, or the upregulation of E-cadherin, reducing the EMT process and therefore the invasiveness capabilities of glioblastoma cells.

Co-treatment of 11PS04 for 2 days did not show any additive effect, but when treatment was prolonged to 4 concomitant days this resulted in an additive effect (Figure 93C). The effect of 11PS04 on cells seemed to be highly time-dependent, and 2 days were not enough to enhance TPGnRH antiproliferative effect. The effect of 11PS04 on miR21 and miR-205-levels became only visible after 24 h of exposure. After 24 hours we can assume a change in the machinery inside the cells (from transcriptional to translational levels). mRNAs directly targeted by miR-21 started to recover their function and began to translate into proteins. This process can take a variable length of time. For example, the MMPs inhibitor TIMP3 is upregulated after miR-21 inhibition. TIMP3 protein can induce apoptosis both by reducing MMPs activity and activating caspases. Zhou and co-workers needed 3 days to measure this apoptotic effect on U87MG transfected cells with an antisense oligonucleotide of miR-21.³⁵⁴ It has been observed in glioblastoma cell lines that after reducing miR-21 expression, the level of RECK (another inhibitor of MMPs), increased consistently. MMPs reduced activity and tumour progression reduction was obtained 4 days after the addition of the studied anti-miR-21.³³⁰

This bring us back to 11PS04's phenotypic effect. Knowing that 24 hours are needed for effective miR-21 inhibition, and assuming another 24 to 72 hours for signalling pathways modifications to become noticeable, we are likely to see the effect of 11PS04 after 72h than after 48 h of treatment. This can explain why 11PS04 do not add to TPGnRH antiproliferative effect in the first 48 h but does after 96 h. To confirm this hypothesis, a time course studio with protein quantification of a direct miRNA target such as TIMP3 would have to be carried out (Western Blot technique). Measurement of protein levels every 24 h would allow us to stablish the time point at which miR-21 inhibition becomes effective.

We cannot ignore either the fact that 11PS04 is dosed twice. The cytotoxicity displayed by 11PS04 alone after 4 days of treatment was twice as potent as after 2 days. Repeated doses of 11PS04 could also explain the additive effect. The second dose of 11PS04 might be reducing further the miR-21 expression, bringing miR-21 levels below a minimum threshold of active oligonucleotides that are unable to interfering with mRNAs' transcription. In this respect, we evaluated the effect of doubling the dose of 11PS04 to 10 μ M (which is equal to the total dose administered on the previous assay). Cell viability was measured the presence and absence of TPGnRH, applying the same protocol of addition for treatments as previously done.

After 48 h since the addition of 11PS04, the cytotoxic effect of 11PS04 increased too, (from ~15% cell viability reduction, Figure 95B to ~40% on Figure 96). 11PS04 did not improved the cytotoxic activity

of TPGnRH after two days in co-treatment, even if the sensitizer was more concentrated. By contrast, after 4 days in co-treatment, the additive effect was evident and cell viability dropped notably below 10% ($7 \pm 2\%$, Figure 96). These results reinforced our first hypothesis that the time of exposure (and not the concentration of sensitizer) is responsible for the antiproliferative effects observed in these experiments. The “delayed” additive effect between the nanofibers and the sensitizer in co-treatment could be due to the time required by the cell machinery to process and express the changes derived from miR-21 inhibition.

Thus, TPGnRH and 11PS04 add their apoptotic and antiproliferative effects, the former by lowering intracellular cAMP levels (via the GnRH-R activating G α i protein-cAMP signalling pathway) and the later by increasing PDCD4 expression (via miR-21 inhibition).^{314,351} Inhibition of miR-21 in U87 MG cells upregulate the transcripts levels of RECK, TIMP3, PTEN and Bax proteins, and recover the apoptotic activity of caspase 9 and 3 (Figure 86).^{330,340,354,356} 11PS04 may help to recover the activity of these antitumoral factors via inhibiting miR21. Any of the above mechanisms could be additive to TPGnRH antiproliferative and apoptotic effect observed (Figure 98). TPGnRH-11PS04 combination appears as a very attractive strategy for GBM treatment. However, more transcriptomic and proteomic studies are needed to fully understand 11PS04 mechanism of action for miR-21 inhibition and its effect at the post-transcriptional level.

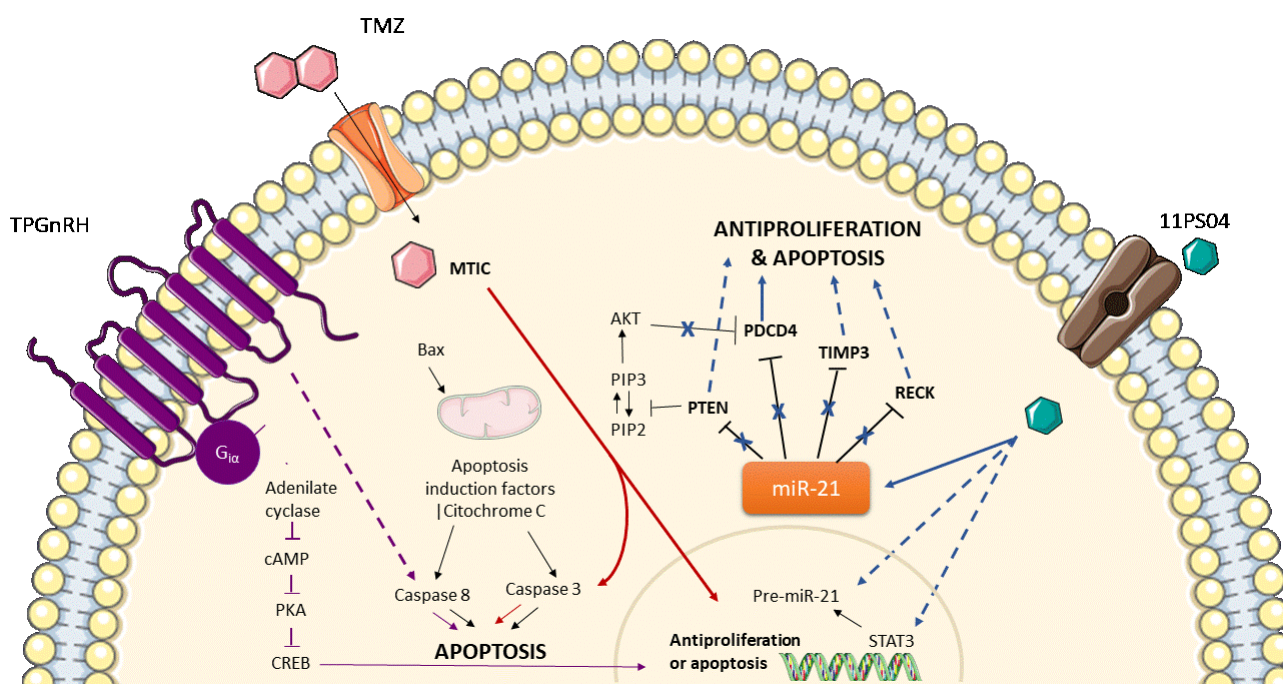


Figure 98 - Molecular pathways induced by TPGnRH-11PS04 and TMZ treatment(s). 11PS04, TPGnRH and TMZ combine their effect upon their interaction with the GnRH-R (in the case of TPGnRH and 11PS04-loaded peptide), or with channel proteins in the cellular membrane. TPGnRH effects are coloured in deep purple. TPGnRH binds to GnRH-R, which is coupled to a $G_{\alpha i}$ protein, and inhibits the adenylate cyclase provoking a decrease in cAMP levels with subsequent antiproliferation and apoptosis. Dashed purple arrows indicate the hypothesised mechanism of action of TPGnRH at low concentrations. TMZ hydrolyses and forms MTIC which alkylates guanine bases of DNA and activates caspase-3 for a global apoptotic effect. TMZ mechanism is indicated by red arrows. 11PS04 reduces the intracellular levels of miR-21 and increases PDCD4 expression upon miR-21 downregulation. Whether if this effect is due to the inhibition of the pro-transcription factor STAT3 or by direct interaction with pre-miR-21 it has not been elucidated yet. Signalling pathways that can be benefited indirectly by miR-21 downregulation after 11PS04 treatment are indicated in blue dashed arrows. Key: cAMP cyclic AMP, PKA protein kinase A, CREB, cAMP response element binding; RECK, reversion-induced cysteine-rich protein with Kazal motifs; TIMP3, tissue inhibitor of metalloproteinases 3; PDCD 4, programmed cell death protein 4; PTEN, phosphatase and tensin homologue; Akt, Protein kinase B; PIP2, phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate, PIP3. Picture made with Servier Medical Arts.

TPGnRH + TMZ + 11PS04

TPGnRH + TMZ + 11PS04 were additive at high concentrations of TMZ (250 μ M, Figure 93D). The triple-treatment assay confirmed that it is possible to combine the 3 compounds to enhance antiproliferative effects with lower concentrations of each therapeutic. TPGnRH doses can be readjusted and reduced ~5-fold if combined with TMZ and 11PS04 without reducing the antiproliferative effect on cells.

TMZ (175 μ M) did not show additive effect with 11PS04, who masked the apoptotic activity of the alkylating agent. However, it did improve TPGnRH effect, which could be explained by the activation of caspase 3 by both compounds. In this experiment, the effect on cells we treated with the 3 components was mainly driven by the effect of TPGnRH and 11PS04. 11PS04-loaded nanofibers alone were as effective as their combination with 175 μ M of TMZ, with cell viability values below 30 % (Figure 95C). Thus, we can conclude that TMZ is needed above 175 μ M so the triple-treatment can be effective. The possibility of adding TMZ externally at different concentrations allows a wider variety of combinations of treatments, using TPGnRH in combination with 11PS04. Indeed, TPGnRH combined with 5 μ M of 11PS04 and TMZ ($\geq$ 175 μ M) could potentially serve as fruitful clinical strategy for the treatment of glioblastoma.

3.4 CONCLUSIONS

Our knowledge of GBM grows as new insights of its specific biomarkers and carcinogenic signalling pathways are discovered. In this work, we utilized two powerful agents developed in our research team, named 11PS04 and TPGnRH, whose combined mechanism of action proved to be able to tackle GBM by targeting different but related anticancer pathways. 11PS04 and TMZ-11PS04 loaded TPGnRH nanofibers were successfully formulated in two different preparations. The formulation with the incorporation of 11PS04 and TMZ together did not lead to an efficient entrapment of high doses of TMZ and therefore its cytotoxic effect was reduced. By contrast, 11PS04-loaded nanofibers showed high antiproliferative activity with respect to glioblastoma cells. Furthermore, 11PS04 was also assessed at higher concentrations, exhibiting almost total inhibition of cell growth. These findings demonstrated the benefits of a combined therapy, and opened up a new potential treatment to be used side by side with existing approved antitumoral drugs in the fight against brain cancer.

3.5 MATERIALS AND METHODS

3.5.1 Materials and Equipment

All reagents employed as starting materials were purchased from commercial suppliers with high purity and were used without further purification (Table 20). Solvents were obtained from Sigma-Aldrich, Acros and Scharlab and were employed straightforward.

Table 21 - Summary table of reagents utilised

Chemical Name	Abbreviation	Purity %	Company
3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide	MTT	97.0	Invitrogen
Arginine	Arg	PS	Novabiochem
Dimethyl Sulfoxide	DMSO	≥99.9	Sigma Aldrich
Dulbecco's Modified Eagle Medium	DMEM	CC	Gibco
Fetal Bovine Serum	FBS	CC	Gibco
Glutamic acid	Glu	PS	Novabiochem
Glycine	Gly	PS	AAPTEC
Histidine	His	PS	Sigma
Leucine	Leu	PS	Novabiochem
Liquified Phenol	-	≥89.0%	Sigma-Aldrich
Ninhydrin	-	≥95.0%	Sigma-Aldrich
N-Methylmorpholine	NMM	99%	Sigma-Aldrich
Non-Essential Amino Acids	NEA	CC	Gibco
O-(Benzotriazol-1-yl)-N,N, N, N'- tetramethyluronium hexafluorophosphate	HBTU	PS	Novabiochem
Palmitic acid N-hydroxysuccinimide ester	PANS	≥ 98	Sigma-Aldrich
Penicillin-Streptomycin	Pen-Strep	CC	Gibco
Phosphate-Buffered Saline	PBS	CC	Gibco
Piperidine	-	99.0	Sigma-Aldrich
Potassium cyanide	KCN	≥99.0%	Sigma-Aldrich
Proline	Proline	PS	Novabiochem®
Rink Amide MBHA Resin (200 µM, 0.59 mmol/g	-	PS	Novabiochem
Serine	Ser	PS	Novabiochem
Temozolomide	TMZ	≥ 98	Sigma Aldrich
Tryptophan	Trp	PS	Novabiochem
Trypan Blue Solution (0.4%)	-	CC	Invitrogen
TrypLE Enzyme 0.25%	Trypsin	CC	Gibco
Tyrosine	Tyr	PS	Novabiochem

Key: cell culture grade, (CC); Peptide Synthesis (PS)

Reactions were monitored by Thin Layer Chromatography (TLC) and spots were visualized by an UV lamp at 254 and 356 nm. In certain occasions, a solution of vanillin or phosphomolybdic acid, with subsequent heating, was used for better traceability of the reaction course. If the crude product was not pure enough, the purification of the synthesised compounds was performed by flash column chromatography with Silica Gel Merck-60 (230-400 mesh).

Peptide synthesis: Resin, Fmoc amino acids and solvents were purchased from Merk (Novabiochem®) and AAPTEC and used without further purification. The peptide was synthesised using a custom-made fritted reaction vessel. Coupling completion was monitored by the Kaiser test.

NMR analysis

Nuclear magnetic resonance (NMR) spectra were recorded in deuterated solvents on Bruker AVANCE-300, Varian INOVA -300 Varian INOVA -400 and Varian INOVA-500 spectrometers. ¹³C-NMR were registered at 75 MHz with complete proton decoupling. The chemical shifts measured are reported in δ (ppm) and the residual signal of the solvent was used as the internal calibration standard. The multiplicity of the signals is reported as follows: s = singlet, d = doublet, t = triplet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet, ddt = doublet of doublet of triplet, br = broad signal. The coupling constant *J* is reported in hertz (Hz).

LC-MS analysis

HPLC-MS analyses were performed on a Waters (2695 HPLC system) apparatus, equipped with a quaternary pump and photodiode array detector, using a Sunfire™ column (C18, 3.5 μ m, 4.6 $\times$ 50 mm) with a solvent gradient of 0.08% formic acid in acetonitrile and 0.01% formic acid. The solvent gradient is specified for each compound in the in the following section .The molecular weight was determined in a quadrupole spectrometer coupled to the HPLC system and with positive electrospray ionization (ES⁺)

TPGnRH peptide formulations were analysed on an Agilent 1100 series apparatus, equipped with a quaternary pump and photodiode array detector (Agilent Technologies, Cheadle, UK). Peptide was eluted using a Phenomenex® column (C18, 100 $\times$ 4.6 mm, 5 μ m) using a gradient elution method with mobile phases consisting in 0.1% (v/v) TFA in H₂O and 0.08% (v/v) TFA in ACN . Peaks were detected at 220 nm. The retention time (RT) of the peak corresponding to each product is reported in minutes.

Elemental analysis and optical rotation analysis

Elemental analysis (EA) was performed in a Heareus CHN-O-RAPID analyser measuring the percentage of Carbon (C), Nitrogen (N), Sulphur (S) and Hydrogen (H).

Optical rotation values were measured on a JASCO P-2000 Digital Polarimeter at the concentration indicated in each case (c 1 corresponds to 10 mg/mL).

Cell culture

Cell culture reagents were obtained from Gibco and Thermo Fisher Scientific (Paisley, UK). All plastics for the cell culture were obtained from Sarstedt Ltd. (Leicester, UK).

3.5.2 Experimental procedures for the synthesis of 11PS04³⁵¹

(*S,Z*)-*tert*-Butyl 4-(3-methoxy-3-oxoprop-1-en-1-yl)-2,2-dimethyloxazolidine-3-carboxylate (2)

To a stirred solution of methyl 2-(bis(2,2,2-trifluoroethoxy)phosphoryl)acetate (1.3 mL, 6.1 mmol) in anhydrous THF (180 mL) under argon at room temperature, a solution of 18-crown-6 (5.7 g, 21.5 mmol) in anhydrous THF (4 mL) was added. The mixture was cooled to -78 °C (cooling bath with the mixture dry ice/acetone) and stirred for 5 minutes prior to adding a 0.5 M solution of potassium bis(trimethylsilyl)amide in toluene (12.3 mL, 6.1 mmol) via a syringe. Stirring was maintained for 15 minutes at this temperature. Next, a solution of (*R*)-*tert*-butyl 4-formyl-2,2-dimethyloxazolidine-3-carboxylate 1 (2.8 g, 5.6 mmol) in anhydrous THF (13 mL) was added. The mixture was stirred for 1 hour at -78 °C and then diluted with a saturated aqueous solution of NH₄Cl (15 mL). This mixture was extracted with Et₂O (3x15 mL), washed with brine (2 x 20 mL), dried over anhydrous MgSO₄, filtered and evaporated *in vacuo*. The crude mixture was subjected to chromatography on silica gel to obtain 1.4 g (90% yield) of pure *Z*-alkene, (*S,Z*)-*tert*-butyl 4-(3-methoxy-3-oxoprop-1-en-1-yl)-2,2-dimethyloxazolidine-3-carboxylate, 2. **¹H NMR** (300 MHz, CDCl₃) δ(ppm): 6.40 - 6.17 (m, 1H), 5.82 (d, 1H, *J* = 11.0 Hz), 5.38 (m, 1H), 4.32-4.17 (m, 1H), 3.76 (m, 1H), 3.70 (s, 3H), 1.61 (s, 3H, *J* = 6.0 Hz), 1.49 (s, 3H, *J* = 13.3 Hz), 1.38 (s, 9H); **¹³C NMR** (75 MHz, CDCl₃) δ (ppm): 166.2, 152.3, 95.3, 80.3, 125.6, 122.5, 68.2, 59.2, 51.9, 28.7, 27.0, 26.8 ppm; **HPLC- MS (ES⁺)**: Gradient MeCN/H₂O 40:60 to 100:0 (5 min), RT: 3.98 min, [M+H]⁺ = 286.3; **EA** calculated for C₁₄H₂₃NO₅: C 58.93, H 8.12, N 4.91 obtained C 59.04, H 8.22, N 5.06; **Optical rotation**: [α]_D = -28 (c = 1, CHCl₃).

(*S*)-*tert*-Butyl (6-oxo-3,6-dihydro-2*H*-pyran-3-yl)carbamate (3)

Trifluoroacetic acid (TFA: 0.5 mL, 6 mmol) was added to a stirred solution of (*S,Z*)-*tert*-butyl 4-(3-methoxy-3-oxoprop-1-en-1-yl)-2,2-dimethyloxazolidine-3-carboxylate via a syringe, 2, (850 mg, 2.95 mmol) in anhydrous CH₂Cl₂ (35 mL) under an argon atmosphere at room temperature. The mixture was stirred overnight and then diluted with dichloromethane, and this mixture was washed with a saturated aqueous solution of NaHCO₃ until a basic pH was obtained. The organic phase was washed with brine, dried over anhydrous MgSO₄, filtered and evaporated *in vacuo* to obtain 508mg of lactone 3, (*S*)-*tert*-Butyl (6-oxo-3,6-dihydro-2*H*-pyran-3-yl)carbamate (80% yield). **¹H NMR** (300 MHz, CDCl₃) δ (ppm): 6.88 (dd, 1H, *J* = 9.5, 4.6 Hz), 6.09 (d, 1H, *J* = 9.8, 0.9 Hz), 4.77 (broad s, 1H), 4.53 - 4.32 (m, 3H), 1.45 (s, 9H); **¹³C NMR** (75 MHz, CDCl₃) δ (ppm): 162.5, 155.7, 144.3, 122.9, 79.2, 70.5, 42.7, 28.2. **HPLC-MS (ES⁺)**: Gradient MeCN/H₂O 10:90 to 100:0 (5 min), RT: 4.7 min, [M+23]⁺ = 236. **EA** calculated for C₁₀H₁₅NO₄: C 56.33, H 7.09, N 6.57 obtained C 56.32, H 7.10, N 6.61; **Optical rotation**: [α]_D = +113 (c = 1.15, CHCl₃) Lit . [α]_D = +105 (c = 1.06, CHCl₃).

(S)-3-Propoxy-N-(6-oxo-3,6-dihydro-2H-pyran-3-yl)benzamide (4)

A solution of lactone **3** (200 mg, 0.9 mmol) in anhydrous dichloromethane (8 mL) was cooled to 0 °C in an argon atmosphere and TFA (1.52 mL, 19.7 mmol) was then added to the solution at room temperature. The mixture was stirred at 0 °C for 5 minutes, removed from the bath, left to reach room temperature slowly and then stirred for 1 hour. When the reaction was completed, the mixture was diluted with dichloromethane and the solvent evaporated at reduced pressure (x4) in order to remove the remaining TFA. The unprotected lactone was dissolved in anhydrous dichloromethane under argon and at room temperature. Then, *N,N*-diisopropylethylamine (DIPEA; 0.5 mL, 2.8 mmol) was added via a syringe, followed by 3-propoxybenzoic acid (202 mg, 1.1 mmol), EDCI (320 mg, 1.7 mmol) and HOBt (151 mg, 1.1 mmol) in anhydrous THF (9 mL). When the reaction was complete, the mixture was treated with a saturated aqueous solution of NH₄Cl (5 mL) and extracted with EtOAc (3 x 10 mL). The organic layer was washed with saturated solution of NaCl (2 x 10 mL), dried over MgSO₄, filtered, and concentrated to dryness. The crude product was purified by silica gel chromatography (hexane:EtOAc 5:1 to 3:1) to obtain the desired compound, **4**, as a white solid (186mg, 72% yield). **¹H NMR** (400 MHz, CDCl₃) δ (ppm): 7.70 (d, *J* = 8.3 Hz, 1H), 7.41 – 7.35 (m, 2H), 7.25 (t, *J* = 8.1 Hz, 1H), 7.00 (ddd, *J* = 8.2, 2.5, 1.1 Hz, 1H), 6.92 (ddd, *J* = 9.7, 5.3, 1.2 Hz, 1H), 6.05 (dd, *J* = 9.7, 1.1 Hz, 1H), 5.01 – 4.88 (m, 1H), 4.51 (dd, *J* = 11.9, 4.1 Hz, 1H), 4.44 (ddd, *J* = 11.9, 3.3, 1.3 Hz, 1H), 3.87 (t, *J* = 6.6 Hz, 2H), 1.75 (q, *J* = 6.9 Hz, 2H), 0.98 (t, *J* = 7.4 Hz, 3H). **¹³C NMR** (101 MHz, CDCl₃) δ (ppm): 167.4, 162.8, 159.8, 143.9, 134.8, 129.9, 123.6, 119.1, 119.0, 113.6, 70.7, 70.1, 42.1, 22.7, 10.6. **HPLC-MS** (ES⁺): Gradient MeCN/H₂O 10:90 to 100:0 (5 min), RT: 4.2 min, [M+1]⁺ = 276. **E.A.** calculated for C₁₅H₁₇NO₄: C 65.44, H 6.22, N 5.09 found C 65.18, H 6.09, N 4.90.

(3aR,7aS)-2-(3-Propoxyphenyl)-7,7a-dihydro-3aH-pyrano[3,4-d]oxazol-6(4H)-one (5; 11PS04)

A solution of compound **4** (155mg, 0.56mmol) was prepared in anhydrous dichloromethane under argon and at 0 °C on ice. Triflic acid (TfOH, 0.25ml, 2.77mmol) was added to this via a syringe and the mixture was stirred at room temperature for 2 hours. When the reaction was complete, the mixture was diluted with dichloromethane, the pH of the aqueous phase was adjusted with a saturated aqueous solution of K₂CO₃ above pH 8, and it was extracted with dichloromethane. The organic extract was dried with anhydrous MgSO₄ and filtered before removal of the solvent. The crude product was purified on a silica gel column eluting with mixtures of hexane/AcOEt to obtain the title compound, **5d**, as a white solid (139mg, 90% yield). **¹H RMN** (300 MHz, CDCl₃) δ (ppm): 7.47 (m, 1H), 7.41 (m, 1H), 7.35 – 7.25 (m, 1H), 7.03 (ddd, *J* = 8.3, 2.7, 1.0 Hz, 1H), 5.24 (ddd, *J* = 9.9, 4.3, 2.7 Hz, 1H), 4.64 (ddd, *J* = 10.0, 3.1, 1.9 Hz, 1H), 4.59 (dd, *J* = 12.3, 2.0 Hz, 1H), 4.42 (dd, *J* = 12.4, 3.2 Hz, 1H), 3.96 (t, *J* = 8.0, 2H), 3.10 (dd, *J* = 16.2, 2.8 Hz, 1H), 2.80 (dd, *J* = 16.2, 4.2 Hz, 1H), 1.82 (m, 2H), 1.06 (t, *J* = 8.0, 3H). **¹³C RMN** (75 MHz, CDCl₃) δ (ppm): 168.8, 165.4, 159.4, 129.6, 127.9, 121.0, 119.4, 114.2, 75.3, 70.0, 68.9, 64.5, 34.5, 22.7, 10.6. **HPLC-MS** (ES⁺): Gradient MeCN/H₂O 10:90 to 100:0 (5 min), RT: 4.3 min, [M+H]⁺=277.3. **E.A.** calculated for C₁₅H₁₇NO₄: C, 65.44%; H, 6.22%; N, 5.09%; obtained: C, 65.28%; H, 6.27%; N, 5.23%. **Optical rotation**: [α]_D = -72 (c = 1, CHCl₃).

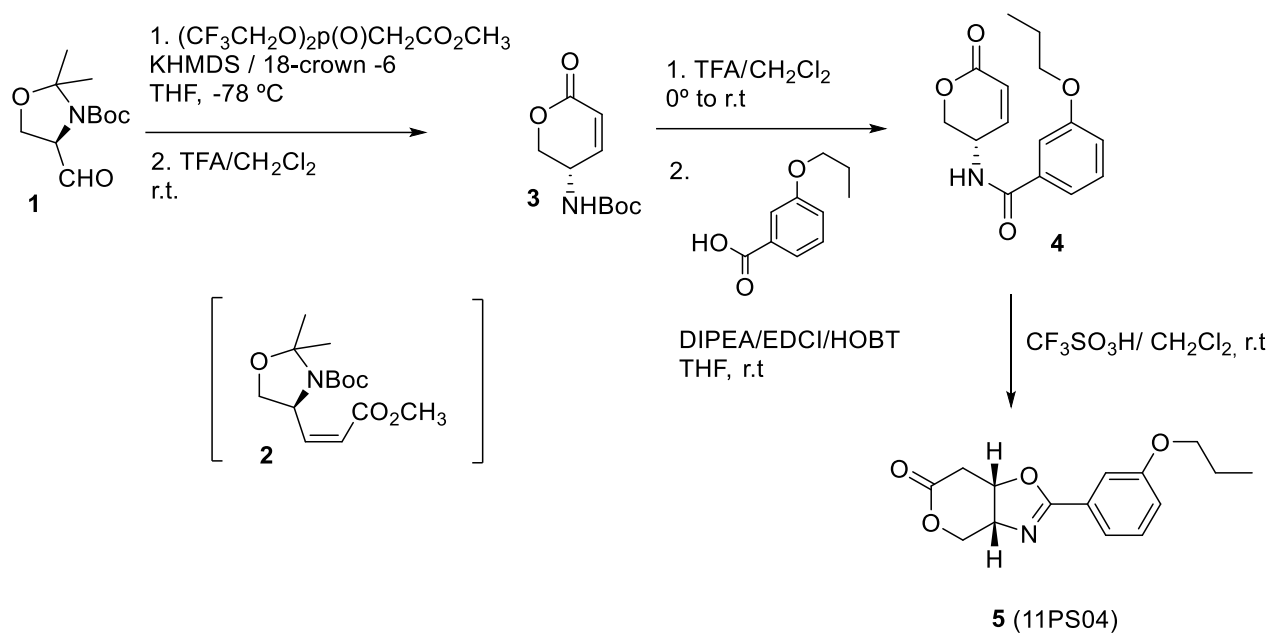


Figure 99 - Schematic representation of the synthesis of 11PS04. Adapted from reference ³⁵¹.

3.5.3 Solid Phase Peptide Synthesis (SPPS)

Synthesis of Tyrosyl-Palmitoyl-Gonadotropin Releasing Hormone (TPGnRH)

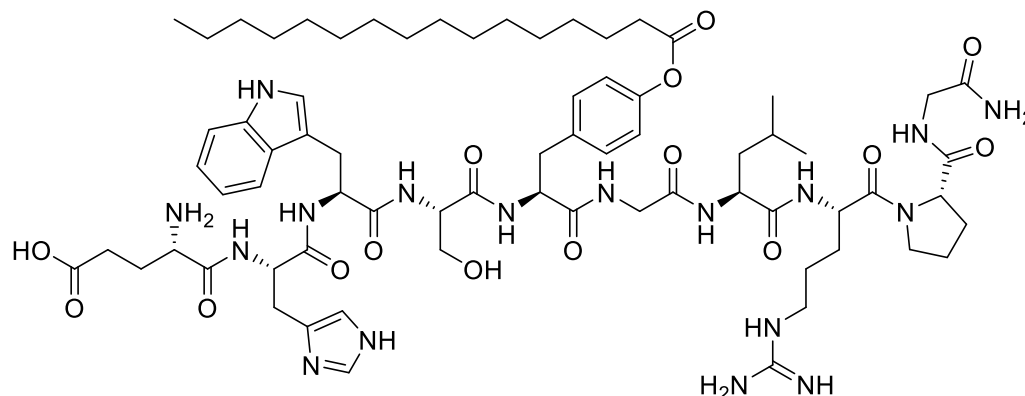


Figure 100 - Chemical structure of TPGnRH peptide (Glu-His-Trp-Ser-Tyr⁵-(O-palmitoyl)-Gly-Leu-Arg-Pro-Gly-CO-NH₂).

Solid phase peptide synthesis of TPGnRH

The peptide synthesis of TPGnRH was performed in solid phase, starting from 0.1695g of Rink amide MBHA (substitution 0.59 mmol/g) at a 0.1 mmol scale. General procedure is shown on Figure 101. Solid phase peptide synthesis was undertaken using an Fmoc/tBu orthogonally protected strategy based on the activation of the carboxyl group of a Fmoc-protected amino acid, **A**, with a coupling agent such as HBTU, **B**, and its addition over a growing chain of amino acids linked to a resin, **C**. The last amino acid of the chain has been deprotected: the α -NH₂ group is ready to react with the pre-activated carboxyl group of the next amino acid, which is added to the chain, obtaining **D** (Figure 101). Amino acids were protected with acid-labile sidechain protecting groups, which can be removed only under highly acidic conditions but remain stable under α -amino N-Fmoc deprotection (which is base-labile) needed for propagation of the peptide chain.

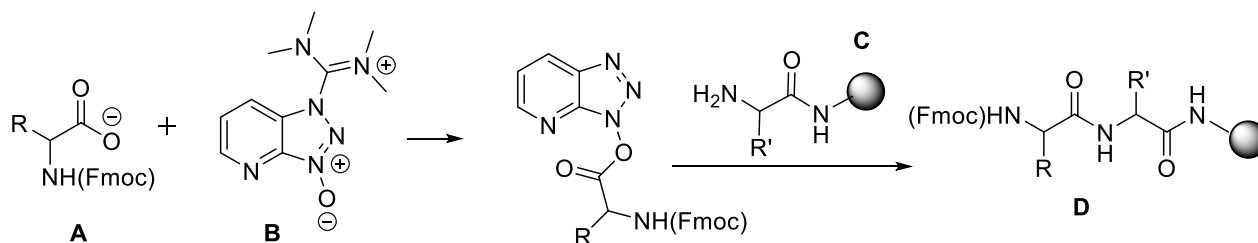


Figure 101 - Schematic representation Fmoc/tBu method for peptide synthesis.

Swelling. After being weighted and transferred into a clean and dry reaction vessel, the resin was swelled in DMF for 45-60 minutes under continuous shaking prior to starting the synthesis. The Fmoc protected Rink amide was treated with 20 % piperidine in DMF for 10 minutes, then beads were rinse with DMF, filtered and drain under vacuum. This process was repeated twice.

Coupling. The corresponding Fmoc-protected amino acid (4.2 eq.) and the coupling agent, HBTU (4 eq.), were weighted separately in SPSS DMF resistant tubes. To activate the N α -protected amino acid, HBTU was added in solid and both were dissolved with 3 mL of 4.45% v/v NMM in DMF. The addition of NMM ensured the basic environment needed to initiate the reaction. Some amino acids such as Fmoc-Arg-Pbf-OH that presented lower solubility required that the mixture was vortexed for few seconds prior to pouring it into the vessel containing the resin. The reaction mixture was left stirring for 30 minutes. In the case of the arginine coupling step, the reaction time was extended to 45 minutes to ensure the complete coupling due to the bulky protecting group present in this amino acid (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine, Arg (Pbf). Couplings were performed twice for each amino acid.

After coupling each amino acid, the Kaiser test was performed³⁵⁷ to confirm the completion of coupling reaction. The Kaiser test is widely used as qualitative proof for the presence of absence of free amino groups. A small amount of resin beads (1-3 mg) were transferred to a filter paper, washed with DCM: MeOH (1:1) to remove the remaining DMF, dried under vacuum and transferred to a small glass tube. Next, one drop of liquified phenol in ethanol (80 w/v), 2 drops of potassium cyanide (0.001M) in pyridine (2% v/v) and 1 drop of ninhydrin ethanolic solution (5% w/v) were added. The suspension was mixed and heated at 120° C for 4-6 minutes, in a heat block (Pierce Reacti-Therm Heating, USA).

A dark blue colouration of resin beads is indicative of the presence of free primary amines while an orange/yellow colour of the beads indicates absence of free primary amines and thus a successful coupling. If coupling was not successful, a third coupling of the amino acid was undertaken prior proceeding to removal of the Fmoc protecting group.

Selective deprotection of Fmoc group. Removal of the Fmoc protecting group was achieved by adding 4 mL of 20% piperidine in DMF to the resin under shaking for 10 minutes. Then, the resin was drained under vacuum, and a second deprotection undertaken. After the second Fmoc-cleavage, the Kaiser test was performed. If deprotection was successful, the resin was rinsed with DMF (8-10 mL) and drained under vacuum. A small quantity of clean DMF (enough to cover the beads) was left while preparing the next coupling reaction.

Lipidation of the tyrosyl hydroxyl group. The esterification of the peptide chain was achieved by the reaction of palmitic acid succinimide ester with the phenolic hydroxyl group of Tyr⁵, as previously described³¹². The process involved two steps: first, the selective acid deprotection of the hydroxy group of tyrosine followed by the palmitoylation with N-hydroxysuccinimide palmitic acid ester.

a. Selective deprotection 2-Chlorotrityl protecting group of Fmoc-Tyr(CITrt)-Gly-Leu-Arg-Pro-Gly-CO-NH-Rink amide resin

The resin was rinsed with DMF and filtered in a glass funnel with filter paper, where it was washed with a mixture DCM: MeOH (1:1). A freshly prepared cleavage solution (20mL) composed of DCM/TFA/TIS [90:5:5 (v/v/v)] was added to the resin beads (10 mL of cleavage mixture per 1g of resin) and allowed to stir in a glass vial for 5 minutes protected from light. Beads were filtered again, and fresh cleavage mixture was added and left to stir for 5 minutes. This process was repeated for a total of 4 times. Removal of the 2-chlorotrityl group was evidenced by a yellow colouration of the cleavage mixture filtrate. After the fourth cleavage the filtrate did not show any colouration indicative of complete removal of the protecting group.

b. Palmitoylation

Immediately after deprotection of the 2-chlorotrityl group of tyrosine, beads were washed with DCM followed by washing with DMF. Beads were transferred in a glass vial with DMF (5-10 mL) and allowed to swell and set over 10-20 minutes. DMF was syringed out leaving only enough DMF to cover the beads by 1mm (1-2 mL). Freshly dissolved palmitic acid N-hydroxysuccinimide ester (0.8 mmol, 8 eq.) was dissolved in 4 mL of DMF and immediately added into the vial containing the beads, followed by 24.2 equivalents (2.425 mmol) of Et₃N. The vial was covered in foil and the reaction mixture was stirred for 24 h at room temperature.

Resin was then left to set, and supernatant was syringed out. Beads were filtered with filter paper and washed by alternating volumes of DCM and DMF. Resin beads were then transferred in the peptide synthesis reaction vessel, washed with DMF and left to swell for 10 minutes prior undertaking the Kaiser test was performed. An orange colour of the sampled beads indicated protection of the reactive groups and SPSS was continued till all amino acids were added as above.

Resin cleavage. Resin was washed with DCM (20-30 mL) and then with DCM: MeOH (20 mL) and left to dry overnight in a pre-weighted glass vial. A freshly prepared mixture of TFA/H₂O/TIS [95:2.5:2.5 (v/v/v)] was added to the resin (10 mL of cleavage mixture per 1g of resin). The reaction mixture was stirred for 4h protected from light. The resin was filtered, and the cleavage solution was collected in a round bottom flask for rota-evaporation under liquid nitrogen for over 4h.

Ether extraction. Cold diethyl ether (-20°, 20-25mL) was slowly added on the crude peptide and a white precipitate was formed. The flask was sealed and kept overnight at -18 °C for maximum peptide precipitation. The suspension was transferred into centrifuge tubes and centrifuged at 1,500 rpm for 5 minutes. Ether was decanted and more fresh cold ether (5-10 mL) was added prior to another cycle of centrifugation. After decanting the supernatant, 3 mL of de-ionized water were added, and the peptide was redispersed. The suspension was snap frozen using liquid nitrogen and lyophilized for at least 24 h.

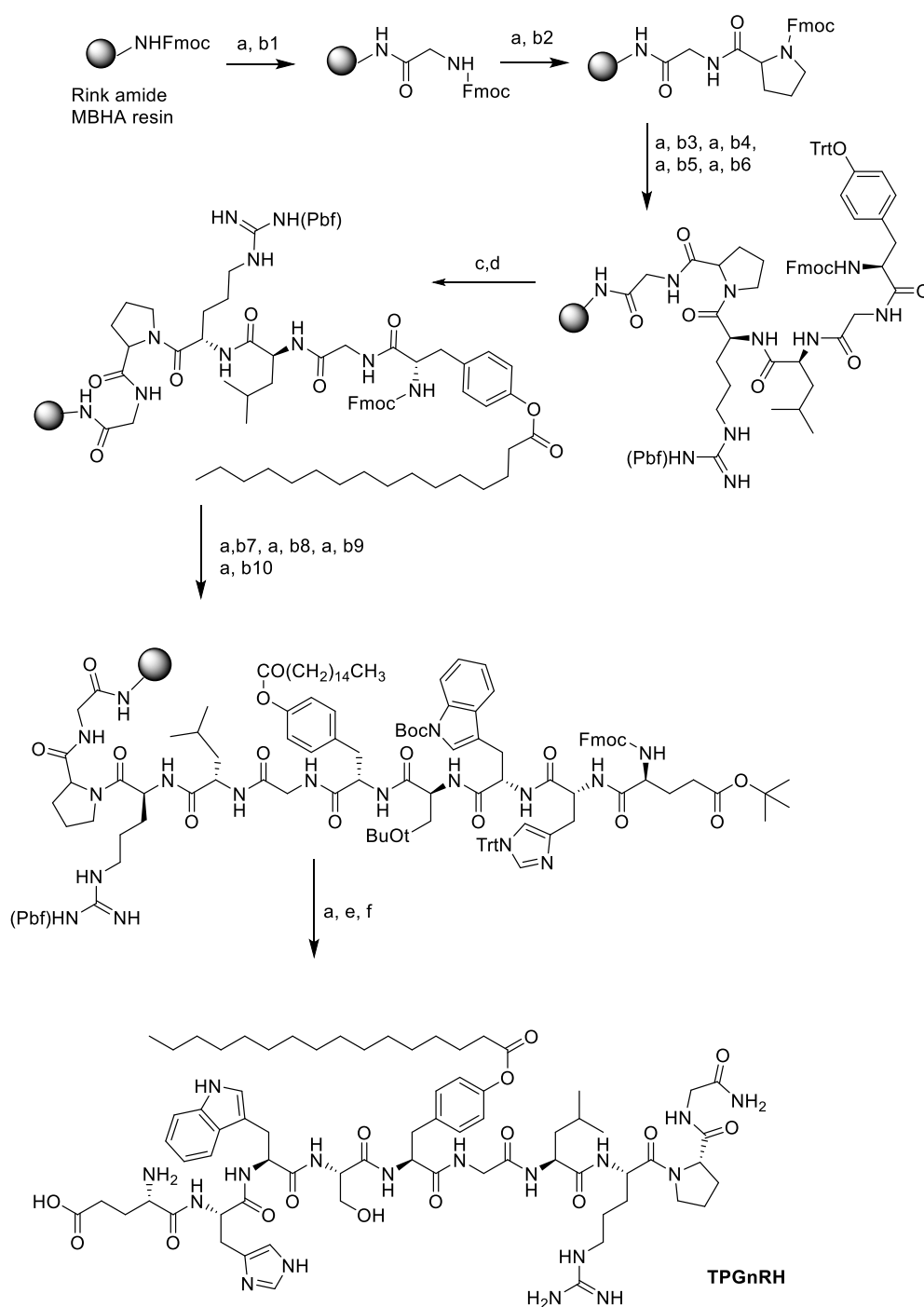


Figure 102 - Schematic SPPS synthesis of tyrosyl-palmitoyl gonadotropin releasing hormone peptide (TPGnRH). **a.** 20% v/v piperidine/DMF, twice. **b1-b10.** Corresponding Fmoc-aa (4.2 eq.), HBTU (4 eq.) dissolved in NMM, 30', twice. **b1** Fmoc-Gly-OH, **b2-** Fmoc-Pro-OH, **b3.** Fmoc-Arg(Pbf)-OH, **b4.** Fmoc-Leu-OH, **b5.** Fmoc-Gly-OH, **b6.** Fmoc-Tyr(2ClTrt)-OH, **b7.** Fmoc-Ser-(tBu)-OH, **b8.** Fmoc-Trp(Boc)-OH, **b9.** Fmoc-His(Trt)-OH, and **b10.** Fmoc-Glu(tBu). **c.** TFA: TIS: DCM (5:5:5 v/v/v), 5' (x4). **d.** Palmitic acid N-hydroxysuccinimide ester (8eq.) and Et₃N (24.2eq) in DMF, 24h **e.** TFA:TIS:H₂O (95:2.5:2.5) for 4 hours, then dry under vacuum. **f.** Precipitation and extraction with cold diethyl ether and freeze drying.

3.5.4 Peptide Characterization

HPLC

Mobile phase consisted of 0.1 % (v/v) TFA in water (eluent A) and 0.08% (v/v) TFA in ACN (eluent B). Flow rate was set at 1.5 mL min⁻¹ at 25°C and the injection volume was 40 µL. Detection of peak by diode array was performed at 220 and 280 nm. OpenLAB software was used to analyse the results (Agilent Technologies, Cheadle, UK). RT: 22.24 minutes.

Table 22 - HPLC gradient method for TPGnRH.

Time (minutes)	0.1% (v/v) TFA in H ₂ O (A)	0.08 % TFA in ACN (B)
0	99	10
5	90	10
15	50	50
18	50	50
28	40	60
33	20	80
38	90	10

Fourier-transform infrared (FTIR) spectroscopy

TPGnRH palmitoylation was confirmed by FTIR spectroscopy. Peaks at 2923 and 2852 cm⁻¹ correspond to the vibration of the palmitoyl moiety, whereas the peak at 1641 cm⁻¹ belongs to the ester linkage between the phenolic tyrosyl residue and palmitoyl moiety. The CO stretch corresponding to the phenolic group of the tyrosine residue appears at 1172 cm⁻¹. At 1633 cm⁻¹ C=O stretch of the amides is observed as well as the peak of the C=C stretch characteristic of aromatic residues at 1515 cm⁻¹.

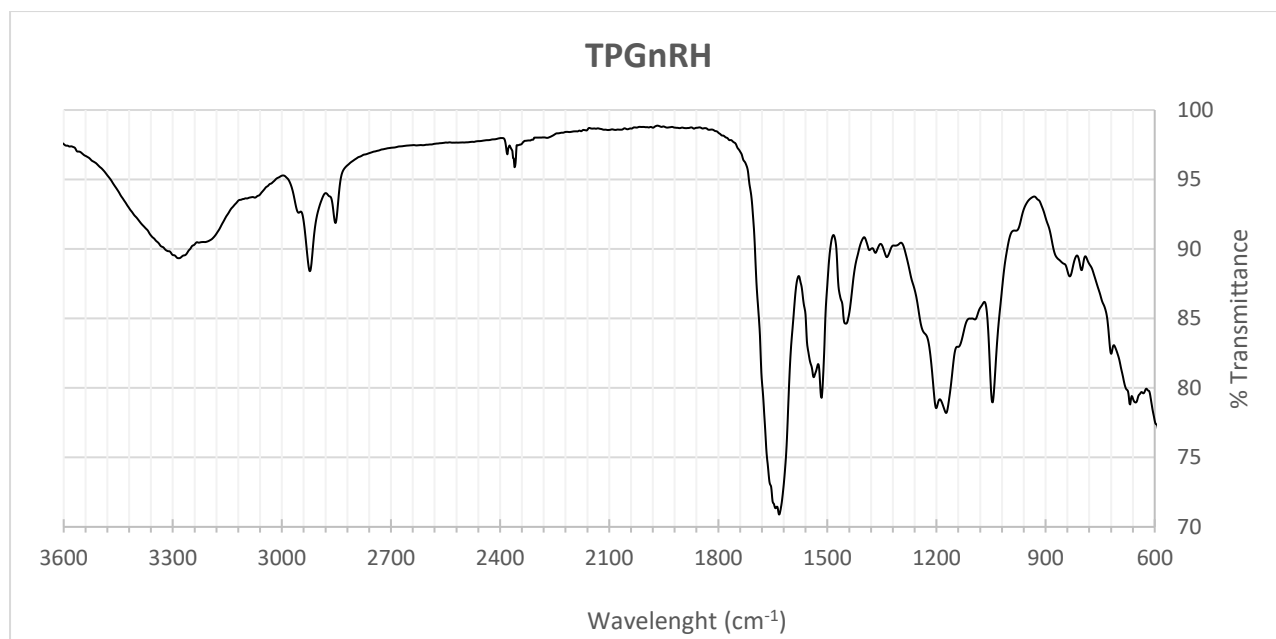


Figure 103 - FTIR spectra of TPGnRH.

3.5.5 Formulation of 11PS04 and TMZ into TPGnRH nanofibers

A total of three formulations were prepared. 11PS04 and Temozolomide were loaded into TPGnRH nanofibers following a *thin-film hydration* method ³¹⁴.

Formulation 1:

Calculates were done for 1 mL of formulation containing 137.5 µg/mL (500 µM) of 11PS04 loaded into 5697 µg/mL (4 mM) of TPGnRH (molar ratio 1:8). TPGnRH (5.63 mg) was weighted in a 6 mm vial, whereas 11PS04 was added as 4.5 µL from a more concentrated stock solution (110 mM, 100% (v/v) in DMSO). The mixture was diluted with 245.5 µL of isopropyl alcohol. The suspension was vortexed, and bath sonicated briefly prior being probe sonicated for 15-20 minutes with the instrument set at 60% of its maximum output (200 watts, Hielscher UP200S, GE). The content of the HPLC open vial was transferred into a 50 mL round bottom flask. Solvent was removed by rota-evaporation with liquid N₂ over 4 h until a thin-film was formed on the side of the flask. The dried thin film was hydrated with 0.5mL of 50mM sodium acetate buffer (pH=4.5, adjusted with NaOH 1M) and suspension was vortexed vigorously to ensure complete film hydration and removal from the glass flask. The formulation was removed and transferred in a clean vial and another 0.5 mL was added to reduce physical losses.

Formulation 2:

11PS04 (137.5 µg/ml, 500 µM), and TMZ (388 µg/ml, 2mM) were loaded into TPGnRH nanofibers (5697 µg/ml, 4mM), the molar ratio was 11PS04:TMZ:TPGnRH (1:4:8). TPGnRH (5.63 mg) was weighted in a 6 mm vial, 11PS04 and TMZ were also added from more concentrated stock solutions. Regarding 11PS04, the stock solution was 110 mM in pure DMSO from which 4.5 µL were added to

the vial containing TPGnRH. In the case of TMZ, 245.5 μL from the stock solution 7.57 mM (1.7 mg in 1.075 mL of IPA) were added followed by vortexing, bath sonication, probe sonication, rota-evaporation, and thin film rehydration as above.

Formulation 3:

In this new formulation, 11PSO4 (110 μg ; 0.4 mM) and TPGnRH nanofibers (1709 μg ; 1.2 mM) were present in a molar ratio 1:3, respectively in 1 mL total volume. 3.6 μL of 11PSO4 were added from a more concentrated solution (110 mM in pure DMSO) to a vial containing TPGnRH (1.7mg) and suspended in 246.4 μL of IPA. Then, the suspension was subjected to vortexing, bath sonication, probe sonication, rota-evaporation and thin film rehydration as above.

3.5.6 Characterization of Formulations

Particle size and zeta potential measurements

Prior to particle and zeta potential measurement (Zetasizer Nano-Zs, Malvern instruments) at 25°C and wavelength of 633 nm, the formulations were diluted [2 μL in 1mL of 10mM phosphate buffer (pH7.4) for F1 and F2 and 10 μL in 1.1 mL of 10mM phosphate buffer (pH7.4) for F3]. All particle size (n=13) zeta potential (n=100) measurements were performed in triplicate and mean and standard deviation was reported. The data were analysed using the Contin method of data analysis.

Transmission electron microscopy (TEM)

TEM images of formulations were obtained by dissolving stock formulations in PBS 0.01M (pH= 7.4). A drop of each formulation was located on the coated side of a copper coated grid and allowed to dry for 5 minutes. Then, samples were negatively stained with uranyl acetate aqueous solution (2% w/v) for 30 sec. Samples were visualised immediately after using Jeol JEM-1400 electron microscope.

Atomic Force Microscopy (AFM)

Formulations were visualized by AFM by diluting 10 μL of each formulation in 1mL of de-ionized water. The aqueous diluted formulations (5 μL) were placed on the surface of muscovite mica (1 cm^2 , Agar Scientific, Essex, UK) and left to dry for 2 min, and dried under air prior to being attached to a nickel disk (1 cm^2) using double-side adhesive tape and placed on the AFM microscope (Park NX10 enclosed in a Park NX10 acoustic enclosure, Park Systems Europe GmbH, Mannheim, Germany). Measurements were performed in air under ambient conditions (T = 23 °C, RH = 21%) using the scanner (max xy = 50 μm). Scanning was performed in tapping mode using triangular Si cantilevers with integrated tips [t = 3.0 - 5.0 μm , l = 115–135 μm , w = 22.5 – 37.5 μm , Vo = 204–497 kHz, k = 10–130 N m^{-1} , R < 10 nm; PointProbe® Plus Non-Contact Tapping Mode High Resonance Frequency Reflex Coating (PPP-NCHR-20) Nanosensors, Neuchâtel, Switzerland] and an RMS amplitude of 0.8V.

The images were processed, and dimensions were measured using Gwyddion 2.55 software (Department of Nanometrology, Czech Metrology Institute).

Quantification of loading using HPLC – Drug loading and encapsulation efficiency

Formulations were diluted with methanol 1: 4 or 1: 10 v/v prior to their analysis. Mobile phase consisted of 0.1 % (v/v) TFA in water (eluent A) and 0.08% (v/v) TFA in ACN (eluent B). Flow rate was set at 1 mL min⁻¹ from 0-4, 1.2 mL min⁻¹ from 4-19 minutes and 1 mL min⁻¹ from 19.1 - 22 minutes (Table 22). Temperature was set at 25°C and the injection volume was 12 µL. Detection of peaks by diode array was performed at 220 nm (for TPGnRH and 11PS04, although the λ_{max} for the latter was nm) and 330 nm (for TMZ). Calibration curves for TPGnRH, 11PS04 and TMZ between 570-57 µg mL⁻¹, 137.5- 5.5 µg mL⁻¹ and 338-33.8 µg mL⁻¹, respectively, were also analysed at these wavelengths, used for quantification and linear curves were obtained [$y = 2,1796x + 16,025$ ($r^2 = 0.9986$) for TPGnRH, $y = 14.435x - 102.12$ ($r^2 = 0.9943$) for TMZ and $y = 10.798x + 9.2653$ ($r^2 = 0.9972$) for 11PS04]. OpenLAB software was used to analyse the results (Agilent Technologies, Cheshire, UK).

Table 23 - Gradient HPLC method for analysis of 11PS04, TMZ and TPGnRH formulations.

Time (minutes)	0.1% (v/v) TFA in H ₂ O (A)	0.08 % TFA in ACN (B)
0	99	1
3	99	1
4	99	10
6	70	30
12	50	50
16	15	85
17	0	100
19	0	100
19.1	99	1
22	99	1

Drug loading (% w/w) and encapsulation efficiency (%) of formulations were and calculated according to equations 3.1 and 3.2.

$$\text{Drug Loading (\%)} = \frac{\text{Weight of drug}}{\text{Weight of TPGnRH}} \times 100$$

Equation 3.1

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Weight of entrapped drug}}{\text{Initial weight of drug}} \times 100$$

Equation 3.2

3.5.7 Antiproliferative Assays in U87 MG cells

Cell culture and cell seeding

Human glioblastoma cell line, U87 MG, was obtained from the European Collection of Cell culture (ECACC, No. 89081402, Lot 11K009, P21 to P28) and cultured in growth medium DMEM supplemented with 10% (v/v) of heat-inactivated FBS, 1% (v/v) of NEAA and 1% Penicillin-Streptomycin.

For all assays, U87 MG cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C, and the medium refreshed every 2-3 days. When cells reached ~80% of confluency, the cells were washed with PBS (~5 mL) and incubated with 2-3 mL of TrypLE Enzyme 0.25% at 37°C for 3 minutes. Then, growth medium (3 mL) was added and the cells were centrifuged (C-28A, Boeco, Hamburg, Germany) at 1,500 rpm for 5 minutes. Supernatant was discarded, and the pellet re-suspended in 1 mL of complete medium.

Cell number was obtained using the Trypan Blue Exclusion Assay²⁶² and the haematocytometer. This method is based on the principle that live cells have intact membrane that exclude dyes such as trypan blue, whilst dead cells take up the dye and are visualised with a blue cytoplasm. In brief, 20 µL of the 1 mL cell suspension was mixed 1: 1 with a solution 0.4% (v/v in PBS) of trypan blue. 10 µL of this mixture were applied to a haematocytometer and visualised on a microscope. Cells with non-coloured cytoplasm were counted. To obtain total the number of viable cells, counted cells were multiplied by 2 (dilution factor of trypan blue mixture) and per 10⁴ as per the haematocytometer dilution.

In all experiments, 96-well plates were seeded with U87 MG cells at 1400 cells/cm² in complete medium and allowed to attach and grow for 3 days in adherent conditions.

Experiments design

After the 3 days, complete change of media and cell treatment took place every 2 days for a total of 6 days. Treatment volume constituted the 10% of the total volume in the well (200 µL). Hence, in all experiments, treatment was added in 20 µL with 180 µL of complete DMEN media.

Non-treated cells were used as negative control and cells treated with PBS [with 0.5% of DMSO (v/v)] were used as vehicle control.

11PS04 and Temozolomide Assay - Protocol 1

An initial experiment was undertaken to assess if addition of 11PS04 can sensitise cells and increased the efficacy of TMZ when combined. 11PS04 stock solution (11 mM, 3 mg mL⁻¹ in pure DMSO) was used to prepare diluted stock dilutions. First, 5 µL of 11 mM stock were mixed with 45 µL of PBS (0.01M, pH 7.4) to obtain a 1.1 mM stock with 10% DMSO v/v. Next, 11PS04 dilution at 100 µM 10% DMSO v/v was freshly prepared by adding 50 µL of 11PS04 1.1 mM to 500 µL of PBS enriched with 10% of DMSO. This 100 µM that was immediately diluted 1 to 20 in complete media to elicit the final concentration in well of 11PS04 (5 µM).

A 0.1 M TMZ stock solution (1.36 mg in 70 µL of pure DMSO) was used to prepare 20-fold concentration of TMZ diluted stocks in PBS (with 10% DMSO v/v) at concentrations ranging 0.5 to 10 mM, which were added to each well (10 µL) for a final concentration of 25- 500 µM.

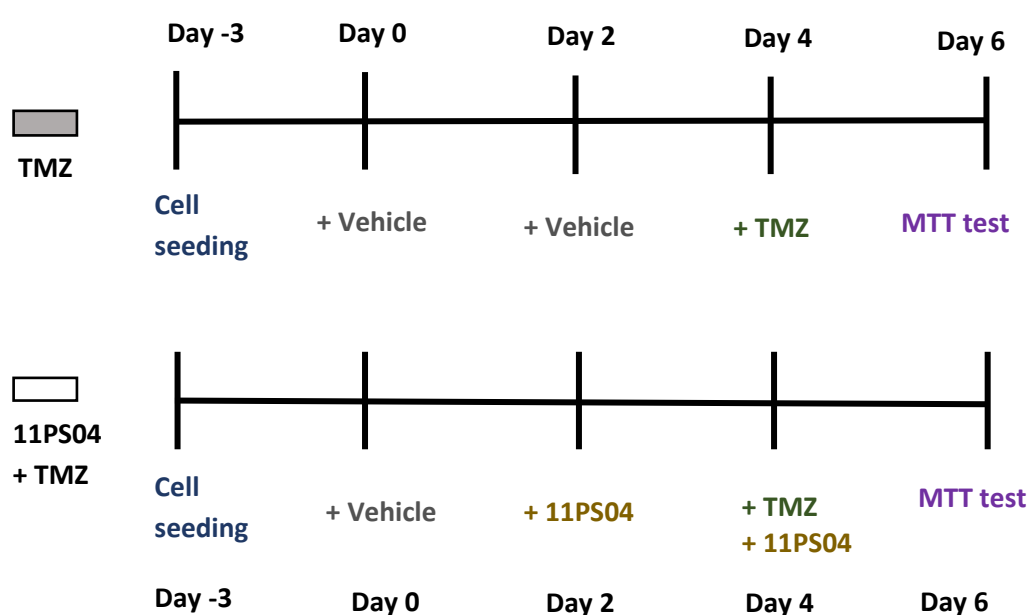


Figure 104 - Schematic representation of TMZ and 11PS04 assay . After 3 days growing, the media were removed and substituted with fresh media. On day 2, the media were again removed and substituted with fresh media, this time containing 5 µM of 11PS04. Two days after (day 4), the media were changed and TMZ was added in a range of concentrations (25-500 µM) in the presence or absence of a new dose of 11PS04. Cell metabolic activity was measured by the MTT test on day 6.

The triple treatment Assay -Protocol 2

An experiment was undertaken with 11PS04, TMZ and TPGnRH to assess their antiproliferative potential when combined. In this assay, each component was externally added. The effect of the pair TMZ -TPGnRH was also evaluated.

Cells were exposed to TPGnRH in a range of concentrations (7 - 70 µM) prepared from more concentrated stock dilutions (140-1400 µM in PBS, pH=7.4). 1400 µM was used as stock solution to

prepare 2-fold serial dilutions by mixing 4 mg of peptide in 2 mL of PBS. 10 μ L of each stock were added per well to achieve the desired concentration.

A fixed dose of 11PS04 (5 μ M) was employed in this assay. Two stocks were prepared:

Stock 1 at 100 μ M - used when 11PS04 was combined with TPGnRH: 11PS04 stock 11 mM was used as stated above on Assay 1 to prepare a diluted stock at 100 μ M (10% DMSO v/v). 10 μ L of this stock were added in a total volume of 200 μ L per well (dilution 1 to 20) to afford the desired final concentration (5 μ M).

Stock 2 at 200 μ M- used when 11PS04 was combined with TMZ and TPGnRH. First, a stock solution 1.1 mM 10% DMSO v/v was prepared as stated in Assay 1. The 200 μ M stock solution was freshly prepared by mixing 60 μ L of 1.1 mM stock with 270 μ L of 10% DMSO v/v in PBS, from where 5 μ L were added to each well (1 to 40 dilution) to achieve the desired 5 μ M.

Cells were treated with TMZ at two different concentrations (100 and 250 μ M). TMZ stock solution 0.1M in pure DMSO was used to prepare two 40-fold diluted stocks at 10 and 4 mM. The 10 mM stock was prepared by mixing 50 μ L of 0.1M stock and 450 μ L of PBS, whereas 4 mM stock solution was prepared by mixing 120 μ L of 10 mM diluted stock with 180 μ L of 10 %DMSO in PBS. From each diluted stock, 5 μ L were added to corresponding wells to achieve the desired final concentration.

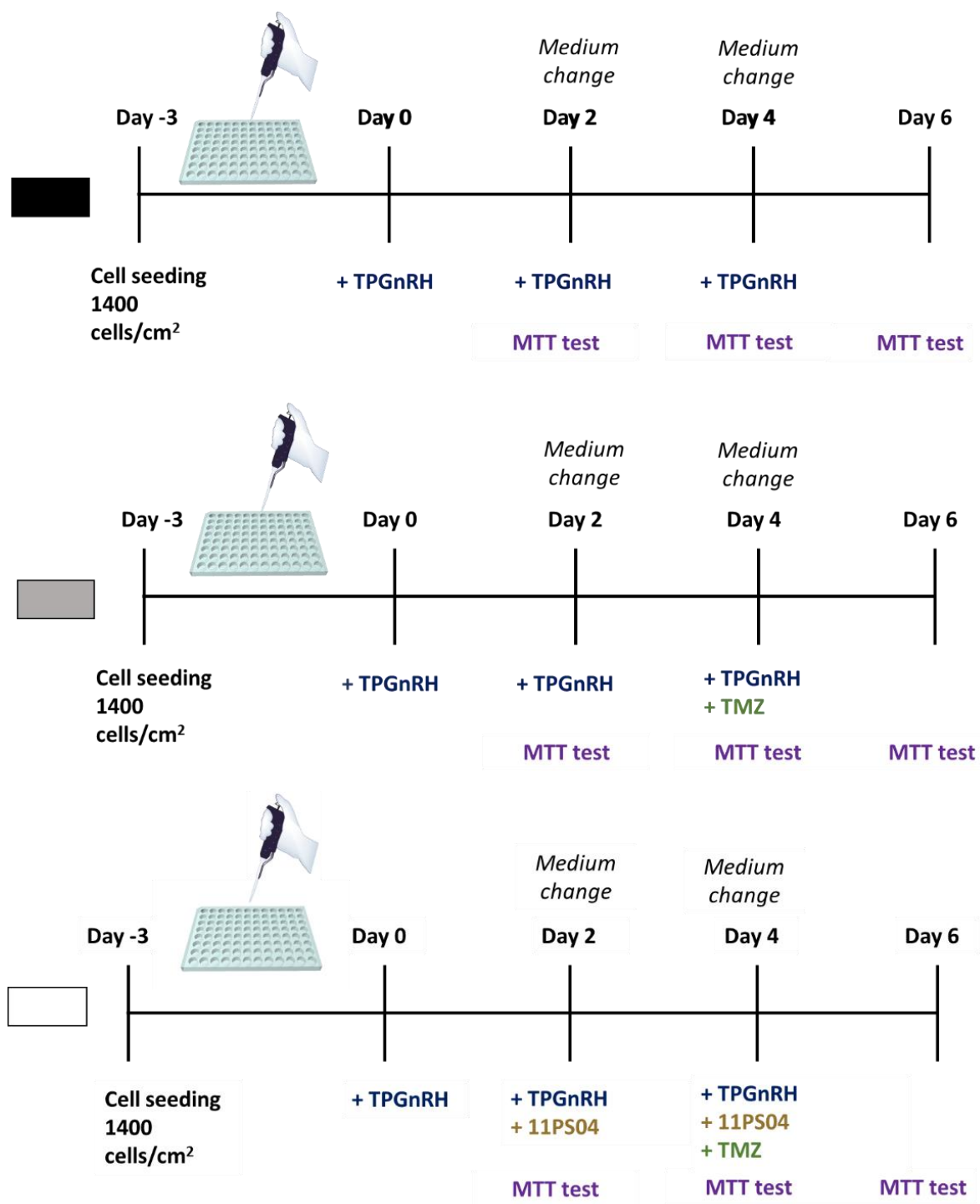


Figure 105 - Schematic representation of Triple Treatment assay . Cells were treated with TPGnRH (7-70 μ M) every two days for 6 days in every test performed. Treatment with only TPGnRH is represented by black bars, treatment with TMZ and TPGnRH in grey colour, and the *triple-treatment* with 11PS04, TMZ and TPGnRH in white colour. Cell viability was assessed by the MTT assay.

11PS04 and 11PS04 and TMZ loaded TPGnRH formulations Assay - Protocol 3

The present experiment was undertaken to assess the antiproliferative effect of two formulations. Formulation 1 consisted on TPGnRH nanofibers loaded with 11PS04 whereas Formulation 2 contained 11PS04-TMZ loaded nanofibers (Formulation 2).

TPGnRH nanofibers stock at 800 μM (1.14 mg mL^{-1}) was used to prepare a diluted stock solution at 400 μM by mixing 200 μL of 800 μM stock with 200 μL of PBS. Cells were treated on Day 0 with 20 μL of freshly prepared 400 μM stock diluted in 180 μL of DMEM.

Cells were treated with 2 μL of 11PS04-loaded nanofibers (Formulation 1; 500 μM of 11PS04 and 4000 μM of TPGnRH) diluted in complete DMEM media (1 to 100) for a final concentration per well of 5 μM and 40 μM , respectively. Likewise, 2 μL of Formulation 2 (11PS04 500 μM , TMZ 2 mM and TPGnRH 4 mM) were freshly prepared and diluted 1 to 100 in complete DMEM media to afford a final concentration per well of 5 μM , 20 μM and 40 μM of 11PS04, TMZ and TPGnRH, respectively.

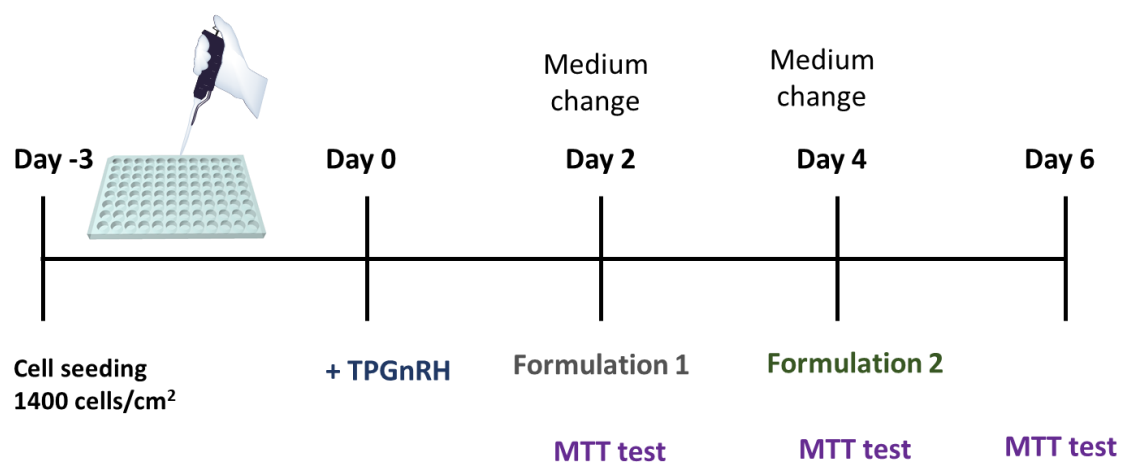


Figure 106 - Schematic representation of assay with 11PS04 loaded nanofibers (Formulation 1) and 11PS04-TMZ loaded nanofibers (Formulation 2). Cells were treated for 2 days with TPGnRH at 40 μM . Formulation 1 (TPGnRH 40 μM ; 11PS04 5 μM) was added on the second day and, two days after, media were removed and refreshed with a new dose of formulation 2 (TPGnRH 40 μM ; 11PS04 5 μM , TMZ 20 μM). Cell viability was assessed by the MTT assay.

Combining 11PS04 loaded nanofibers with TMZ Assay – Protocol 4

This experiment was undertaken to evaluate the antiproliferative efficacy of 11PS04-loaded nanofibers (Formulation 3, 400 μM 11PS04, 1.2 mM TPGnRH) when combined with TMZ externally added.

TPGnRH nanofibers stock at 800 μM (1.14 mg mL^{-1}) was used to prepare a diluted stock solution at 300 μM by mixing 510 μL of 800 μM stock with 850 μL of PBS, from which 10 μL were immediately added to each test well diluted in 180 μL of DMEN for a final concentration of 15 μM of peptide.

A freshly prepared diluted stock was made by diluting formulation 3 in PBS at ratio formulation: PBS 1:4. Then, 10 μL of this diluted stock were immediately added to test wells, eliciting a final concentration of 5 μM and 15 μM of 11PS04 and TPGnRH, respectively. This treatment was added on the 2nd and 4th day.

A 20-fold diluted stock of TMZ at 3.5 mM was prepared by mixing 50 μL of 35 mM (100% DMSO) stock of TMZ with 450 μL of PBS. The 3.5 mM stock was diluted further in DMEN 1 to 20 to afford the desired final concentration per well (175 μM). Freshly prepare physical mixtures of 11PS04 (5 μM) with TMZ (175 μM) and TMZ (175 μM) with TPGnRH nanofibers (15 μM) were used as control.

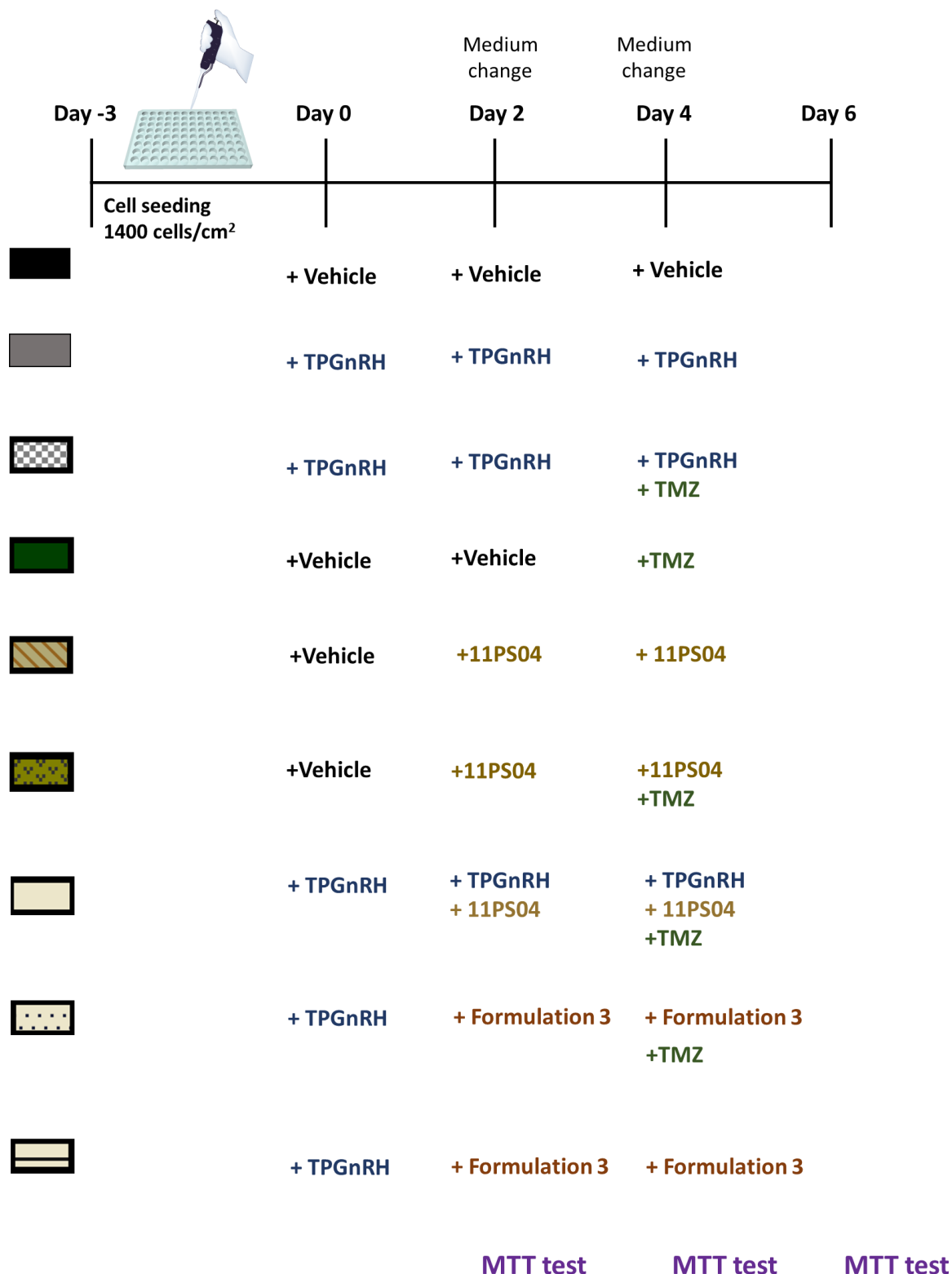


Figure 107 - Schematic representation of the applied treatments with 11PS04-loaded nanofibers (Formulation 3) and TMZ. Treatments started at day 0 with periodical refreshment of the medium and addition of the corresponding treatments every two days. TPGnRH was added at a final concentration of 15 μ M, 11PS04 at 5 μ M and TMZ 175 μ M. Cell viability was measured by the MTT assay every 2 days.

Doubling the dose of 11PS04 Assay – Protocol 5

An experiment was undertaken to assess the antiproliferative efficacy of TPGnRH nanofibers combined with double amount of 11PS04, this is, 10 μ M (each compound externally added).

TPGnRH nanofibers stock at 800 μ M (1.14 mg mL⁻¹) was used to prepare a diluted stock solution at 300 μ M by mixing 51 μ L of 800 μ M stock with 85 μ L of PBS, from which 10 μ L were immediately added to each test well diluted in 180 μ L of DMEN for a final concentration of 15 μ M of peptide. A 10-fold concentration stock of 11PS04 was prepared from 2 mM stock (in pure DMSO) by diluting 10 μ L in 90 μ L of PBS to elicit a 200 μ M diluted stock solution, from which 10 μ L were added to each well for a final concentration of 10 μ M.

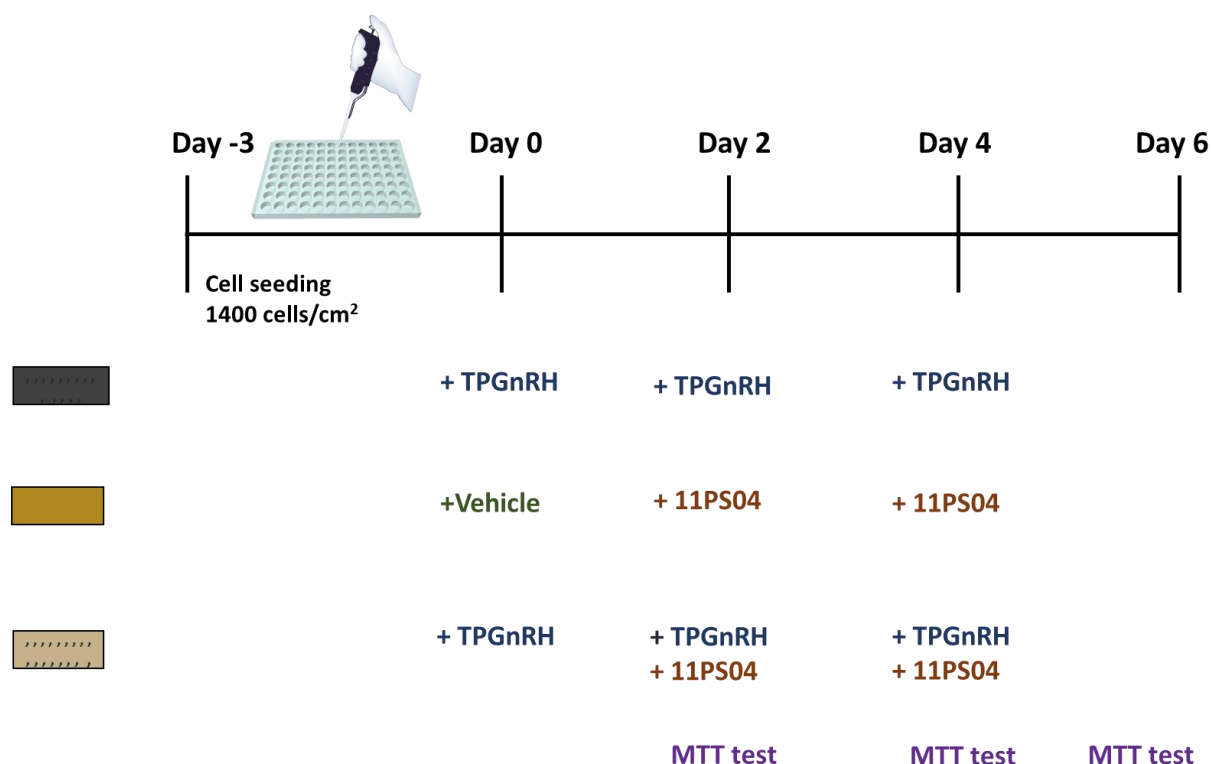


Figure 108 - Schematic representation of the addition protocol followed in assay 5. Vehicle contains 0.5% (v/v) of DMSO in PBS. The final concentration of each treatment was 15 μ M and 5 μ M for TPGnRH and 11PS04, respectively.

Cell metabolic activity

Cell metabolic activity was measured by the MTT assay. In this colorimetric test, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced by NADH-dependent oxidoreductase enzyme to form formazan, a purple precipitate that can be measured and is directly related with the number of viable cells. An MTT stock solution (5 mg/mL in PBS) was bath sonicated for x minutes and filtered sterilised via a 0.22 µm nylon filter. The filtrate was stored in the freezer protected from light.

In all experiments, after 2, 4 and 6 days of treatment, MTT assay was performed by adding 20 µL of MTT solution to each well (without removing supernatant), after which cells were incubated for 4 h at 37°C. Then, media were removed and 100 µL of DMSO were added to dissolve the formazan crystals. Plates were covered in foil and rotated in a gyratory shaker for 15 min to help the dissolution process of crystals. UV absorbance was measured at 570 and 690 nm using Multiskan Go microplate spectrophotometer (Thermo Scientific, Paisley, UK). Cell metabolic activity can be calculated by subtracting the values at 690 nm 570 nm to remove background, and dividing the values by the control per 100 to express as a % relative to control (Equation 3.3):

$$\text{Cell metabolic activity} = \frac{(Abs_{570 \text{ nm sample}} - Abs_{690 \text{ nm sample}})}{(Abs_{570 \text{ nm control}} - Abs_{690 \text{ nm control}})} \times 100$$

Equation 3.3

Where Abs_{570 nm sample} is optical density (OD) values registered at 570 nm on test wells and Abs_{570 nm control} are OD obtained for control wells; likewise, Abs_{690 sample} and Abs₆₉₀ are OD values of control and samples are obtained as background

3.5.8 Statistics

Experimental data was analysed with GraphPad Prism 8 (GraphPad Inc., California, USA) software. Results were expressed as mean ± SD and statistical significance was set at 5% and assessed using the t-test or One-way ANOVA or Two-ANOVA using the Dunnett's and/or Tukey's post-hoc tests for multiple group comparisons.

REFERENCES

- (1) WHO | Global action plan on the public health response to dementia 2017 - 2025 http://www.who.int/mental_health/neurology/dementia/action_plan_2017_2025/en/ (accessed May 5, 2020).
- (2) WHO. Dementia: a public health priority <https://www.who.int/news-room/fact-sheets/detail/dementia> (accessed Apr 18, 2020).
- (3) Plan Integral de Alzheimer y otras demencias (2019-2023) https://www.msbs.gob.es/profesionales/saludPublica/docs/Plan_Integral_Alzheimer_Octubre_2019.pdf (accessed Jul 5, 2020).
- (4) Kovacs, G. G. Tauopathies. In *Handbook of Clinical Neurology*; Elsevier B.V, 2018; Vol. 145, pp 355–368.
- (5) Goedert, M.; Yamaguchi, Y.; Mishra, S. K.; Higuchi, M.; Sahara, N. Tau Filaments and the Development of Positron Emission Tomography Tracers. *Front. Neurol.* **2018**, *9*, 70.
- (6) Hall, B.; Mak, E.; Cervenka, S.; Aigbirhio, F. I.; Rowe, J. B.; O’Brien, J. T. In Vivo Tau PET Imaging in Dementia: Pathophysiology, Radiotracer Quantification, and a Systematic Review of Clinical Findings. *Ageing Res. Rev.* **2017**, *36*, 50–63.
- (7) Braak, H.; Braak, E. Neuropathological Stageing of Alzheimer-Related Changes. *Acta Neuropathol.* **1991**, *82*, 239–259.
- (8) Braak, H.; Jellinger, K.; Braak, E.; Bohl, J. Allocortical Neurofibrillary Changes in Progressive Supranuclear Palsy. *Acta Neuropathol.* **1992**, *84* (5), 478–483.
- (9) Dickson, D. W. Neuropathologic Differentiation of Progressive Supranuclear Palsy and Corticobasal Degeneration. *J. Neurol.* **1999**, *246*, II6–II15.
- (10) Spillantini, M. G.; Goedert, M. Tau Pathology and Neurodegeneration. *Lancet Neurol.* **2013**, *12* (6), 609–622.
- (11) Sun, X.; Chen, W. D.; Wang, Y. D. β -Amyloid: The Key Peptide in the Pathogenesis of Alzheimer’s Disease. *Front. Pharmacol.* **2015**, *6* (221).
- (12) Serrano-Pozo, A.; Frosch, M. P.; Masliah, E.; Hyman, B. T. Neuropathological Alterations in Alzheimer Disease. *Cold Spring Harb. Perspect. Med.* **2011**, *1* (a006189).
- (13) Dickson, T. C.; Vickers, J. C. The Morphological Phenotype of Beta-Amyloid Plaques and Associated Neuritic Changes in Alzheimer’s Disease. *Neuroscience* **2001**, *105* (1), 99–107.
- (14) Gallardo, G.; Holtzman, D. M. Amyloid- β and Tau at the Crossroads of Alzheimer’s Disease. In *Tau Biology*; Takashima, A., Wolozin, B., Buee, L., Eds.; Springer Singapore: Singapore, 2019; pp 187–203.
- (15) Vassar, R.; Kovacs, D. M.; Yan, R.; Wong, P. C. The β -Secretase Enzyme BACE in Health and Alzheimer’s Disease: Regulation, Cell Biology, Function, and Therapeutic Potential. *J. Neurosci.* **2009**, *29* (41), 12787–12794.

- (16) Sisodia, S. S.; St George-Hyslop, P. H. γ -Secretase, Notch, A β and Alzheimer's Disease: Where Do the Presenilins Fit In? *Nat. Rev. Neurosci.* **2002**, 3 (4), 281–290.
- (17) Vassar, R.; Kuhn, P.-H.; Haass, C.; Kennedy, M. E.; Rajendran, L.; Wong, P. C.; Lichtenthaler, S. F. Function, Therapeutic Potential and Cell Biology of BACE Proteases: Current Status and Future Prospects. *J. Neurochem.* **2014**, 130 (1), 4–28.
- (18) Yatin, S. M.; Aksenov, M.; Butterfield, D. A. The Antioxidant Vitamin E Modulates Amyloid β -Peptide-Induced Creatine Kinase Activity Inhibition and Increased Protein Oxidation: Implications for the Free Radical Hypothesis of Alzheimer's Disease. *Neurochem. Res.* **1999**, 24 (3), 427–435.
- (19) Mark, R. J.; Hensley, K.; Butterfield, D. A.; Mattson, M. P. Amyloid β -Peptide Impairs Ion-Motive ATPase Activities: Evidence for a Role in Loss of Neuronal Ca²⁺ Homeostasis and Cell Death. *J. Neurosci.* **1995**, 15 (9), 6239–6249.
- (20) Varadarajan, S.; Yatin, S.; Aksenova, M.; Butterfield, D. A. Review: Alzheimer's Amyloid β -Peptide-Associated Free Radical Oxidative Stress and Neurotoxicity. *J. Struct. Biol.* **2000**, 130 (2–3), 184–208.
- (21) Ezeani, M.; Omabe, M. A New Perspective of Lysosomal Cation Channel-Dependent Homeostasis in Alzheimer's Disease. *Mol. Neurobiol.* **2016**, 53 (3), 1672–1678.
- (22) Heneka, M. T.; Golenbock, D. T.; Latz, E. Innate Immunity in Alzheimer's Disease. *Nat. Immunol.* **2015**, 16, 229–236.
- (23) Weggen, S.; Eriksen, J. L.; Das, P.; Sagi, S. A.; Wang, R.; Pietrzik, C. U.; Findlay, K. A.; Smith, T. E.; Murphy, M. P.; Bulters, T.; Kang, D. E.; Marquez-Sterling, N.; Golde, T. E.; Koo, E. H. A Subset of NSAIDs Lower Amyloidogenic A β 42 Independently of Cyclooxygenase Activity. *Nature* **2001**, 414 (6860), 212–216.
- (24) Neniskyte, U.; Neher, J. J.; Brown, G. C. Neuronal Death Induced by Nanomolar Amyloid β Is Mediated by Primary Phagocytosis of Neurons by Microglia. *J. Biol. Chem.* **2011**, 286 (46), 39904–39913.
- (25) Liu, S.; Liu, Y.; Hao, W.; Wolf, L.; Kiliaan, A. J.; Penke, B.; R be, C. E.; Walter, J.; Heneka, M. T.; Hartmann, T.; Menger, M. D.; Fassbender, K. TLR2 Is a Primary Receptor for Alzheimer's Amyloid β Peptide To Trigger Neuroinflammatory Activation. *J. Immunol.* **2012**, 188 (3), 1098–1107.
- (26) Shepherd, C.; McCann, H.; Halliday, G. M. Variations in the Neuropathology of Familial Alzheimer's Disease. *Acta Neuropathol.* **2009**, 118 (1), 37–52.
- (27) Mullan, M.; Crawford, F.; Axelman, K.; Houlden, H.; Lilius, L.; Winblad, B.; Lannfelt, L. A Pathogenic Mutation for Probable Alzheimer's Disease in the APP Gene at the N-Terminus of β -Amyloid. *Nat. Genet.* **1992**, 1 (5), 345–347.
- (28) Di Fede, G.; Catania, M.; Morbin, M.; Rossi, G.; Suardi, S.; Mazzoleni, G.; Merlin, M.; Giovagnoli, A. R.; Prioni, S.; Erbetta, A.; Falcone, C.; Gobbi, M.; Colombo, L.; Bastone, A.; Beeg, M.; Manzoni, C.; Francescucci, B.; Spagnoli, A.; Cant , L.; Del Favero, E.; Levy, E.; Salmona, M.; Tagliavini, F. A Recessive Mutation in the APP Gene with Dominant-Negative Effect on Amyloidogenesis. *Science (80-.)*. **2009**, 323 (5920), 1473–1477.

- (29) Schmechel, D. E.; Saunders, A. M.; Strittmatter, W. J.; Crain, B. J.; Hulette, C. M.; Joo, S. H.; Pericak-Vance, M. A.; Goldgaber, D.; Roses, A. D. Increased Amyloid β -Peptide Deposition in Cerebral Cortex as a Consequence of Apolipoprotein E Genotype in Late-Onset Alzheimer Disease. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90* (20), 9649–9653.
- (30) Corder, E. H.; Saunders, A. M.; Strittmatter, W. J.; Schmechel, D. E.; Gaskell, P. C.; Small, G. W.; Roses, A. D.; Haines, J. L.; Pericak-Vance, M. A. Gene Dose of Apolipoprotein E Type 4 Allele and the Risk of Alzheimer's Disease in Late Onset Families. *Science* (80-.). **1993**, *261* (5123), 921–923.
- (31) Suh, J.; Choi, S. H.; Romano, D. M.; Gannon, M. A.; Lesinski, A. N.; Kim, D. Y.; Tanzi, R. E. ADAM10 Missense Mutations Potentiate β -Amyloid Accumulation by Impairing Prodomain Chaperone Function. *Neuron* **2013**, *80* (2), 385–401.
- (32) Scheuner, D.; Eckman, C.; Jensen, M.; Song, X.; Citron, M.; Suzuki, N.; Bird, T. D.; Hardy, J.; Hutton, M.; Kukull, W.; Larson, E.; Levy-Lahad, E.; Viitanen, M.; Peskind, E.; Poorkaj, P.; Schellenberg, G.; Tanzi, R.; Wasco, W.; Lannfelt, L.; Selkoe, D.; Younkin, S. Secreted Amyloid β -Protein Similar to That in the Senile Plaques of Alzheimer's Disease Is Increased in Vivo by the Presenilin 1 and 2 and APP Mutations Linked to Familial Alzheimer's Disease. *Nat. Med.* **1996**, *2* (8), 864–870.
- (33) Karran, E.; Mercken, M.; Strooper, B. De. The Amyloid Cascade Hypothesis for Alzheimer's Disease: An Appraisal for the Development of Therapeutics. *Nat. Rev. Drug Discov.* **2011**, *10* (9), 698–712.
- (34) Perrin, R. J.; Fagan, A. M.; Holtzman, D. M. Multimodal Techniques for Diagnosis and Prognosis of Alzheimer's Disease. *Nature*. October 15, 2009, pp 916–922.
- (35) Brier, M. R.; Gordon, B.; Friedrichsen, K.; McCarthy, J.; Stern, A.; Christensen, J.; Owen, C.; Aldea, P.; Su, Y.; Hassenstab, J.; Cairns, N. J.; Holtzman, D. M.; Fagan, A. M.; Morris, J. C.; Benzinger, T. L.; Ances, B. M. Tau and A-Beta Imaging, CSF Measures, and Cognition in Alzheimer's Disease. *Sci. Transl. Med.* **2016**, *8* (338), 1–10.
- (36) NatureResearch. Alternative splicing - Latest research and news | Nature <https://www.nature.com/subjects/alternative-splicing> (accessed Apr 18, 2020).
- (37) Verwilt, P.; Kim, H. S.; Kim, S.; Kang, C.; Kim, J. S. Shedding Light on Tau Protein Aggregation: The Progress in Developing Highly Selective Fluorophores. *Chem. Soc. Rev.* **2018**, *47* (7), 2249–2265.
- (38) Bazrgar, M.; Khodabakhsh, P.; Mohagheghi, F.; Prudencio, M.; Ahmadiani, A. Brain MicroRNAs Dysregulation: Implication for Missplicing and Abnormal Post-Translational Modifications of Tau Protein in Alzheimer's Disease and Related Tauopathies. *Pharmacol. Res.* **2020**, *155* (104729).
- (39) Arendt, T.; Stieler, J. T.; Holzer, M. Tau and Tauopathies. *Brain Res. Bull.* **2016**, *126*, 238–292.
- (40) Wang, Y.; Mandelkow, E. Tau in Physiology and Pathology. *Nat. Rev. Neurosci.* **2016**, *17* (1), 5–21.
- (41) Hanger, D. P.; Anderton, B. H.; Noble, W. Tau Phosphorylation: The Therapeutic Challenge for Neurodegenerative Disease. *Trends in Molecular Medicine*. March 2009, pp 112–119.

- (42) Clark, A. R.; Ohlmeyer, M. Protein Phosphatase 2A as a Therapeutic Target in Inflammation and Neurodegeneration. *Pharmacology and Therapeutics*. Elsevier Inc. September 1, 2019, pp 181–201.
- (43) Maeda, S.; Takashima, A. Tau Oligomers. In *Tau Biology*; Takashima, A., Wolozin, B., Buee, L., Eds.; Springer Singapore: Singapore, 2019; pp 373–380.
- (44) Friedhoff, P.; Schneider, A.; Mandelkow, E.-M.; Mandelkow, E. Rapid Assembly of Alzheimer-like Paired Helical Filaments from Microtubule-Associated Protein Tau Monitored by Fluorescence in Solution. *Biochemistry* **1998**, *37*, 10223–10230.
- (45) Berriman, J.; Serpell, L. C.; Oberg, K. A.; Fink, A. L.; Goedert, M.; Crowther, R. A. Tau Filaments from Human Brain and from in Vitro Assembly of Recombinant Protein Show Cross- β Structure. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100* (15), 9034–9038.
- (46) Fitzpatrick, A. W. P.; Falcon, B.; He, S.; Murzin, A. G.; Murshudov, G.; Garringer, H. J.; Crowther, R. A.; Ghetti, B.; Goedert, M.; Scheres, S. H. W. Cryo-EM Structures of Tau Filaments from Alzheimer's Disease. *Nature* **2017**, *547* (7662), 185–190.
- (47) Chiti, F.; Dobson, C. M. Protein Misfolding, Functional Amyloid, and Human Disease. *Annu. Rev. Biochem.* **2006**, *75* (1), 333–366.
- (48) Eisenberg, D.; Jucker, M. The Amyloid State of Proteins in Human Diseases. *Cell*. March 16, 2012, pp 1188–1203.
- (49) Sunde, M.; Serpell, L. C.; Bartlam, M.; Fraser, P. E.; Pepys, M. B.; Blake, C. C. F. Common Core Structure of Amyloid Fibrils by Synchrotron X-Ray Diffraction. *J. Mol. Biol.* **1997**, *273* (3), 729–739.
- (50) Fitzpatrick, A. W. P.; Debelouchina, G. T.; Bayro, M. J.; Clare, D. K.; Caporini, M. A.; Bajaj, V. S.; Jaroniec, C. P.; Wang, L.; Ladizhansky, V.; Müller, S. A.; Macphee, C. E.; Waudby, C. A.; Mott, H. R.; De Simone, A.; Knowles, P. J.; Saibil, H. R.; Vendruscolo, M.; Orlova, E. V.; Griffin, R. G.; Dobson, C. M. Atomic Structure and Hierarchical Assembly of a Cross- β Amyloid Fibril. *PNAS* **2013**, *110* (14), 5468–5473.
- (51) Knowles, T. P.; Fitzpatrick, A. W.; Meehan, S.; Mott, H. R.; Vendruscolo, M.; Dobson, C. M.; Welland, M. E. Role of Intermolecular Forces in Defining Material Properties of Protein Nanofibrils. *Science* (80-.). **2007**, *318* (5858), 1900–1903.
- (52) Baldwin, A. J.; Knowles, T. P. J.; Tartaglia, G. G.; Fitzpatrick, A. W.; Devlin, G. L.; Shammass, S. L.; Waudby, C. A.; Mossuto, M. F.; Meehan, S.; Gras, S. L.; Christodoulou, J.; Anthony-Cahill, S. J.; Barker, P. D.; Vendruscolo, M.; Dobson, C. M. Metastability of Native Proteins and the Phenomenon of Amyloid Formation. *J. Am. Chem. Soc.* **2011**, *133* (36), 14160–14163.
- (53) Fitzpatrick, A. W. P.; Falcon, B.; He, S.; Murzin, A. G.; Murshudov, G.; Garringer, H. J.; Crowther, R. A.; Ghetti, B.; Goedert, M.; Scheres, S. H. W. Cryo-EM Structures of Tau Filaments from Alzheimer's Disease. *Nature* **2017**, *547* (7662), 185–190.
- (54) Crick, F. H. C.; Rich, A. Structure of Polyglycine II. *Nature* **1955**, *176* (4486), 780–781.
- (55) Crowther, R. A. Straight and Paired Helical Filaments in Alzheimer Disease Have a Common Structural Unit. *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88* (6), 2288–2292.

- (56) Wischik, C. M.; Novak, M.; Edwards, P. C.; Klug, A.; Tichelaar, W.; Crowther, R. A. Structural Characterization of the Core of the Paired Helical Filament of Alzheimer Disease. *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85* (13), 4884–4888.
- (57) Braak, H.; Del Tredici, K. The Pathological Process Underlying Alzheimer's Disease in Individuals under Thirty. *Acta Neuropathol.* **2011**, *121*, 171–181.
- (58) Ashe, K. H.; Zahs, K. R. Review Probing the Biology of Alzheimer's Disease in Mice. *Neuron* **2010**, *66* (5), 631–645.
- (59) Higuchi, M. Tau PET Imaging. In *Tau Biology*; Takashima, A., Wolozin, B., Buee, L., Eds.; Springer Singapore: Singapore, 2019; pp 217–230.
- (60) Ametamey, S. M.; Honer, M.; Schubiger, P. A. Molecular Imaging with PET. *Chem. Rev.* **2008**, *108*, 1501–1516.
- (61) Li, Z.; Conti, P. S. Radiopharmaceutical Chemistry for Positron Emission Tomography. *Adv. Drug Deliv. Rev.* **2010**, *62*, 1031–1051.
- (62) Pike, V. W.; Pike, V. W. Considerations in the Development of Reversibly Binding PET Radio-Ligands for Brain Imaging. *Curr. Med. Chem.* **2016**, *23*, 1818–1869.
- (63) Kawada, K.; Iwamoto, M.; Sakai, Y. Mechanisms Underlying 18 F-Fluorodeoxyglucose Accumulation in Colorectal Cancer. *World J. Radiol.* **2016**, *8* (11), 880.
- (64) Alberca Serrano, R.; López Pousa, S. *Patogenia y Fisiopatología de La Enfermedad de Alzheimer*; Editorial Médica Panamericana, 2012.
- (65) Villemagne, V. L.; Doré, V.; Burnham, S. C.; Masters, C. L.; Rowe, C. C. Imaging Tau and Amyloid- β Proteinopathies in Alzheimer Disease and Other Conditions. *Nat. Rev. Neurol.* **2018**, *14* (4), 225–236.
- (66) Schafer, K. N.; Kim, S.; Matzavinos, A.; Kuret, J. Selectivity Requirements for Diagnostic Imaging of Neurofibrillary Lesions in Alzheimer's Disease: A Simulation Study. *Neuroimage* **2012**, *60* (3), 1724–1733.
- (67) Declercq, L.; Rombouts, F.; Koole, M.; Fierens, K.; Mariën, J.; Langlois, X.; Andrés, J. I.; Schmidt, M.; MacDonald, G.; Moechars, D.; Vanduffel, W.; Tousseyn, T.; Vandenberghe, R.; Van Laere, K.; Verbruggen, A.; Bormans, G. Preclinical Evaluation of 18F-JNJ64349311, a Novel PET Tracer for Tau Imaging. *J. Nucl. Med.* **2017**, *58* (6), 975–981.
- (68) Gobbi, L. C.; Knust, H.; Körner, M.; Honer, M.; Czech, C.; Belli, S.; Muri, D.; Edelmann, M. R.; Hartung, T.; Erbsmehl, I.; Grall-Ulsemmer, S.; Koblet, A.; Rueher, M.; Steiner, S.; Ravert, H. T.; Mathews, W. B.; Holt, D. P.; Kuwabara, H.; Valentine, H.; Dannals, R. F.; Wong, D. F.; Borroni, E. Identification of Three Novel Radiotracers for Imaging Aggregated Tau in Alzheimer's Disease with Positron Emission Tomography. *J. Med. Chem.* **2017**, *60* (17), 7350–7370.
- (69) Chien, D. T.; Szardenings, A. K.; Bahri, S.; Walsh, J. C.; Mu, F.; Xia, C.; Shankle, W. R.; Lerner, A. J.; Su, M. Y.; Elizarov, A.; Kolb, H. C. Early Clinical PET Imaging Results with the Novel PHF-Tau Radioligand [F18]-T808. *J. Alzheimer's Dis.* **2014**, *38* (1), 171–184.
- (70) Betthausen, T. J.; Lao, P. J.; Murali, D.; Barnhart, T. E.; Furumoto, S.; Okamura, N.; Stone, C. K.; Johnson, S. C.; Christian, B. T. In Vivo Comparison of Tau Radioligands 18F-THK-5351 and 18F-

- THK-5317. *J. Nucl. Med.* **2017**, *58* (6), 996–1002.
- (71) Harada, R.; Okamura, N.; Furumoto, S.; Tago, T.; Yanai, K.; Arai, H.; Kudo, Y. Characteristics of Tau and Its Ligands in PET Imaging. *Biomolecules* **2016**, *6* (1), 7.
- (72) Lockhart, A. Imaging Alzheimer's Disease Pathology: One Target, Many Ligands. *Drug Discovery Today*. December 2006, pp 1093–1099.
- (73) Zhang, W.; Arteaga, J.; Cashion, D. K.; Chen, G.; Gangadharath, U.; Gomez, L. F.; Kasi, D.; Lam, C.; Liang, Q.; Liu, C.; Mocharla, V. P.; Mu, F.; Sinha, A.; Katrin Szardenings, A.; Wang, E.; Walsh, J. C.; Xia, C.; Yu, C.; Zhao, T.; Kolb, H. C. A Highly Selective and Specific PET Tracer for Imaging of Tau Pathologies. *J. Alzheimer's Dis.* **2012**, *31* (3), 601–612.
- (74) Maruyama, M.; Shimada, H.; Suhara, T.; Shinotoh, H.; Ji, B.; Maeda, J.; Zhang, M.-R.; Trojanowski, J. Q.; Lee, V. M.-Y.; Ono, M.; Masamoto, K.; Takano, H.; Sahara, N.; Iwata, N.; Okamura, N.; Furumoto, S.; Kudo, Y.; Chang, Q.; Saido, T. C.; Takashima, A.; Lewis, J.; Jang, M.-K.; Aoki, I.; Ito, H.; Higuchi, M. Imaging of Tau Pathology in a Tauopathy Mouse Model and in Alzheimer Patients Compared to Normal Controls. *Neuron* **2013**, *79* (6), 1094–1108.
- (75) Chandra, A.; Valkimadi, P.; Pagano, G.; Cousins, O.; Dervenoulas, G.; Politis, M. Applications of Amyloid, Tau, and Neuroinflammation PET Imaging to Alzheimer's Disease and Mild Cognitive Impairment. *Hum. Brain Mapp.* **2019**, *40* (18), 5424–5442.
- (76) Agüero, C.; Dhaynaut, M.; Normandin, M. D.; Amaral, A. C.; Guehl, N. J.; Neelamegam, R.; Marquie, M.; Johnson, K. A.; El Fakhri, G.; Frosch, M. P.; Gomez-Isla, T. Autoradiography Validation of Novel Tau PET Tracer [18F]-MK-6240 on Human Postmortem Brain Tissue. *Acta Neuropathol. Commun.* **2019**, *7* (1), 37.
- (77) Sanabria Bohórquez, S.; Marik, J.; Ogasawara, A.; Tinianow, J. N.; Gill, H. S.; Barret, O.; Tamagnan, G.; Alagille, D.; Ayalon, G.; Manser, P.; Bengtsson, T.; Ward, M.; Williams, S. P.; Kerchner, G. A.; Seibyl, J. P.; Marek, K.; Weimer, R. M. [18F]GTP1 (Genentech Tau Probe 1), a Radioligand for Detecting Neurofibrillary Tangle Tau Pathology in Alzheimer's Disease. *Eur. J. Nucl. Med. Mol. Imaging* **2019**, *46* (10), 2077–2089.
- (78) Shimada, H.; Kitamura, S.; ONO, M.; Kimura, Y.; Ichise, M.; Takahata, K.; Moriguchi, S.; Kubota, M.; Ishii, T.; Takado, Y.; Seki, C.; Hirano, S.; Shinotoh, H.; Sahara, N.; Tempest, P.; Tamagnan, G.; Seibyl, J.; Barret, O.; Alagille, D.; Zhang, M.-R.; Kuwabara, S.; Jang, M.-K.; Marek, K.; Suhara, T.; Higuchi, M. [P3-378]: FIRST-IN-HUMAN PET STUDY WITH ¹⁸F-AM-PBB3 AND ¹⁸F-PM-PBB3. *Alzheimer's Dement.* **2017**, *13* (7S_Part_23), P1104–P1104.
- (79) Arriagada, P. V.; Growdon, J. H.; Hedley-Whyte, E. T.; Hyman, B. T. Neurofibrillary Tangles but Not Senile Plaques Parallel Duration and Severity of Alzheimer's Disease. *Neurology* **1992**, *42* (3), 631–639.
- (80) Li, Z.; Conti, P. S. Radiopharmaceutical Chemistry for Positron Emission Tomography. *Adv. Drug Deliv. Rev.* **2010**, *62* (11), 1031–1051.
- (81) Villemagne, V. L.; Fodero-Tavoletti, M. T.; Masters, C. L.; Rowe, C. C. Tau Imaging: Early Progress and Future Directions. *Lancet Neurol.* **2015**, *14* (1), 114–124.
- (82) Bernard-Gauthier, V.; Aliaga, A.; Aliaga, A.; Boudjemeline, M.; Hopewell, R.; Kostikov, A.; Rosa-Neto, P.; Thiel, A.; Schirrmacher, R. Syntheses and Evaluation of Carbon-11- and Fluorine-18-Radiolabeled Pan-Tropomyosin Receptor Kinase (Trk) Inhibitors: Exploration of the 4-Aza-2-

- Oxindole Scaffold as Trk PET Imaging Agents. *ACS Chem. Neurosci.* **2015**, *6* (2), 260–276.
- (83) Declercq, L.; Celen, S.; Lecina, J.; Ahamed, M.; Tousseyn, T.; Moechars, D.; Alcazar, J.; Ariza, M.; Fierens, K.; Bottelbergs, A.; Mariën, J.; Vandenberghe, R.; Andres, I. J.; Van Laere, K.; Verbruggen, A.; Bormans, G. Comparison of New Tau PET-Tracer Candidates With [18F]T808 and [18F]T807. *Mol. Imaging* **2016**, *15*, 1–15.
- (84) Rival, Y.; Grassy, G.; Michel, G. Synthesis and Antibacterial Activity of Some Imidazo[1,2-a]Pyrimidine Derivatives. *Chem. Pharm. Bull.* **1992**, *40* (5), 1170–1176.
- (85) Fisher, M. H.; Lusi, A. Imidazo[1,2-a]Pyridine Anthelmintic and Antifungal Agents. *J. Med. Chem.* **1972**, *15* (9), 982–985.
- (86) Kaminski, J. J.; Dowejko, A. M. Antiulcer Agents. 6. Analysis of the in Vitro Biochemical and in Vivo Gastric Antisecretory Activity of Substituted Imidazo[1,2-a]Pyridines and Related Analogues Using Comparative Molecular Field Analysis and Hypothetical Active Site Lattice Methodologies. *J. Med. Chem.* **1997**, *40* (4), 427–436.
- (87) Kotovskaya, S. K.; Baskakova, Z. M.; Charushin, V. N.; Chupakhin, O. N.; Belanov, E. F.; Bormotov, N. I.; Balakhnin, S. M.; Serova, O. A. Synthesis and Antiviral Activity of Fluorinated Pyrido [1,2-a] Benzimidazoles. *Pharm. Chem. J.* **2005**, *39* (11), 574–578.
- (88) Rupert, K. C.; Henry, J. R.; Dodd, J. H.; Wadsworth, S. A.; Cavender, D. E.; Olini, G. C.; Fahmy, B.; Siekierka, J. J. Imidazopyrimidines, Potent Inhibitors of P38 MAP Kinase. *Bioorganic Med. Chem. Lett.* **2003**, *13* (3), 347–350.
- (89) Humphries, A. C.; Gancia, E.; Gilligan, M. T.; Goodacre, S.; Hallett, D.; Merchant, K. J.; Thomas, S. R. 8-Fluoroimidazo[1,2-a]Pyridine: Synthesis, Physicochemical Properties and Evaluation as a Bioisosteric Replacement for Imidazo[1,2-a]Pyrimidine in an Allosteric Modulator Ligand of the GABAA Receptor. *Bioorganic Med. Chem. Lett.* **2006**, *16* (6), 1518–1522.
- (90) Davey, D.; Erhardt, P. W.; Lumma, W. C.; Wiggins, J.; Cantor, E.; Sullivan, M.; Pang, D. Cardiotonic Agents. 1. Novel 8-Aryl-Substituted Imidazo[1,2-a]- and -[1,5-a]Pyridines and Imidazo[1,5-a]Pyridinones as Potential Positive Inotropic Agents. *J. Med. Chem.* **1987**, *30* (8), 1337–1342.
- (91) Fookes, C. J. R.; Pham, T. Q.; Mattner, F.; Greguric, I.; Loc'h, C.; Liu, X.; Berghofer, P.; Shepherd, R.; Gregoire, M. C.; Katsifis, A. Synthesis and Biological Evaluation of Substituted [18F] Imidazo[1,2-a]Pyridines and [18F]Pyrzolo[1,5-a]Pyrimidines for the Study of the Peripheral Benzodiazepine Receptor Using Positron Emission Tomography. *J. Med. Chem.* **2008**, *51* (13), 3700–3712.
- (92) Mizushige, K.; Ueda, T.; Yukiiri, K.; Suzuki, H. Olprinone: A Phosphodiesterase III Inhibitor with Positive Inotropic and Vasodilator Effects. *Cardiovasc. Drug Rev.* **2002**, *20* (3), 163–174.
- (93) Almirante, L.; Polo, L.; Mugnaini, A.; Provixciali, E.; Plugarli, P.; Biacxotti, A.; Gamba, A.; Murmaxx, W. Derivatives of Imidazole. I. Synthesis and Reactions of Imidazo[1,2-a]Pyridines with Analgesic, Antiinflammatory, Antipyretic, and Anticonvulsant Activity. *J. Med. Chem.* **1965**, *8* (3), 305–312.
- (94) Langer, S. Z.; Arbilla, S.; Benavides, J.; Scatton, B. Zolpidem and Alpidem: Two Imidazopyridines with Selectivity for Omega 1- and Omega 3-Receptor Subtypes. *Adv. Biochem. Psychopharmacol.* **1990**, *46*, 61–72.

- (95) GUDMUNDSSON, K.; BOGGS, S. D. CHEMICAL COMPOUNDS. WO/2006/026703, March 9, 2006.
- (96) Bagdi, A. K.; Santra, S.; Monir, K.; Hajra, A. Synthesis of Imidazo[1,2-a]Pyridines: A Decade Update. *Chem. Commun. (Camb)*. **2015**, 51 (9), 1555–1575.
- (97) Pericherla, K.; Kaswan, P.; Pandey, K.; Kumar, A. Recent Developments in the Synthesis of Imidazo[1,2-a]Pyridines. *Synth*. **2015**, 47 (7), 887–912.
- (98) Zhu, D.-J.; Chen, J.-X.; Liu, M.-C.; Ding, J.-C.; Wu, H.-Y. *Catalyst-and Solvent-Free Synthesis of Imidazo[1,2-a]Pyridines*; 2009; Vol. 20.
- (99) Yadav, J. S.; Subba Reddy, B. V.; Gopal Rao, Y.; Srinivas, M.; Narsaiah, A. V. Cu(OTf)₂-Catalyzed Synthesis of Imidazo[1,2-a]Pyridines from α -Diazoketones and 2-Aminopyridines. *Tetrahedron Lett*. **2007**, 48 (43), 7717–7720.
- (100) Xie, Y. Y.; Chen, Z. C.; Zheng, Q. G. Organic Reactions in Ionic Liquids: Ionic Liquid-Accelerated Cyclocondensation of α -Tosyloxyketones with 2-Aminopyridine. *Synthesis (Stuttg)*. **2002**, 2002 (11), 1505–1508.
- (101) Liu, Z.; Chen, Z.-C.; Zheng, Q.-G. Hypervalent Iodine in Synthesis. 94. A Facile Synthesis of 2-Substituted-Imidazo[1,2-a]Pyridines by Cyclocondensation of Alkynyl(Phenyl) Iodonium Salts and 2-Aminopyridine. *Synth. Commun*. **2004**, 34 (2), 361–367.
- (102) Wu, Z.; Pan, Y.; Zhou, X. Synthesis of 3-Arylimidazo[1,2-a]Pyridines by a Catalyst-Free Cascade Process. *Synthesis (Stuttg)*. **2011**, 2011 (14), 2255–2260.
- (103) Yan, H.; Yang, S.; Gao, X.; Zhou, K.; Ma, C.; Yan, R.; Huang, G. Iron(II)-Catalyzed Denitration Reaction: Synthesis of 3-Methyl-2-Arylimidazo[1,2-a]Pyridine Derivatives from Aminopyridines and 2-Methylnitroolefins. *Synlett* **2012**, 23 (20), 2961–2964.
- (104) Santra, S.; Bagdi, A. K.; Majee, A.; Hajra, A. Iron(III)-Catalyzed Cascade Reaction between Nitroolefins and 2-Aminopyridines: Synthesis of Imidazo[1,2-a]Pyridines and Easy Access towards Zolimidine. *Adv. Synth. Catal*. **2013**, 355 (6), 1065–1070.
- (105) Yan, H.; Wang, Y.; Pan, C.; Zhang, H.; Yang, S.; Ren, X.; Li, J.; Huang, G. Iron(III)-Catalyzed Denitration Reaction: One-Pot Three-Component Synthesis of Imidazo[1,2-a]Pyridine Derivatives. *European J. Org. Chem*. **2014**, 2754–2763.
- (106) DiMauro, E. F.; Kennedy, J. M. Rapid Synthesis of 3-Amino-Imidazopyridines by a Microwave-Assisted Four-Component Coupling in One Pot. *J. Org. Chem*. **2007**, 72 (3), 1013–1016.
- (107) Chernyak, N.; Gevorgyan, V. General and Efficient Copper-Catalyzed Three-Component Coupling Reaction towards Imidazoheterocycles: One-Pot Synthesis of Alpidem and Zolpidem. *Angew. Chemie - Int. Ed*. **2010**, 49 (15), 2743–2746.
- (108) Wang, H.; Wang, Y.; Liang, D.; Liu, L.; Zhang, J.; Zhu, Q. Copper-Catalyzed Intramolecular Dehydrogenative Aminooxygenation: Direct Access to Formyl-Substituted Aromatic N-Heterocycles. *Angew. Chemie - Int. Ed*. **2011**, 50 (25), 5678–5681.
- (109) Chandra Mohan, D.; Nageswara Rao, S.; Adimurthy, S. Synthesis of Imidazo[1,2-a]Pyridines: “Water-Mediated” Hydroamination and Silver-Catalyzed Aminooxygenation. *J. Org. Chem*. **2013**, 78 (3), 1266–1272.

- (110) Wang, H.; Wang, Y.; Peng, C.; Zhang, J.; Zhu, Q. A Direct Intramolecular C-H Amination Reaction Cocatalyzed by Copper(II) and Iron(III) as Part of an Efficient Route for the Synthesis of Pyrido[1,2-*a*]Benzimidazoles from N-Aryl-2-Aminopyridines. *J. Am. Chem. Soc.* **2010**, *132* (38), 13217–13219.
- (111) Masters, K.; Rauws, T. R. M.; Yadav, A. K.; Herrebout, W. A.; Van der Veken, B.; Maes, B. U. W. On the Importance of an Acid Additive in the Synthesis of Pyrido[1,2-*a*]Benzimidazoles by Direct Copper-Catalyzed Amination. *Chem. – A Eur. J.* **2011**, *17* (23), 6315–6320.
- (112) Donohoe, T. J.; Kabeshov, M. A.; Rathi, A. H.; Smith, I. E. D. Direct Preparation of Thiazoles, Imidazoles, Imidazopyridines and Thiazolidines from Alkenes. *Org. Biomol. Chem.* **2012**, *10* (5), 1093–1101.
- (113) Yan, R. L.; Yan, H.; Ma, C.; Ren, Z. Y.; Gao, X. A.; Huang, G. S.; Liang, Y. M. Cu(I)-Catalyzed Synthesis of Imidazo[1,2-*a*]Pyridines from Aminopyridines and Nitroolefins Using Air as the Oxidant. *J. Org. Chem.* **2012**, *77* (4), 2024–2028.
- (114) Bagdi, A. K.; Rahman, M.; Santra, S.; Majee, A.; Hajra, A. Copper-Catalyzed Synthesis of Imidazo[1,2-*a*]Pyridines through Tandem Imine Formation-Oxidative Cyclization under Ambient Air: One-Step Synthesis of Zolimidine on a Gram-Scale. *Adv. Synth. Catal.* **2013**, *355* (9), 1741–1747.
- (115) Chandra Mohan, D.; Reddy Donthiri, R.; Nageswara Rao, S.; Adimurthy, S. Copper(I) Iodide-Catalysed Aerobic Oxidative Synthesis of Imidazo[1,2-*a*]Pyridines from 2-Aminopyridines and Methyl Ketones. *Adv. Synth. Catal.* **2013**, *355* (11–12), 2217–2221.
- (116) Monir, K.; Kumar Bagdi, A.; Mishra, S.; Majee, A.; Hajra, A. Copper(II)-Catalyzed Aerobic Oxidative Coupling between Chalcone and 2-Aminopyridine *via* C–H Amination: An Expedient Synthesis of 3-Aroylimidazo[1,2-*a*]Pyridines. *Adv. Synth. Catal.* **2014**, *356* (5), 1105–1112.
- (117) Zhu, H.; Shao, N.; Chen, T.; Zou, H. Functionalized Heterocyclic Scaffolds Derived from Morita-Baylis-Hillman Acetates. *Chem. Commun.* **2013**, *49* (70), 7738–7740.
- (118) Bakherad, M.; Nasr-Isfahani, H.; Keivanloo, A.; Doostmohammadi, N. Pd-Cu Catalyzed Heterocyclization during Sonogashira Coupling: Synthesis of 2-Benzylimidazo[1,2-*a*]Pyridine. *Tetrahedron Lett.* **2008**, *49* (23), 3819–3822.
- (119) Enguehard, C.; Hervet, M.; Théry, I.; Renou, J.-L.; Fauvelle, F.; Gueiffier, A. (Hetero)Arylation of 6-Halogenoimidazo[1,2-*a*]Pyridines Differently Substituted at C(2): Influence of the 2-Substituent on TheSuzuki Cross-Coupling Reaction. *Helv. Chim. Acta* **2001**, *84* (12), 3610–3615.
- (120) Elahi, F.; Adeel, M.; Tahir, M. N. *4-(2-Fluoropyridin-5-Yl)Phenol*; International Union of Crystallography, 2012; Vol. 68.
- (121) Kim, J.; Lee, D.; Park, C.; So, W.; Jo, M.; Ok, T.; Kwon, J.; Kong, S.; Jo, S.; Kim, Y.; Choi, J.; Kim, H. C.; Ko, Y.; Choi, I.; Park, Y.; Yoon, J.; Moon, J.; Ju, K.; Kim, J.; Han, S.-J.; Kim, T.-H.; Cechetto, J.; Nam, J.; Sommer, P.; Liuzzi, M.; Lee, J.; No, Z. Discovery of Phenylaminopyridine Derivatives as Novel HIV-1 Non- Nucleoside Reverse Transcriptase Inhibitors. *ACS Med. Chem. Lett.* **2012**, 678–682.
- (122) Mou, J.; Park, A.; Cai, Y.; Yuan, J.; Yuan, C. Structure and Activity Relationship Study of E6 as a Novel Necroptosis Inducer. *Bioorg. Med. Chem. Lett.* **2015**, 3057–3061.

- (123) Sherwood, J.; Clark, J. H.; Fairlamb, I. J. S.; Slattery, J. M. Solvent Effects in Palladium Catalysed Cross-Coupling Reactions. *Green Chem.* **2019**, *21* (9), 2164–2213.
- (124) FisherScientific. Palladium-Catalysed Coupling Chemistry. 2010, pp 1–15.
- (125) Primas, N.; Bouillon, A.; Lancelot, J.-C.; El-Kashef, H.; Rault, S. Synthesis of 5-Arylthiazoles. Comparative Study between Suzuki Cross-Coupling Reaction and Direct Arylation. *Tetrahedron* **2009**, *65* (29), 5739–5746.
- (126) Teulade, J.-C.; Moreau, E.; Chezal, J.-M.; Dechambre, C.; Canitrot, D.; Blache, Y.; Lartigue, C.; Chavignon, O. Efficient Synthesis of New Polyfunctionalized Thiadiazacacenaphthylenes from Imidazo[1,2-a]Pyridines. *Heterocycles* **2002**, *57* (1), 21–38.
- (127) Wang, Y.; Frett, B.; Li, H.-Y. Efficient Access to 2, 3-Diarylimidazo [1, 2-a] Pyridines via a One-Pot, Ligand-Free, Palladium-Catalyzed Three-Component Reaction under Microwave Irradiation. *Org. Lett.* **2014**, *16* (11), 3016–3019.
- (128) Kurti, L.; Czako, B. *Strategic Applications of Named Reactions in Organic Synthesis: Background and Detailed Mechanisms*; Elsevier, 2006; Vol. 44.
- (129) Goud, N. S.; Joshi, R. K.; Bharath, R. D.; Kumar, P. Fluorine-18: A Radionuclide with Diverse Range of Radiochemistry and Synthesis Strategies for Target Based PET Diagnosis. *Eur. J. Med. Chem.* **2020**, 187.
- (130) Cai, L.; Lu, S.; Pike, V. W. Chemistry with [18F]Fluoride Ion. *European J. Org. Chem.* **2008**, No. 17, 2853–2873.
- (131) Lasne, M.-C.; Perrio, C.; Rouden, J.; Barré, L.; Roeda, D.; Dolle, F.; Crouzel, C. Chemistry of β + -Emitting Compounds Based on Fluorine-18. In *Contrast Agents II*; Springer, Berlin, Heidelberg, 2002; pp 201–258.
- (132) Goedert, M.; Spillantini, M. G. Ordered Assembly of Tau Protein and Neurodegeneration. In *Tau Biology*; Takashima, A., Wolozin, B., Buee, L., Eds.; Springer Singapore: Singapore, 2019; pp 3–21.
- (133) Kidd, M. Paired Helical Filaments in Electron Microscopy of Alzheimer's Disease. *Nature* **1963**, *197* (4863), 192–193.
- (134) Xu, S.; Brunden, K. R.; Trojanowski, J. Q.; Lee, V. M. Y. Characterization of Tau Fibrillization in Vitro. *Alzheimer's Dement.* **2010**, *6* (2), 110–117.
- (135) Crespo, R.; Koudstaal, W.; Apetri, A. *In Vitro* Assay for Studying the Aggregation of Tau Protein and Drug Screening. *J. Vis. Exp.* **2018**, No. 141, 1–10.
- (136) Wilson, D. M.; Binder, L. I. Free Fatty Acids Stimulate the Polymerization of Tau and Amyloid β Peptides: In Vitro Evidence for a Common Effector of Pathogenesis in Alzheimer's Disease. *Am. J. Pathol.* **1997**, *150* (6), 2181–2195.
- (137) Pérez, M.; Valpuesta, J. M.; Medina, M.; Montejo de Garcini, E.; Avila, J. Polymerization of τ into Filaments in the Presence of Heparin: The Minimal Sequence Required for τ - τ Interaction. *J. Neurochem.* **1996**, *67* (3), 1183–1190.
- (138) Arnaut, L. G.; Formosinho, S. J.; Burrows, H. *Chemical Kinetics : From Molecular Structure to Chemical Reactivity*; Elsevier, 2007.

- (139) Wang, W.; Nema, S.; Teagarden, D. Protein Aggregation-Pathways and Influencing Factors. *International Journal of Pharmaceutics*. Elsevier May 10, 2010, pp 89–99.
- (140) Maeda, S.; Sahara, N.; Saito, Y.; Murayama, M.; Yoshiike, Y.; Kim, H.; Miyasaka, T.; Murayama, S.; Ikai, A.; Takashima, A. Granular Tau Oligomers as Intermediates of Tau Filaments. *Biochemistry* **2007**, *46*, 3856–3861.
- (141) Despres, C.; Byrne, C.; Qi, H.; Cantrelle, F.; Huvent, I.; Chambraud, B. Identification of the Tau Phosphorylation Pattern That Drives Its Aggregation. *PNAS Early Ed.* **2017**, 1–6.
- (142) Sui, D.; Liu, M.; Kuo, M.-H. In Vitro Aggregation Assays Using Hyperphosphorylated Tau Protein. *J. Vis. Exp.* **2015**, No. 95, 1–9.
- (143) Baggett, D. W.; Nath, A. The Rational Discovery of a Tau Aggregation Inhibitor. *Biochemistry* **2018**, *57* (42), 6099–6107.
- (144) Takashima, A.; Wolozin, B.; Buee, L. *Tau Biology*; Takashima, A., Wolozin, B., Buee, L., Eds.; Springer Singapore: Singapore, 2019.
- (145) Sigurdsson, E. M.; Calero, M.; Gasset, M. *Methods in Molecular Biology: Amyloid Proteins*; 2008; Vol. 457.
- (146) Principle and Protocol of Surface Plasmon Resonance (SPR) - Creative BioMart <https://www.creativebiomart.net/resource/principle-and-protocol-of-surface-plasmon-resonance-spr-361.htm> (accessed May 28, 2020).
- (147) Zhang, X.; Hou, Y.; Peng, C.; Wang, C.; Wang, X.; Liang, Z.; Lu, J.; Chen, B.; Dai, J.; Liu, B.; Cui, M. Oligoethyleneoxy-Modified 99m Tc-Labeled β -Amyloid Imaging Probes with Improved Brain Pharmacokinetics for Single-Photon Emission Computed Tomography. *J. Med. Chem.* **2018**, *61* (3), 1330–1339.
- (148) Fascio, M. L.; Errea, M. I.; D'Accorso, N. B. Imidazothiazole and Related Heterocyclic Systems. Synthesis, Chemical and Biological Properties. *Eur. J. Med. Chem.* **2015**, *90*, 666–683.
- (149) Kamal, A.; Khanna, G. B. R.; Krishnaji, T.; Ramu, R. A New Facile Chemoenzymatic Synthesis of Levamisole. *Bioorganic Med. Chem. Lett.* **2005**, *15* (3), 613–615.
- (150) Mas Prio, J.; Torrens Jover, A. Substituted N-Imidazo[2,1-b]Thiazole-5-Sulfonamide Derivatives as 5-Ht6 Ligands. US20110105555A1, May 8, 2009.
- (151) Ahswell, M. A.; Tandon, M. Synthesis of Imidazooxazole and Imidazothiazole Inhibitors of P38 MAP Kinase. US8178672B2, October 19, 2005.
- (152) Li, Y.; Giulianotti, M.; Houghten, R. A. High Throughput Synthesis of 2,3,6-Trisubstituted-5,6-Dihydroimidazo[2,1-b] Thiazole Derivatives. *Tetrahedron Lett.* **2011**, *52* (6), 696–698.
- (153) Taher, A. T.; Georgey, H. H.; El-Subbagh, H. I. Novel 1,3,4-Heterodiazole Analogues: Synthesis and in-Vitro Antitumor Activity. *Eur. J. Med. Chem.* **2012**, *47* (1), 445–451.
- (154) Swathi, N.; Durai Ananda Kumar, T.; Haribabu, K.; Subrahmanyam, C. V. S. Synthesis and Biological Evaluation of Substituted Thiazolamines, Imidazo [2,1b] Thiazol-6-(5h)-Ones, Thiazolo [3,2-a] Pyrimidin-5-Ones and Thiazolyl Thioureas. *Indian J. Heterocycl. Chem.* **2012**, *21* (3), 263–268.

- (155) Park, J. H.; El-Gamal, M. I.; Lee, Y. S.; Oh, C. H. New Imidazo[2,1-b]Thiazole Derivatives: Synthesis, in Vitro Anticancer Evaluation, and in Silico Studies. *Eur. J. Med. Chem.* **2011**, *46* (12), 5769–5777.
- (156) Kamali, T. A.; Bakherad, M.; Nasrollahzadeh, M.; Farhangi, S.; Habibi, D. Synthesis of 6-Substituted Imidazo[2,1-b]Thiazoles via Pd/Cu-Mediated Sonogashira Coupling in Water. *Tetrahedron Lett.* **2009**, *50* (39), 5459–5462.
- (157) Zhu, Y.-S.; Shi, B.; Fang, R.; Wang, X.; Jing, H. Palladium-Catalyzed Microwave-Assisted Direct Arylation of Imidazo[2,1-b]Thiazoles with Aryl Bromides: Synthesis and Mechanistic Study. *Org. Biomol. Chem.* **2014**, *12* (30), 5773.
- (158) Andreani, A.; Mireia, R.; Daniela, B.; Lell, i G. Substituted 6-Phenylimidazo[2,1-b]Thiazoles and Thiazolines as Potential Cardiogenic Agents. *Eur. J. Med. Chem.* **1984**, *19*, 219–222.
- (159) Verwilt, P.; Kim, H. S.; Kim, S.; Kang, C.; Kim, J. S. Shedding Light on Tau Protein Aggregation: The Progress in Developing Highly Selective Fluorophores. *Chem. Soc. Rev.* **2018**, *47*, 2249.
- (160) Arosio, P.; Knowles, T. P. J.; Linse, S. On the Lag Phase in Amyloid Fibril Formation. *Phys. Chem. Chem. Phys.* **2015**, *17* (12), 7606–7618.
- (161) Levine, H. Stopped-Flow Kinetics Reveal Multiple Phases of Thioflavine T Binding to Alzheimer β (1–40) & Insulin Amyloid. *Neurobiol. Aging* **2003**, *15* (2), S80.
- (162) Harry Levine. Quantification of Beta-Sheet Amyloid Fibril Structures with Thioflavin T. *Methods Enzymol.* **1999**, *309* (18), 274–284.
- (163) Bouchard, M.; Zurdo, J.; Nettleton, E. J.; Dobson, C. M.; Robinson, C. V. Formation of Insulin Amyloid Fibrils Followed by FTIR Simultaneously with CD and Electron Microscopy. *Protein Sci.* **2000**, *9*, 1960–1967.
- (164) Kalhor, H. R.; Yahyazadeh, A. Investigating the Effects of Amino Acid-Based Surface Modification of Carbon Nanoparticles on the Kinetics of Insulin Amyloid Formation. *Colloids Surfaces B Biointerfaces* **2019**, *176*, 471–479.
- (165) Baggett, D. W.; Nath, A. The Rational Discovery of a Tau Aggregation Inhibitor. *Biochemistry* **2018**, *57* (42), 6099–6107.
- (166) Lockhart, A.; Ye, L.; Judd, D. B.; Merritt, A. T.; Lowe, P. N.; Morgenstern, J. L.; Hong, G.; Gee, A. D.; Brown, J. Evidence for the Presence of Three Distinct Binding Sites for the Thioflavin T Class of Alzheimer's Disease PET Imaging Agents on β -Amyloid Peptide Fibrils. *J. Biol. Chem.* **2005**, *280* (9), 7677–7684.
- (167) Jensen, J. R.; Cisek, K.; Honson, N. S.; Kuret, J. Ligand Polarizability Contributes to Tau Fibril Binding Affinity. *Bioorganic Med. Chem.* **2011**, *19* (17), 5147–5154.
- (168) Lemoine, L.; Gillberg, P. G.; Svedberg, M.; Stepanov, V.; Jia, Z.; Huang, J.; Nag, S.; Tian, H.; Ghetti, B.; Okamura, N.; Higuchi, M.; Halldin, C.; Nordberg, A. Comparative Binding Properties of the Tau PET Tracers THK5117, THK5351, PBB3, and T807 in Postmortem Alzheimer Brains. *Alzheimer's Res. Ther.* **2017**, *9* (1).
- (169) Lemoine, L.; Saint-Aubert, L.; Marutle, A.; Antoni, G.; Eriksson, J. P.; Ghetti, B.; Okamura, N.; Nennesmo, I.; Gillberg, P. G.; Nordberg, A. Visualization of Regional Tau Deposits Using (3)H-

- THK5117 in Alzheimer Brain Tissue. *Acta Neuropathol. Commun.* **2015**, 3 (1), 40.
- (170) Murugan, N. A.; Nordberg, A.; Ågren, H. Different Positron Emission Tomography Tau Tracers Bind to Multiple Binding Sites on the Tau Fibril: Insight from Computational Modeling. *ACS Chem. Neurosci.* **2018**, 9 (7), 1757–1767.
- (171) Marabotti, A.; Balestreri, L.; Cozzini, P.; Mozzarelli, A.; Kellogg, G. E.; Abraham, D. J. HINT Predictive Analysis of Binding between Retinol Binding Protein and Hydrophobic Ligands. *Bioorganic Med. Chem. Lett.* **2000**, 10 (18), 2129–2132.
- (172) Kellogg, G. E.; Burnett, J. C.; Abraham, D. J. Very Empirical Treatment of Solvation and Entropy: A Force Field Derived from Log Po/W. *J. Comput. Aided. Mol. Des.* **2001**, 15 (4), 381–393.
- (173) Cozzini, P.; Fornabaio, M.; Marabotti, A.; Abraham, D. J.; Kellogg, G. E.; Mozzarelli, A. Simple, Intuitive Calculations of Free Energy of Binding for Protein-Ligand Complexes. 1. Models without Explicit Constrained Water. *J. Med. Chem.* **2002**, 45 (12), 2469–2483.
- (174) Ono, M.; Sahara, N.; Kumata, K.; Ji, B.; Ni, R.; Koga, S.; Dickson, D. W.; Trojanowski, J. Q.; Lee, V. M. Y.; Yoshida, M.; Hozumi, I.; Yoshiyama, Y.; Van Swieten, J. C.; Nordberg, A.; Suhara, T.; Zhang, M. R.; Higuchi, M. Distinct Binding of PET Ligands PBB3 and AV-1451 to Tau Fibril Strains in Neurodegenerative Tauopathies. *Brain* **2017**, 140 (3), 764–780.
- (175) Amin, K.; Dannenfelser, R. M. In Vitro Hemolysis: Guidance for the Pharmaceutical Scientist. *J. Pharm. Sci.* **2006**, 95 (6), 1173–1176.
- (176) Xi, J. B.; Fang, Y. F.; Frett, B.; Zhu, M. L.; Zhu, T.; Kong, Y. N.; Guan, F. J.; Zhao, Y.; Zhang, X. W.; Li, H. yu; Ma, M. L.; Hu, W. Structure-Based Design and Synthesis of Imidazo[1,2-a]Pyridine Derivatives as Novel and Potent Nek2 Inhibitors with in Vitro and in Vivo Antitumor Activities. *Eur. J. Med. Chem.* **2017**, 126 (2017), 1083–1106.
- (177) Price, T. W.; Firth, G.; Eling, C. J.; Kinnon, M.; Long, N. J.; Sturge, J.; Stasiuk, G. J. A 18F Radiolabelled Zn(II) Sensing Fluorescent Probe. *Chem. Commun.* **2018**, 54 (26), 3227–3230.
- (178) Glycine-HCl Buffer (0.1 M, pH 3.0) Preparation and Recipe | AAT Bioquest <https://www.aatbio.com/resources/buffer-preparations-and-recipes/glycine-hcl-buffer-ph-3> (accessed Jul 21, 2020).
- (179) Katebi, B.; Mahdavimehr, M.; Meratan, A. A.; Ghasemi, A.; Nemat-Gorgani, M. Protective Effects of Silibinin on Insulin Amyloid Fibrillation, Cytotoxicity and Mitochondrial Membrane Damage. *Arch. Biochem. Biophys.* **2018**, 659 (August), 22–32.
- (180) Pickhardt, M.; Larbig, G.; Khlistunova, I.; Coksezen, A.; Meyer, B.; Mandelkow, E. M.; Schmidt, B.; Mandelkow, E. Phenylthiazolyl-Hydrazide and Its Derivatives Are Potent Inhibitors of Tau Aggregation and Toxicity in Vitro and in Cells. *Biochemistry* **2007**, 46, 10016–10023.
- (181) Rojo, L. E.; Alzate-Morales, J.; Saavedra, I. N.; Davies, P.; MacCioni, R. B. Selective Interaction of Lansoprazole and Astemizole with Tau Polymers: Potential New Clinical Use in Diagnosis of Alzheimer's Disease. *J. Alzheimer's Dis.* **2010**.
- (182) Dellafiora, L.; Mena, P.; Cozzini, P.; Brighenti, F.; Del Rio, D. Modelling the Possible Bioactivity of Ellagitannin-Derived Metabolites. in Silico Tools to Evaluate Their Potential Xenoestrogenic Behavior. *Food Funct.* **2013**, 4 (10), 1442–1451.

- (183) Ehrlich, V. A.; Dellaflora, L.; Mollergues, J.; Dall'Asta, C.; Serrant, P.; Marin-Kuan, M.; Lo Piparo, E.; Schilter, B.; Cozzini, P. Hazard Assessment through Hybrid in Vitro/in Silico Approach: The Case of Zearalenone. *Altex- Altern. to Anim. Exp.* **2015**, 32 (4), 275–286.
- (184) Evans, B. C.; Nelson, C. E.; Yu, S. S.; Beavers, K. R.; Kim, A. J.; Li, H.; Nelson, H. M.; Duvall, C. L. Ex Vivo Red Blood Cell Hemolysis Assay for the Evaluation of PH-Responsive Endosomolytic Agents for Cytosolic Delivery of Biomacromolecular Drugs. *J. Vis. Exp.* **2013**, No. March, 7–11.
- (185) Lu, J.; Tan, M.; Cai, Q. The Warburg Effect in Tumor Progression: Mitochondrial Oxidative Metabolism as an Anti-Metastasis Mechanism. *Cancer Lett.* **2015**, 356 (2), 156–164.
- (186) Glycolysis - CNX, Biology - OpenStax https://cnx.org/contents/GFy_h8cu@11.10:tYtpI6rX@6/Glycolysis (accessed Apr 28, 2020).
- (187) Oxidative Phosphorylation - CNX, Biology - OpenStax https://cnx.org/contents/GFy_h8cu@11.6:7oTVAgZ@7/Oxidative-Phosphorylation (accessed Apr 28, 2020).
- (188) Ciclo del ácido cítrico y fosforilación oxidativa - CNX, Biology - OpenStax <https://cnx.org/contents/56AW05H8@13.4:K9cY927B@10/Ciclo-del-ácido-cítrico-y-fosforilación-oxidativa>.
- (189) Warburg, O. On the Origin of Cancer Cells. *Science (80-.)*. **1956**, 123 (3191), 309–314.
- (190) Hsu, P. P.; Sabatini, D. M. Cancer Cell Metabolism: Warburg and Beyond. *Cell* **2008**, 134 (5), 703–707.
- (191) Granchi, C.; Bertini, S.; Macchia, M.; Minutolo, F. Inhibitors of Lactate Dehydrogenase Isoforms and Their Therapeutic Potentials. *Curr. Med. Chem.* **2010**, 17 (7), 672–697.
- (192) Sørensen, B. S.; Alsner, J.; Overgaard, J.; Horsman, M. R. Hypoxia Induced Expression of Endogenous Markers in Vitro Is Highly Influenced by PH. *Radiother. Oncol.* **2007**, 83 (3), 362–366.
- (193) Sørensen, B. S.; Hao, J.; Overgaard, J.; Vorum, H.; Honoré, B.; Alsner, J.; Horsman, M. R. Influence of Oxygen Concentration and PH on Expression of Hypoxia Induced Genes. *Radiother. Oncol.* **2005**, 76 (2), 187–193.
- (194) Neilands, J. B. The Purity of Crystalline Lactic Dehydrogenase. *Science (80-.)*. **1952**, 115 (2980), 143–144.
- (195) McClendon, S.; Zhadin, N.; Callender, R. The Approach to the Michaelis Complex in Lactate Dehydrogenase: The Substrate Binding Pathway. *Biophys. J.* **2005**, 89 (3), 2024–2032.
- (196) McClendon, S.; Vu, D. M.; Clinch, K.; Callender, R.; Dyer, R. B. Structural Transformations in the Dynamics of Michaelis Complex Formation in Lactate Dehydrogenase. *Biophys. J.* **2005**, 89 (1), 7–9.
- (197) Read, J. A.; Winter, V. J.; Eszes, C. M.; Sessions, R. B.; Brady, R. L. Structural Basis for Altered Activity of M- and H-Isozyme Forms of Human Lactate Dehydrogenase. *Proteins* **2001**, 43 (2), 175–185.
- (198) Rao, S. T.; Rossmann, M. G. Comparison of Super-Secondary Structures in Proteins. *J. Mol. Biol.* **1973**, 76 (2), 241–256.

- (199) Tan, B.; Young, D. A.; Lu, Z. H.; Wang, T.; Meier, T. I.; Shepard, R. L.; Roth, K.; Zhai, Y.; Huss, K.; Kuo, M. S.; Gillig, J.; Parthasarathy, S.; Burkholder, T. P.; Smith, M. C.; Geeganage, S.; Zhao, G. Pharmacological Inhibition of Nicotinamide Phosphoribosyltransferase (NAMPT), an Enzyme Essential for NAD⁺ Biosynthesis, in Human Cancer Cells: Metabolic Basis and Potential Clinical Implications. *J. Biol. Chem.* **2013**, *288* (5), 3500–3511.
- (200) Kroemer, G.; Pouyssegur, J. Tumor Cell Metabolism: Cancer's Achilles' Heel. *Cancer Cell* **2008**, *13* (6), 472–482.
- (201) Harris, R. A.; Tindale, L.; Cumming, R. C. Age-Dependent Metabolic Dysregulation in Cancer and Alzheimer's Disease. *Biogerontology* **2014**, *15* (6), 559–577.
- (202) Moeller, B. J.; Richardson, R. A.; Dewhirst, M. W. Hypoxia and Radiotherapy: Opportunities for Improved Outcomes in Cancer Treatment. *Cancer Metastasis Rev.* **2007**, *26* (2), 241–248.
- (203) Brown, J. M.; Wilson, W. R. Exploiting Tumour Hypoxia in Cancer Treatment. *Nat. Rev. Cancer* **2004**, *4* (6), 437–447.
- (204) Lopez-Lazaro, M. Role of Oxygen in Cancer: Looking Beyond Hypoxia. *Anticancer. Agents Med. Chem.* **2012**, *9* (5), 517–525.
- (205) De Milioto, A.; Canese, R.; Marino, M. L.; Borghi, M.; Iero, M.; Villa, A.; Venturi, G.; Lozupone, F.; Iessi, E.; Logozzi, M.; Mina, P. Della; Santinami, M.; Rodolfo, M.; Podo, F.; Rivoltini, L.; Fais, S. PH-Dependent Antitumor Activity of Proton Pump Inhibitors against Human Melanoma Is Mediated by Inhibition of Tumor Acidity. *Int. J. Cancer* **2010**, *127* (1), 207–219.
- (206) Di, H.; Zhang, X.; Guo, Y.; Shi, Y.; Fang, C.; Yuan, Y.; Wang, J.; Shang, C.; Guo, W.; Li, C. Silencing LDHA Inhibits Proliferation, Induces Apoptosis and Increases Chemosensitivity to Temozolomide in Glioma Cells. *Oncol. Lett.* **2018**, *15* (4), 5131–5136.
- (207) Sullivan, R.; Graham, C. H. Hypoxia-Driven Selection of the Metastatic Phenotype. *Cancer Metastasis Rev.* **2007**, *26* (2), 319–331.
- (208) Rong, Y.; Wu, W.; Ni, X.; Kuang, T.; Jin, D.; Wang, D.; Lou, W. Lactate Dehydrogenase A Is Overexpressed in Pancreatic Cancer and Promotes the Growth of Pancreatic Cancer Cells. *Tumor Biol.* **2013**, *34* (3), 1523–1530.
- (209) Hu, S.; Jiang, Q.; Luo, D.; Zhao, L.; Fu, X.; Chen, Y.; Song, X.; Li, L.; Zhao, H.; He, Y.; Peng, B. MiR-200b Is a Key Regulator of Tumor Progression and Metabolism Targeting Lactate Dehydrogenase A in Human Malignant Glioma. *Oncotarget* **2016**, *7* (30), 48423–48431.
- (210) Xian, Z. Y.; Liu, J. M.; Chen, Q. K.; Chen, H. Z.; Ye, C. J.; Xue, J.; Yang, H. Q.; Li, J. L.; Liu, X. F.; Kuang, S. J. Inhibition of LDHA Suppresses Tumor Progression in Prostate Cancer. *Tumor Biol.* **2015**, *36* (10), 8093–8100.
- (211) Brand, A.; Singer, K.; Koehl, G. E.; Kolitzus, M.; Schoenhammer, G.; Thiel, A.; Matos, C.; Bruss, C.; Klobuch, S.; Peter, K.; Kastenberger, M.; Bogdan, C.; Schleicher, U.; Mackensen, A.; Ullrich, E.; Fichtner-Feigl, S.; Kesselring, R.; Mack, M.; Ritter, U.; Schmid, M.; Blank, C.; Dettmer, K.; Oefner, P. J.; Hoffmann, P.; Walenta, S.; Geissler, E. K.; Pouyssegur, J.; Villunger, A.; Steven, A.; Seliger, B.; Schreml, S.; Haferkamp, S.; Kohl, E.; Karrer, S.; Berneburg, M.; Herr, W.; Mueller-Klieser, W.; Renner, K.; Kreutz, M. LDHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance by T and NK Cells. *Cell Metab.* **2016**, *24* (5), 657–671.

- (212) Fantin, V. R.; St-Pierre, J.; Leder, P. Attenuation of LDH-A Expression Uncovers a Link between Glycolysis, Mitochondrial Physiology, and Tumor Maintenance. *Cancer Cell* **2006**, 9 (6), 425–434.
- (213) Haupt, S.; Berger, M.; Goldberg, Z.; Haupt, Y. Apoptosis - The P53 Network. *J. Cell Sci.* **2003**, 116, 4077–4085.
- (214) McConkey, D. J.; Orrenius, S. The Role of Calcium in the Regulation of Apoptosis. *Biochem. Biophys. Res. Commun.* **1997**, 239, 357–366.
- (215) Sheng, S. L.; Liu, J. J.; Dai, Y. H.; Sun, X. G.; Xiong, X. P.; Huang, G. Knockdown of Lactate Dehydrogenase A Suppresses Tumor Growth and Metastasis of Human Hepatocellular Carcinoma. *FEBS J.* **2012**, 279 (20), 3898–3910.
- (216) Maekawa, M.; Sudo, K.; Kanno, T.; Li, S. S. L. Molecular Characterization of Genetic Mutation in Human Lactate Dehydrogenase-A (M) Deficiency. *Biochem. Biophys. Res. Commun.* **1990**, 168 (2), 677–682.
- (217) Kanno, T.; Sudo, K.; Maekawa, M.; Nishimura, Y.; Ukita, M.; Fukutake, K. Lactate Dehydrogenase M-Subunit Deficiency: A New Type of Hereditary Exertional Myopathy. *Clin. Chim. Acta* **1988**, 173 (1), 89–98.
- (218) Doherty, J. R.; Cleveland, J. L.; Doherty, J. R.; Cleveland, J. L. Targeting Lactate Metabolism for Cancer Therapeutics. *J. Clin. Invest.* **2013**, 123 (9), 3685–3692.
- (219) Zhang, S. L.; He, Y.; Tam, K. Y. Targeting Cancer Metabolism to Develop Human Lactate Dehydrogenase (HLDH)5 Inhibitors. *Drug Discov. Today* **2018**, 23 (7), 1407–1415.
- (220) Fortunato, S.; Bononi, G.; Granchi, C.; Minutolo, F. An Update on Patents Covering Agents That Interfere with the Cancer Glycolytic Cascade. *ChemMedChem* **2018**, 13 (21), 2251–2265.
- (221) Woodford, M. R.; Chen, V. Z.; Backe, S. J.; Bratslavsky, G.; Mollapour, M. Structural and Functional Regulation of Lactate Dehydrogenase-A in Cancer. *Future Medicinal Chemistry*. Future Medicine Ltd. March 1, 2020, pp 439–455.
- (222) Deck, L. M.; Royer, R. E.; Chamblee, B. B.; Hernandez, V. M.; Malone, R. R.; Torres, J. E.; Hunsaker, L. A.; Piper, R. C.; Makler, M. T.; Vander Jagt, D. L. Selective Inhibitors of Human Lactate Dehydrogenases and Lactate Dehydrogenase from the Malarial Parasite Plasmodium Falciparum. *J. Med. Chem.* **1998**, 41 (20), 3879–3887.
- (223) Connors, R.; Schambach, F.; Read, J.; Cameron, A.; Sessions, R. B.; Vivas, L.; Easton, A.; Croft, S. L.; Brady, R. L. Mapping the Binding Site for Gossypol-like Inhibitors of Plasmodium Falciparum Lactate Dehydrogenase. *Mol. Biochem. Parasitol.* **2005**, 142 (2), 137–148.
- (224) Le, A.; Cooper, C. R.; Gouw, A. M.; Dinavahi, R.; Maitra, A.; Deck, L. M.; Royer, R. E.; Vander Jagt, D. L.; Semenza, G. L.; Dang, C. V. Inhibition of Lactate Dehydrogenase A Induces Oxidative Stress and Inhibits Tumor Progression. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, 107 (5), 2037–2042.
- (225) Ward, R. A.; Brassington, C.; Breeze, A. L.; Caputo, A.; Critchlow, S.; Davies, G.; Goodwin, L.; Hassall, G.; Greenwood, R.; Holdgate, G. A.; Mrosek, M.; Norman, R. A.; Pearson, S.; Tart, J.; Tucker, J. A.; Vogtherr, M.; Whittaker, D.; Wingfield, J.; Winter, J.; Hudson, K. Design and Synthesis of Novel Lactate Dehydrogenase a Inhibitors by Fragment-Based Lead Generation. *J. Med. Chem.* **2012**, 55 (7), 3285–3306.

- (226) Jinhua Chen Z.; Ding, C.; Peter, B.; Labadie, D.; GAO, Z.; Purkey, S.; Edward, K. W. L. H. Piperidine-Dione Derivatives. WO2015140133A1, 2015.
- (227) Andrea, F. D.; Vagelli, G.; Granchi, C.; Guazzelli, L.; Tuccinardi, T.; Poli, G.; Iacopini, D.; Minutolo, F.; Bussolo, V. Di. Synthesis and Biological Evaluation of New Glycoconjugated LDH Inhibitors as Anticancer Agents. *Molecules* **2019**, *24*, 2–18.
- (228) Minutolo, F.; Macchia, M.; Granchi, C.; Bussolo, V. DI; Giannaccini, G.; Lucacchini, A.; Hergenrother, P. J.; Calvaresi, E. C. Inhibiteurs de Dérivés d'indole de l'enzyme Lactate Déhydrogénase (LDH). WO2013092753 A1, March 24, 2013.
- (229) David J. Maloney, Ajit Jadhav, Ganesha Rai Bantukallu, K. R.; Brimacombe, B. T.; Mott, A. G.; Waterson, Gary Allen Sulikowski, K. K. Small Molecule Inhibitors of Lactate Dehydrogenase and Methods of Use Thereof. WO2016109559A2, December 29, 2015.
- (230) Galezowski, M.; Gondela, A.; Levenets, O.; Fogt, J.; Dudek, L.; Obara, A.; Sowinska, M.; Krol, M.; Biela, A.; Rzymiski, T. Pyrazolidine Derivatives and Related Compounds. WO2017055396, April 6, 2017.
- (231) Rai, G.; Brimacombe, K. R.; Mott, B. T.; Urban, D. J.; Hu, X.; Yang, S. M.; Lee, T. D.; Cheff, D. M.; Kouznetsova, J.; Benavides, G. A.; Pohida, K.; Kuenstner, E. J.; Luci, D. K.; Lukacs, C. M.; Davies, D. R.; Dranow, D. M.; Zhu, H.; Sulikowski, G.; Moore, W. J.; Stott, G. M.; Flint, A. J.; Hall, M. D.; Darley-Usmar, V. M.; Neckers, L. M.; Dang, C. V.; Waterson, A. G.; Simeonov, A.; Jadhav, A.; Maloney, D. J. Discovery and Optimization of Potent, Cell-Active Pyrazole-Based Inhibitors of Lactate Dehydrogenase (LDH). *J. Med. Chem.* **2017**, *60* (22), 9184–9204.
- (232) Brown, K. K.; Chai, D.; Christopher, S.; Dodson, K. J.; Duffy, A.; Shaw, N. Substituted Quinoline Derivatives That Are Inhibitors of the Activity of Lactate Dehydrogenase A. WO2013096153A1, December 17, 2012.
- (233) Kohlmann, A.; Zech, S. G.; Li, F.; Zhou, T.; Squillace, R. M.; Commodore, L.; Greenfield, M. T.; Lu, X.; Miller, D. P.; Huang, W. S.; Qi, J.; Thomas, R. M.; Wang, Y.; Zhang, S.; Dodd, R.; Liu, S.; Xu, R.; Xu, Y.; Miret, J. J.; Rivera, V.; Clackson, T.; Shakespeare, W. C.; Zhu, X.; Dalgarno, D. C. Fragment Growing and Linking Lead to Novel Nanomolar Lactate Dehydrogenase Inhibitors. *J. Med. Chem.* **2013**, *56* (3), 1023–1040.
- (234) Copeland, R. A. Chapter 4 Protein–Ligand Binding Equilibria. In *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*; 2000; Vol. 7, pp 76–108.
- (235) Copeland, R. A. *ENZYMES A Practical Introduction to Structure, Mechanism, and Data Analysis*, 2nd ed.; Wiley-Blackwell Publishing Ltd, 2000; Vol. 7.
- (236) Copeland, R. A. *Evaluation of Enzyme Inhibitors in Drug Discovery*; John Wiley & Sons, Ltd, 2013.
- (237) Copeland, R. A. Chapter 5-Kinetics of Single-Substrate Enzyme Reactions. In *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*; 2000; Vol. 7, pp 109–145.
- (238) Asche, C. Antitumour Quinones. *Mini-Reviews Med. Chem.* **2005**, *5* (5), 449–467.
- (239) Sreelatha, T.; Kandhasamy, S.; Dinesh, R.; Shruthy, SureshShweta, S.; Mukesh, D.; Karunagaran, D.; Balaji, R.; Mathivanan, N.; Perumal, P. T. Synthesis and SAR Study of Novel Anticancer and Antimicrobial Naphthoquinone Amide Derivatives. *Bioorganic Med. Chem.*

- Lett.* **2014**, 24 (15), 3647–3651.
- (240) Dos Santos, E. V. M.; Carneiro, J. W. D. M.; Ferreira, V. F. Quantitative Structure-Activity Relationship in Aziridiny-1,4- Naphthoquinone Antimalarials: Study of Theoretical Correlations by the PM3 Method. *Bioorganic Med. Chem.* **2004**, 12 (1), 87–93.
- (241) Paul, A.; Viswanathan, G. K.; Mahapatra, S.; Balboni, G.; Pacifico, S.; Gazit, E.; Segal, D. Antagonistic Activity of Naphthoquinone-Based Hybrids toward Amyloids Associated with Alzheimer's Disease and Type-2 Diabetes. *ACS Chem. Neurosci.* **2019**, 10 (8), 3510–3520.
- (242) Babula, P.; Adam, V.; Havel, L.; Kizek, R. [Naphthoquinones and Their Pharmacological Properties]. *Ceska Slov. Farm.* **2007**, 56 (3), 114–120.
- (243) Colucci, M. A.; Couch, G. D.; Moody, C. J. Natural and Synthetic Quinones and Their Reduction by the Quinone Reductase Enzyme NQO1: From Synthetic Organic Chemistry to Compounds with Anticancer Potential. *Org. Biomol. Chem.* **2008**, 6 (4), 637–656.
- (244) Garuti, L.; Roberti, M.; Pizzirani, D. Nitrogen-Containing Heterocyclic Quinones: A Class of Potential Selective Antitumor Agents. *Mini-Reviews Med. Chem.* **2007**, 7 (5), 481–489.
- (245) Katritzky, A. R.; Huang, L.; Sakhuja, R. Efficient Syntheses of Naphthoquinone-Dipeptides. *Synthesis (Stuttg.)* **2010**, No. 12, 2011–2016.
- (246) Granchi, C.; Roy, S.; Giacomelli, C.; MacChia, M.; Tuccinardi, T.; Martinelli, A.; Lanza, M.; Betti, L.; Giannaccini, G.; Lucacchini, A.; Funel, N.; León, L. G.; Giovannetti, E.; Peters, G. J.; Palchadhuri, R.; Calvaresi, E. C.; Hergenrother, P. J.; Minutolo, F. Discovery of N-Hydroxyindole-Based Inhibitors of Human Lactate Dehydrogenase Isoform A (LDH-A) as Starvation Agents against Cancer Cells. *J. Med. Chem.* **2011**, 54 (6), 1599–1612.
- (247) Copeland, R. A. Chapter 8-Reversible Inhibitors. In *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*; 2000; Vol. 7, pp 266–304.
- (248) Copeland, R. A. Reversible Modes of Inhibitor Interactions with Enzymes. In *Evaluation of Enzyme Inhibitors in Drug Discovery*; John Wiley & Sons, Ltd, 2013; pp 57–121.
- (249) GraphPad Prism 7 Curve Fitting Guide - Key concepts: Enzyme inhibition https://www.graphpad.com/guides/prism/7/curve-fitting/reg_key_concepts_enzyme_inhibition.htm (accessed May 11, 2020).
- (250) Tuccinardi, T.; Poli, G.; Corchia, I.; Granchi, C.; Lapillo, M.; Macchia, M.; Minutolo, F.; Ortore, G.; Martinelli, A. A Virtual Screening Study for Lactate Dehydrogenase 5 Inhibitors by Using a Pharmacophore-Based Approach. *Mol. Inform.* **2016**, 434–439.
- (251) Powers, J. L.; Kiesman, N. E.; Tran, C. M.; Brown, J. H.; Bevilacqua, V. L. H. Laboratory Exercises Lactate Dehydrogenase Kinetics and Inhibition Using a Microplate Reader* h S. *Biochem. Mol. Biol. Educ.* **2007**, 35 (4), 287–292.
- (252) Cameron, A.; Read, J.; Tranter, R.; Winter, V. J.; Sessions, R. B.; Brady, L. L.; Vivas, L.; Easton, A.; Kendrick, H.; Croft, S. L.; Barros, D.; Lavandera, J. L.; Martin, J. J.; Risco, F.; García-Ochoa, S.; Gamo, F. J.; Sanz, L.; Leon, L.; Ruiz, J. R.; Gabarró, R.; Mallon, A.; Gómez De Las Heras, F. Identification and Activity of a Series of Azole-Based Compounds with Lactate Dehydrogenase-Directed Anti-Malarial Activity. *J. Biol. Chem.* **2004**, 279 (30), 31429–31439.

- (253) Granchi, C.; Calvaresi, E. C.; Tuccinardi, T.; Paterni, I.; Macchia, M.; Martinelli, A.; Hergenrother, P. J.; Minutolo, F. Assessing the Differential Action on Cancer Cells of LDH-A Inhibitors Based on the N-Hydroxyindole-2-Carboxylate (NHI) and Malonic (Mal) Scaffolds. *Org. Biomol. Chem.* **2013**, *11* (38), 6588–6596.
- (254) Crane, C. A.; Austgen, K.; Haberthur, K.; Hofmann, C.; Moyes, K. W.; Avanesyan, L.; Fong, L.; Campbell, M. J.; Cooper, S.; Oakes, S. A.; Parsa, A. T.; Lanier, L. L. Immune Evasion Mediated by Tumor-Derived Lactate Dehydrogenase Induction of NKG2D Ligands on Myeloid Cells in Glioblastoma Patients. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (35), 12823–12828.
- (255) Kim, J.; Han, J.; Jang, Y.; Kim, S. J.; Lee, M. J.; Ryu, M. J.; Kweon, G. R.; Heo, J. Y. High-Capacity Glycolytic and Mitochondrial Oxidative Metabolisms Mediate the Growth Ability of Glioblastoma. *Int. J. Oncol.* **2015**, *47* (3), 1009–1016.
- (256) Xie, H.; Valera, V. A.; Merino, M. J.; Amato, A. M.; Signoretti, S.; Linehan, W. M.; Sukhatme, V. P.; Seth, P. LDH-A Inhibition, a Therapeutic Strategy for Treatment of Hereditary Leiomyomatosis and Renal Cell Cancer. *Mol. Cancer Ther.* **2009**, *8* (3), 626–635.
- (257) Le, A.; Cooper, C. R.; Gouw, A. M.; Dinavahi, R.; Maitra, A.; Deck, L. M.; Royer, R. E.; Vander Jagt, D. L.; Semenza, G. L.; Dang, C. V. Inhibition of Lactate Dehydrogenase A Induces Oxidative Stress and Inhibits Tumor Progression. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (5), 2037–2042.
- (258) Vichai, V.; Kirtikara, K. Sulforhodamine B Colorimetric Assay for Cytotoxicity Screening. *Nat. Protoc.* **2006**, *1* (3), 1112–1116.
- (259) Sultan Aslantürk, Ö. In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages. *IntechOpen* **2018**, 1–19.
- (260) Chakrabarti, A. C. Permeability of Membranes to Amino Acids and Modified Amino Acids: Mechanisms Involved in Translocation. *Amino Acids* **1994**, *6* (3), 213–229.
- (261) Beaumont, K.; Webster, R.; Gardner, I.; Dack, K. Design of Ester Prodrugs to Enhance Oral Absorption of Poorly Permeable Compounds: Challenges to the Discovery Scientist. *Curr. Drug Metab.* **2005**, *4* (6), 461–485.
- (262) Strober, W. Trypan Blue Exclusion Test of Cell Viability. In *Current Protocols in Immunology*; John Wiley & Sons, Inc., 2001.
- (263) Orellana, E. A.; Kasinski, A. L. Sulforhodamine B (SRB) Assay in Cell Culture to Investigate Cell Proliferation. *Bio-Protocol* **2016**, *6* (21), e1984.
- (264) Maiese, K. “Micromanaging” Glioblastoma Multiforme: The Potential of MicroRNAs, Circular RNAs, and the Hippo Pathway as Novel Treatment Strategies. *Curr. Neurovasc. Res.* **2018**, *15* (3), 173–174.
- (265) Llaguno, S. R. A.; Parada, L. F. Cell of Origin of Glioma: Biological and Clinical Implications. *Br. J. Cancer* **2016**, *115* (12), 1445–1450.
- (266) Louis, D. N.; Ohgaki, H.; Wiestler, O. D.; Cavenee, W. K.; Burger, P. C.; Jouvett, A.; Scheithauer, B. W.; Kleihues, P. The 2007 WHO Classification of Tumours of the Central Nervous System. *Acta Neuropathol.* **2007**, *114* (2), 97–109.
- (267) Perry, A.; Wesseling, P. Histologic Classification of Gliomas. In *Handbook of Clinical Neurology*;

- Elsevier, 2016; Vol. 134, pp 71–95.
- (268) Wesseling, P.; Capper, D. WHO 2016 Classification of Gliomas. *Neuropathol. Appl. Neurobiol.* **2018**, *44* (2), 139–150.
- (269) Ostrom, Q. T.; Bauchet, L.; Davis, F. G.; Deltour, I.; Fisher, J. L.; Langer, C. E.; Pekmezci, M.; Schwartzbaum, J. A.; Turner, M. C.; Walsh, K. M.; Wrensch, M. R.; Barnholtz-Sloan, J. S. The Epidemiology of Glioma in Adults: A State of the Science Review. *Neuro. Oncol.* **2014**, *16* (7), 896–913.
- (270) Ohgaki, H.; Kleihues, P. The Definition of Primary and Secondary Glioblastoma. *Clin. Cancer Res.* **2013**, *19* (4), 764–772.
- (271) Stupp, R.; Mason, W. P.; van den Bent, M. J.; Weller, M.; Fisher, B.; Taphoorn, M. J. B.; Belanger, K.; Brandes, A. A.; Marosi, C.; Bogdahn, U.; Curschmann, J.; Janzer, R. C.; Ludwin, S. K.; Gorlia, T.; Allgeier, A.; Lacombe, D.; Cairncross, J. G.; Eisenhauer, E.; Mirimanoff, R. O. Radiotherapy plus Concomitant and Adjuvant Temozolomide for Glioblastoma. *N. Engl. J. Med.* **2005**, *352* (10), 987–996.
- (272) Tamimi, A. F.; Juweid, M. Epidemiology and Outcome of Glioblastoma. In *Glioblastoma*; Codon Publications, 2017; pp 143–153.
- (273) Louis, D. N.; Perry, A.; Reifenberger, G.; von Deimling, A.; Figarella-Branger, D.; Cavenee, W. K.; Ohgaki, H.; Wiestler, O. D.; Kleihues, P.; Ellison, D. W. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: A Summary. *Acta Neuropathol.* **2016**, *131* (6), 803–820.
- (274) Yan, H.; Parsons, D. W.; Jin, G.; McLendon, R.; Rasheed, B. A.; Yuan, W.; Kos, I.; Batinic-Haberle, I.; Jones, S.; Riggins, G. J.; Friedman, H.; Friedman, A.; Reardon, D.; Herndon, J.; Kinzler, K. W.; Velculescu, V. E.; Vogelstein, B.; Bigner, D. D. IDH1 and IDH2 Mutations in Gliomas. *N. Engl. J. Med.* **2009**, *360* (8), 765–773.
- (275) Nobusawa, S.; Watanabe, T.; Kleihues, P.; Ohgaki, H. IDH1 Mutations as Molecular Signature and Predictive Factor of Secondary Glioblastomas. *Clin. Cancer Res.* **2009**, *15* (19), 6002–6007.
- (276) Batash, R.; Asna, N.; Schaffer, P.; Francis, N.; Schaffer, M. Glioblastoma Multiforme, Diagnosis and Treatment; Recent Literature Review. *Curr. Med. Chem.* **2017**, *24* (27), 3002–3009.
- (277) Yong, R. L.; Lonser, R. R. Surgery for Glioblastoma Multiforme: Striking a Balance. *World Neurosurg.* **2011**, *76* (6), 528–530.
- (278) Laperriere, N.; Zuraw, L.; Cairncross, G. Radiotherapy for Newly Diagnosed Malignant Glioma in Adults: A Systematic Review. *Radiother. Oncol.* **2002**, *64* (3), 259–273.
- (279) Stevens, M. F. G.; Vaughan, ;) K; Stevens ; Ege, M. F. G.; Gilbert, K.; Horton, J. K. *Antitumor Imidazotetrazines. 1. Synthesis and Chemistry of 8-Carbamoyl-3-(2-Chloroethyl)Imidazo[5,1-d]-1,2,3,5-Tetrazin-4(3H)-One, a Novel Broad-Spectrum Antitumor Agent*; 1984; Vol. 27.
- (280) Hickman, J. A.; Stevens, M. F. G.; Gibson, N. W.; Langdon, S. P.; Fixăçmes, C.; Atassi, G.; Lunt, E.; Tilson, R. M. *Experimental Antitumor Activity against Murine Tumor Model Systems of 8-Carbamoyl-3-(2-Chloroethyl)Imidazo[5,1-Ci]-1,2,3,5-Tetrazin-4(3H)-One (Mitozolomide), a Novel Broad-Spectrum Agent1*; 1985; Vol. 45.

- (281) Moody, C. L.; Wheelhouse, R. T. The Medicinal Chemistry of Imidazotetrazine Prodrugs. *Pharmaceuticals* **2014**, *7* (7), 797–838.
- (282) Portnow, J.; Badie, B.; Chen, M.; Liu, A.; Blanchard, S.; Synold, T. W. The Neuropharmacokinetics of Temozolomide in Patients with Resectable Brain Tumors: Potential Implications for the Current Approach to Chemoradiation. *Clin. Cancer Res.* **2009**, *15* (22), 7092–7098.
- (283) Wesolowski, J. R.; Rajdev, P.; Mukherji, S. K. Temozolomide (Temodar). *Am. J. Neuroradiol.* **2010**, *31* (8), 1383–1384.
- (284) Friedman, H. S.; Kerby, T.; Calvert, H. Temozolomide and Treatment of Malignant Glioma. *Clin. Cancer Res.* **2000**, *6* (7), 2585–2597.
- (285) Gilant, E.; Kaza, M.; Szlagowska, A.; Serafin-Byczak, K.; Rudzki, P. J. Validated HPLC Method for Determination of Temozolomide in Human Plasma. *Acta Pol. Pharm. - Drug Res.* **2012**, *69* (6), 1347–1355.
- (286) Borhani, S.; Mozdarani, H.; Somayyeh, B.; Bakhshandeh, M.; Nosrati, H. In Vitro Radiosensitizing Effects of Temozolomide on U87MG Cell Lines of Human Glioblastoma Multiforme. *Iran. J. Med. Sci.* **2017**, *42* (3), 258–265.
- (287) Bower, M.; Newlands, E. S.; Bleeher, N. M.; Brada, M.; Begent, R. J. H.; Calvert, H.; Colquhoun, I.; Lewis, P.; Brampton, M. H. Multicentre CRC Phase II Trial of Temozolomide in Recurrent or Progressive High-Grade Glioma. *Cancer Chemother. Pharmacol.* **1997**, *40* (6), 484–488.
- (288) O'Reilly, S. M.; Newlands, E. S.; Brampton, M.; Glaser, M. G.; Rice-Edwards, J. M.; Illingworth, R. D.; Richards, P. G.; Kennard, C.; Colquhoun, I. R.; Lewis, P.; Stevens, M. F. G. Temozolomide: A New Oral Cytotoxic Chemotherapeutic Agent with Promising Activity against Primary Brain Tumours. *Eur. J. Cancer* **1993**, *29* (7), 940–942.
- (289) Songtao, Q.; Lei, Y.; Si, G.; Yanqing, D.; Huixia, H.; Xuelin, Z.; Lanxiao, W.; Fei, Y. IDH Mutations Predict Longer Survival and Response to Temozolomide in Secondary Glioblastoma. *Cancer Sci.* **2012**, *103* (2), 269–273.
- (290) Sturm, D.; Witt, H.; Hovestadt, V.; Khuong-Quang, D. A.; Jones, D. T. W.; Konermann, C.; Pfaff, E.; Tönjes, M.; Sill, M.; Bender, S.; Kool, M.; Zapatka, M.; Becker, N.; Zucknick, M.; Hielscher, T.; Liu, X. Y.; Fontebasso, A. M.; Ryzhova, M.; Albrecht, S.; Jacob, K.; Wolter, M.; Ebinger, M.; Schuhmann, M. U.; van Meter, T.; Frühwald, M. C.; Hauch, H.; Pekrun, A.; Radlwimmer, B.; Niehues, T.; Von Komorowski, G.; Dürken, M.; Kulozik, A. E.; Madden, J.; Donson, A.; Foreman, N. K.; Drissi, R.; Fouladi, M.; Scheurlen, W.; von Deimling, A.; Monoranu, C.; Roggendorf, W.; Herold-Mende, C.; Unterberg, A.; Kramm, C. M.; Felsberg, J.; Hartmann, C.; Wiestler, B.; Wick, W.; Milde, T.; Witt, O.; Lindroth, A. M.; Schwartzentruber, J.; Faury, D.; Fleming, A.; Zakrzewska, M.; Liberski, P. P.; Zakrzewski, K.; Hauser, P.; Garami, M.; Klekner, A.; Bogner, L.; Morrissy, S.; Cavalli, F.; Taylor, M. D.; van Sluis, P.; Koster, J.; Versteeg, R.; Volckmann, R.; Mikkelsen, T.; Aldape, K.; Reifenberger, G.; Collins, V. P.; Majewski, J.; Korshunov, A.; Lichter, P.; Plass, C.; Jabado, N.; Pfister, S. M. Hotspot Mutations in H3F3A and IDH1 Define Distinct Epigenetic and Biological Subgroups of Glioblastoma. *Cancer Cell* **2012**, *22* (4), 425–437.
- (291) Abe, H.; Natsumeda, M.; Kanemaru, Y.; Watanabe, J.; Tsukamoto, Y.; Okada, M.; Yoshimura, J.; Oishi, M.; Fujii, Y. MGMT Expression Contributes to Temozolomide Resistance in H3K27M-Mutant Diffuse Midline Gliomas and MGMT Silencing to Temozolomide Sensitivity in IDH-

Mutant Gliomas. *Neurol Med Chir* **2018**, 58, 290–295.

- (292) Bender, S.; Tang, Y.; Lindroth, A. M.; Hovestadt, V.; Jones, D. T. W.; Kool, M.; Zapatka, M.; Northcott, P. A.; Sturm, D.; Wang, W.; Radlwimmer, B.; Højfeldt, J. W.; Truffaux, N.; Castel, D.; Schubert, S.; Ryzhova, M.; Şeker-Cin, H.; Gronych, J.; Johann, P. D.; Stark, S.; Meyer, J.; Milde, T.; Schuhmann, M.; Ebinger, M.; Monoranu, C. M.; Ponnuswami, A.; Chen, S.; Jones, C.; Witt, O.; Collins, V. P.; vonDeimling, A.; Jabado, N.; Puget, S.; Grill, J.; Helin, K.; Korshunov, A.; Lichter, P.; Monje, M.; Plass, C.; Cho, Y. J.; Pfister, S. M. Reduced H3K27me3 and DNA Hypomethylation Are Major Drivers of Gene Expression in K27M Mutant Pediatric High-Grade Gliomas. *Cancer Cell* **2013**, 24 (5), 660–672.
- (293) Wu, J. P.; Wang, J.; Abeywardane, A.; Andersen, D.; Emmanuel, M.; Gautschi, E.; Goldberg, D. R.; Kashem, M. A.; Lukas, S.; Mao, W.; Martin, L.; Morwick, T.; Moss, N.; Pargellis, C.; Patel, U. R.; Patnaude, L.; Peet, G. W.; Skow, D.; Snow, R. J.; Ward, Y.; Werneburg, B.; White, A. The Discovery of Carboline Analogs as Potent MAPKAP-K2 Inhibitors. *Bioorganic Med. Chem. Lett.* **2007**, 17 (16), 4664–4669.
- (294) Fernandes, C.; Costa, A.; Osório, L.; Lago, R. C.; Linhares, P.; Carvalho, B.; Caeriro, C. Current Standards of Care in Glioblastoma Therapy. In *Glioblastoma*; Codon Publications, 2017; pp 197–241.
- (295) Fleming, A. B.; Saltzman, W. M. Pharmacokinetics of the Carmustine Implant. *Clin. Pharmacokinet.* **2002**, 41 (6), 403–419.
- (296) Lin, S. H.; Kleinberg, L. R. Carmustine Wafers: Localized Delivery of Chemotherapeutic Agents in CNS Malignancies. *Expert Rev. Anticancer Ther.* **2008**, 8 (3), 343–359.
- (297) Brem, H.; Piantadosi, S.; Burger, P. C.; Walker, M.; Selker, R.; Vick, N. A.; Black, K.; Sisti, M.; Brem, S.; Mohr, G.; Muller, P.; Morawetz, R.; Schold, S. C. Placebo-Controlled Trial of Safety and Efficacy of Intraoperative Controlled Delivery by Biodegradable Polymers of Chemotherapy for Recurrent Gliomas. *Lancet* **1995**, 345 (8956), 1008–1012.
- (298) Westphal, M.; Ram, Z.; Riddle, V.; Hilt, D.; Bortey, E. Gliadel® Wafer in Initial Surgery for Malignant Glioma: Long-Term Follow-up of a Multicenter Controlled Trial. *Acta Neurochir. (Wien)*. **2006**, 148, 269–275.
- (299) Mukherji, S. K. Bevacizumab (Avastin). *American Journal of Neuroradiology*. February 2010, pp 235–236.
- (300) Gilbert, M. R.; Dignam, J.; Won, M.; Blumenthal, D. T.; Vogelbaum, M. A.; Aldape, K. D.; Colman, H.; Chakravarti, A.; Jeraj, R.; Armstrong, T. S.; Wefel, J. S.; Brown, P. D.; Jaeckle, K. A.; Schiff, D.; Atkins, J. N.; Brachman, D.; Werner-Wasik, M.; Komaki, R.; Sulman, E. P.; Mehta, M. P. RTOG 0825: Phase III Double-Blind Placebo-Controlled Trial Evaluating Bevacizumab (Bev) in Patients (Pts) with Newly Diagnosed Glioblastoma (GBM). *J. Clin. Oncol.* **2013**, 31 (18_suppl), 1–1.
- (301) Wick, W.; Cloughesy, T. F.; Nishikawa, R.; Mason, W.; Saran, F.; Henriksson, R.; Hilton, M.; Kerloeguen, Y.; Chinot, O. L. Tumor Response Based on Adapted Macdonald Criteria and Assessment of Pseudoprogression (PsPD) in the Phase III AVAglio Trial of Bevacizumab (Bv) plus Temozolomide (T) plus Radiotherapy (RT) in Newly Diagnosed Glioblastoma (GBM). *J. Clin. Oncol.* **2013**, 31 (15_suppl), 2002–2002.
- (302) Gil-Gil, M. J.; Mesia, C.; Rey, M.; Bruna, J. Bevacizumab for the Treatment of Glioblastoma. *Clin.*

Med. Insights Oncol. **2013**, *7*, 123–135.

- (303) Kim, M. M.; Umemura, Y.; Leung, D. Bevacizumab and Glioblastoma Past, Present, and Future Directions. *Cancer J. (United States)* **2018**, *24* (4), 180–186.
- (304) Castro, B. A.; Aghi, M. K. Bevacizumab for Glioblastoma: Current Indications, Surgical Implications, and Future Directions. *Neurosurg. Focus* **2014**, *37* (6), E9.
- (305) Schrag, D.; Dueck, A. C.; Naughton, M. J.; Niedzwiecki, D.; Earle, C.; Shaw, J. E.; Grothey, A.; Hochster, H. S.; Blanke, C. D.; Venook, A. P. Cost of Chemotherapy for Metastatic Colorectal Cancer with Either Bevacizumab or Cetuximab: Economic Analysis of CALGB/SWOG 80405. *J. Clin. Oncol.* **2015**, *33* (15_suppl), 6504–6504.
- (306) Limonta, P.; Marelli, M. M.; Motta, M.; Luciano, M.; Moretti, R. M. GnRH Receptors in Cancer: From Cell Biology to Novel Targeted Therapeutic Strategies. *Endocr. Rev.* **2012**, *33* (5), 784–811.
- (307) Conn, P. M. Gonadotropin-Releasing Hormone and Its Analogs. *Annu. Rev. Biochem.* **1994**, *45*, 391–405.
- (308) Naor, Z. Frontiers in Neuroendocrinology Signaling by G-Protein-Coupled Receptor (GPCR): Studies on the GnRH Receptor. *Front. Neuroendocrinol.* **2009**, *30* (1), 10–29.
- (309) Marelli, M. M.; Moretti, R. M.; Mai, S.; Müller, O.; Groeninghen, J. C. V. A. N.; Limonta, P. Novel Insights into GnRH Receptor Activity : Role in the Control of Human Glioblastoma Cell Proliferation. *Oncol. Rep.* **2009**, *21*, 1277–1282.
- (310) Jayaram, S.; Gupta, M. K.; Raju, R.; Gautam, P.; Sirdeshmukh, R. Multi-Omics Data Integration and Mapping of Altered Kinases to Pathways Reveal Gonadotropin Hormone Signaling in Glioblastoma. *Omi. A J. Integr. Biol.* **2016**, *20* (12), 736–746.
- (311) Olberg, D. E.; Hausner, S. H.; Bauer, N.; Klaveness, J.; Indrevoll, B.; Andressen, K. W.; Dahl, M.; Levy, F. O.; Sutcliffe, J. L.; Haraldsen, I. Radiosynthesis of High Affinity Fluorine-18 Labeled GnRH Peptide Analogues: In Vitro Studies and in Vivo Assessment of Brain Uptake in Rats. *Medchemcomm* **2015**, *6* (4), 708–714.
- (312) Lalatsa, A.; Lee, V.; Malkinson, J. P.; Zloh, M.; Schätzlein, A. G.; Uchegbu, I. F. A Prodrug Nanoparticle Approach for the Oral Delivery of a Hydrophilic Peptide, Leucine5-Enkephalin, to the Brain. *Mol. Pharm.* **2012**, *9* (6), 1665–1680.
- (313) Lalatsa, A.; Schätzlein, A. G.; Garrett, N. L.; Moger, J.; Briggs, M.; Godfrey, L.; Iannitelli, A.; Freeman, J.; Uchegbu, I. F. Chitosan Amphiphile Coating of Peptide Nanofibres Reduces Liver Uptake and Delivers the Peptide to the Brain on Intravenous Administration. *J. Control. Release* **2015**, *197*, 87–96.
- (314) Lalatsa, A.; Leite, D. Peptide Nanofibers. WO/2019/193327, October 10, 2019.
- (315) Meyer, C.; Sims, A. H.; Morgan, K.; Harrison, B.; Muir, M.; Bai, J.; Faratian, D.; Millar, R. P.; Langdon, S. P. Transcript and Protein Profiling Identifies Signaling, Growth Arrest, Apoptosis, and NF-KB Survival Signatures Following GNRH Receptor Activation. *Endocr. Relat. Cancer* **2013**, *20* (1), 123–136.
- (316) Pucci, B.; Kasten, M.; Giordano, A. Cell Cycle and Apoptosis. *Neoplasia* **2000**, *2* (4), 291–299.

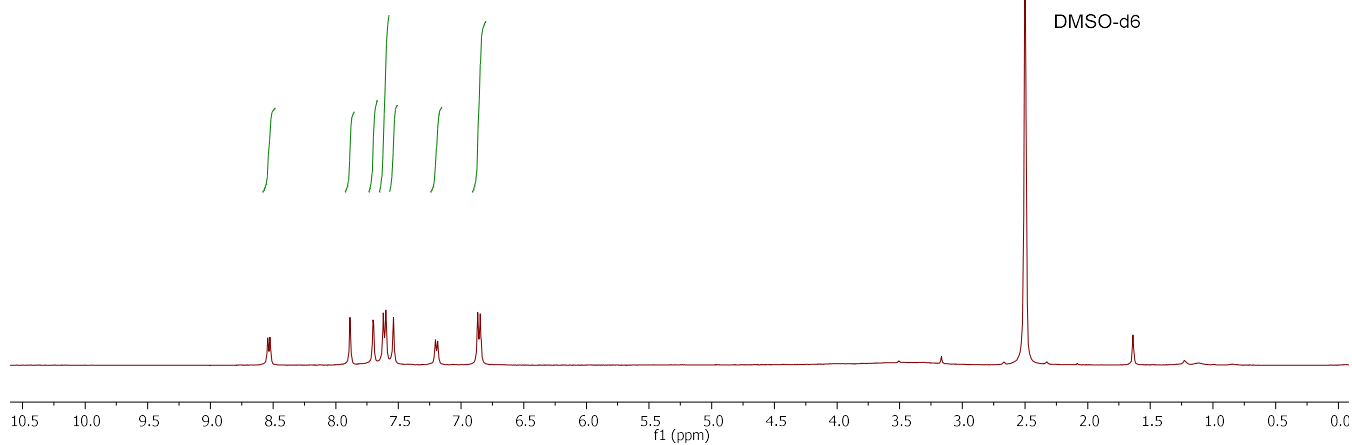
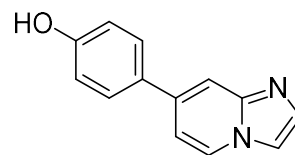
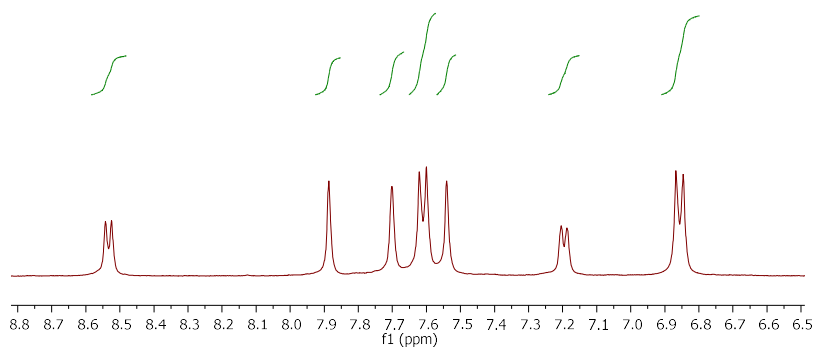
- (317) Miller, J. H. Synthesis of Proteins. In *Encyclopedia of Genetics*; Elsevier, 2001; p 1567.
- (318) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and Specific Genetic Interference by Double-Stranded RNA in *Caenorhabditis Elegans*. *Nature* **1998**, *391* (6669), 806–811.
- (319) Chan, J. A.; Krichevsky, A. M.; Kosik, K. S. MicroRNA-21 Is an Antiapoptotic Factor in Human Glioblastoma Cells. *Cancer Res.* **2005**, *65* (14), 6029–6033.
- (320) Di Leva, G.; Garofalo, M.; Croce, C. M. MicroRNAs in Cancer. *Annu. Rev. Pathol. Mech. Dis.* **2014**, *9* (1), 287–314.
- (321) Feng, Y. H.; Tsao, C. J. Emerging Role of MicroRNA-21 in Cancer (Review). *Biomed. Reports* **2016**, *5*, 395–402.
- (322) Fulci, V.; Chiaretti, S.; Goldoni, M.; Azzalin, G.; Carucci, N.; Tavoraro, S.; Castellano, L.; Magrelli, A.; Citarella, F.; Messina, M.; Maggio, R.; Peragine, N.; Santangelo, S.; Mauro, F. R.; Landgraf, P.; Tuschl, T.; Weir, D. B.; Chien, M.; Russo, J. J.; Ju, J.; Sheridan, R.; Sander, C.; Zavolan, M.; Guarini, A.; Foà, R.; Macino, G. Quantitative Technologies Establish a Novel MicroRNA Profile of Chronic Lymphocytic Leukemia. *Blood* **2007**, *109* (11), 4944–4951.
- (323) Feng, Y.-H.; Wu, C.-L.; Tsao, C.-J.; Chang, J.-G.; Lu, P.-J.; Yeh, K.-T.; Uen, Y.-H.; Lee, J.-C.; Shiau, A.-L. Dereglated Expression of Sprouty2 and MicroRNA-21 in Human Colon Cancer: Correlation with the Clinical Stage of the Disease. *Cancer Biol. Ther.* **2011**, *11* (1), 111–121.
- (324) Jansson, M. D.; Lund, A. H. MicroRNA and Cancer. *Mol. Oncol.* **2012**, *6* (6), 590–610.
- (325) Denli, A. M.; Tops, B. B. J.; Plasterk, R. H. A.; Ketting, R. F.; Hannon, G. J. Processing of Primary MicroRNAs by the Microprocessor Complex. *Nature* **2004**, *432* (7014), 231–235.
- (326) Li, X.; Wei, Y.; Wang, Z. MicroRNA-21 and Hypertension. *Hypertens. Res.* **2018**, *41*, 649–661.
- (327) Medina, P. P.; Nolde, M.; Slack, F. J. OncomiR Addiction in an in Vivo Model of MicroRNA-21-Induced Pre-B-Cell Lymphoma. *Nature* **2010**, *467* (7311), 86–90.
- (328) Volinia, S.; Calin, G. A.; Liu, C. G.; Ambs, S.; Cimmino, A.; Petrocca, F.; Visone, R.; Iorio, M.; Roldo, C.; Ferracin, M.; Prueitt, R. L.; Yanaihara, N.; Lanza, G.; Scarpa, A.; Vecchione, A.; Negrini, M.; Harris, C. C.; Croce, C. M. A MicroRNA Expression Signature of Human Solid Tumors Defines Cancer Gene Targets. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (7), 2257–2261.
- (329) Sheedy, F. J. Turning 21: Induction of MiR-21 as a Key Switch in the Inflammatory Response. *Front. Immunol.* **2015**, *6*, 19.
- (330) Gabriely, G.; Wurdinger, T.; Kesari, S.; Esau, C. C.; Burchard, J.; Linsley, P. S.; Krichevsky, A. M. MicroRNA 21 Promotes Glioma Invasion by Targeting Matrix Metalloproteinase Regulators. *Mol. Cell. Biol.* **2008**, *28* (17), 5369–5380.
- (331) Chen, J.; Xu, T.; Chen, C. The Critical Roles of MiR-21 in Anti-Cancer Effects of Curcumin. *Ann. Transl. Med.* **2015**, *3* (21), 9.
- (332) Benitez, J. A.; Ma, J.; D'Antonio, M.; Boyer, A.; Camargo, M. F.; Zanca, C.; Kelly, S.; Khodadadi-Jamayran, A.; Jameson, N. M.; Andersen, M.; Miletic, H.; Saberi, S.; Frazer, K. A.; Cavenee, W. K.; Furnari, F. B. PTEN Regulates Glioblastoma Oncogenesis through Chromatin-Associated Complexes of DAXX and Histone H3.3. *Nat. Commun.* **2017**, *8* (15223).

- (333) Han, M.; Liu, M.; Wang, Y.; Chen, X.; Xu, J.; Sun, Y.; Zhao, L.; Qu, H.; Fan, Y.; Wu, C. Antagonism of MiR-21 Reverses Epithelial-Mesenchymal Transition and Cancer Stem Cell Phenotype through AKT/ERK1/2 Inactivation by Targeting PTEN. *PLoS One* **2012**, *7* (6), 1–11.
- (334) Göke, R.; Barth, P.; Schmidt, A.; Samans, B.; Lankat-Buttgereit, B. Programmed Cell Death Protein 4 Suppresses CDK1/Cdc2 via Induction of P21Waf1/Cip1. *Am. J. Physiol. - Cell Physiol.* **2004**, *287* (6), C1541-6.
- (335) Leupold, J. H.; Yang, H. S.; Colburn, N. H.; Asangani, I.; Post, S.; Allgayer, H. Tumor Suppressor Pcd4 Inhibits Invasion/Intravasation and Regulates Urokinase Receptor (u-PAR) Gene Expression via Sp-Transcription Factors. *Oncogene* **2007**, *26* (31), 4550–4562.
- (336) Wang, Q.; Yang, H.-S. The Role of Pcd4 in Tumour Suppression and Protein Translation. *Biol. Cell* **2018**, *110* (8), 169–177.
- (337) Krichevsky, A. M.; Gabriely, G. MiR-21: A Small Multi-Faceted RNA. *J. Cell. Mol. Med.* **2009**, *13* (1), 39–53.
- (338) Nakada, M.; Okada, Y.; Yamashita, J. The Role of Matrix Metalloproteinases in Glioma Invasion. *Front. Biosci.* **2003**, *8*, e261–e269.
- (339) Havasi, A.; Li, Z.; Wang, Z.; Martin, J. L.; Botla, V.; Ruchalski, K.; Schwartz, J. H.; Borkan, S. C. Hsp27 Inhibits Bax Activation and Apoptosis via a Phosphatidylinositol 3-Kinase-Dependent Mechanism. *J. Biol. Chem.* **2008**, *283* (18), 12305–12313.
- (340) Shi, L.; Chen, J.; Yang, J.; Pan, T.; Zhang, S.; Wang, Z. MiR-21 Protected Human Glioblastoma U87MG Cells from Chemotherapeutic Drug Temozolomide Induced Apoptosis by Decreasing Bax/Bcl-2 Ratio and Caspase-3 Activity. *Brain Res.* **2010**, *1352*, 255–264.
- (341) Zhu, S.; Wu, H.; Wu, F.; Nie, D.; Sheng, S.; Mo, Y.-Y. MicroRNA-21 Targets Tumor Suppressor Genes in Invasion and Metastasis. *Cell Res. /* **2008**, *18*, 350–359.
- (342) Ganguly, D.; Fan, M.; Yang, C. H.; Zbytek, B.; Finkelstein, D.; Roussel, M. F.; Pfeffer, L. M. The Critical Role That STAT3 Plays in Glioma-Initiating Cells: STAT3 Addiction in Glioma. *Oncotarget* **2018**, *9* (31), 22095–22112.
- (343) Li, C.; Zheng, H.; Hou, W.; Bao, H.; Xiong, J.; Che, W.; Gu, Y.; Sun, H.; Liang, P. Long Non-Coding RNA Linc00645 Promotes TGF- β -Induced Epithelial–Mesenchymal Transition by Regulating MiR-205-3p-ZEB1 Axis in Glioma. *Cell Death Dis.* **2019**, *10* (10), 1–17.
- (344) Rich, J. N.; Eyler, C. E. Cancer Stem Cells in Brain Tumor Biology. In *Cold Spring Harbor Symposia on Quantitative Biology*; NIH Public Access, 2008; Vol. 73, pp 411–420.
- (345) Shang, C.; Guo, Y.; Hong, Y.; Liu, Y. hui; Xue, Y. xue. MiR-21 up-Regulation Mediates Glioblastoma Cancer Stem Cells Apoptosis and Proliferation by Targeting FASLG. *Mol. Biol. Rep.* **2015**, *42* (3), 721–727.
- (346) Xiao, Y.; FengMing, L.; Man, H.; Qiong, W.; JinHuan, W. Downregulation of Serum MicroRNA-205 as a Potential Diagnostic and Prognostic Biomarker for Human Glioma. *J. Neursurgery* **2016**, *124* (1), 122–128.
- (347) Zhang, P.; Sun, Y.; Ma, L. ZEB1: At the Crossroads of Epithelial-Mesenchymal Transition, Metastasis and Therapy Resistance. *Cell Cycle* **2015**, *14* (4), 481–487.

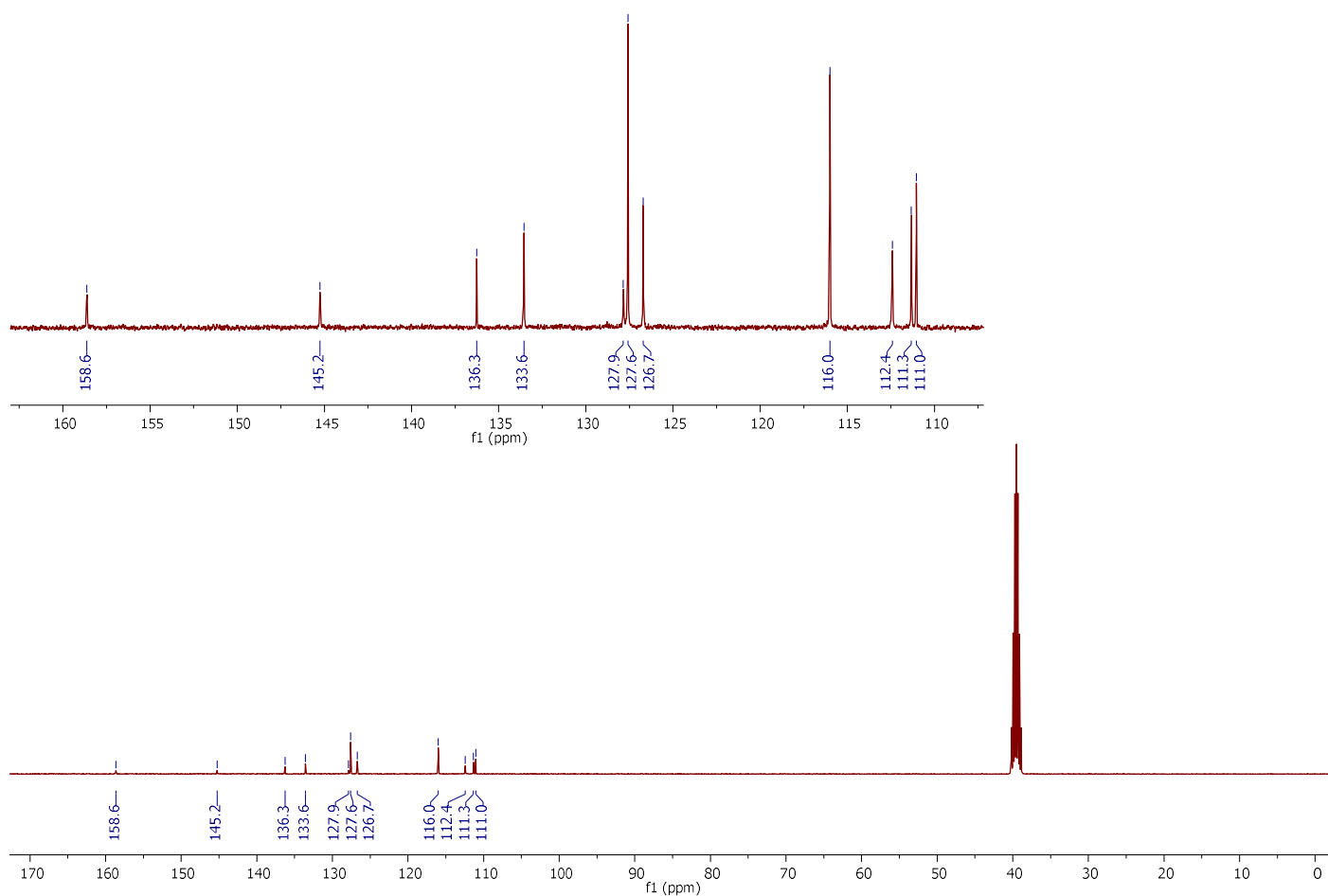
- (348) Seo, Y. E.; Suh, H. W.; Bahal, R.; Josowitz, A.; Zhang, J.; Song, E.; Cui, J.; Noorbakhsh, S.; Jackson, C.; Bu, T.; Piotrowski-Daspi, A.; Bindra, R.; Saltzman, W. M. Nanoparticle-Mediated Intratumoral Inhibition of MiR-21 for Improved Survival in Glioblastoma. *Biomaterials* **2019**, *201*, 87–98.
- (349) Purow, B. W.; Haque, R. M.; Noel, M. W.; Su, Q.; Burdick, M. J.; Lee, J.; Sundaresan, T.; Pastorino, S.; Park, J. K.; Mikolaenko, I.; Maric, D.; Eberhart, C. G.; Fine, H. A. Expression of Notch-1 and Its Ligands, Delta-like-1 and Jagged-1, Is Critical for Glioma Cell Survival and Proliferation. *Cancer Res.* **2005**, *65* (6), 2353–2363.
- (350) Candace A, G.; Daoua, M.-C.; Moser, R. P.; H. Ross, A. Gamma-Secretase Inhibitors Enhance Temozolomide Treatment of Human Gliomas by Inhibiting Neurosphere Repopulation and Xenograft Recurrence. *Cancer Res.* **2010**, *10* (17), 6870–6879.
- (351) Aguado, T.; Romero-revilla, J. A.; Granados, R.; Campuzano, S.; Torrente-rodríguez, R. M.; Cuesta, Á. M.; Albiñana, V.; Botella, L. M.; Santamaría, S.; Garcia-sanz, J. A.; Pingarrón, J. M.; Sánchez-Sancho, F.; Sánchez-Puelles, J.-M.; María, L.; Santamaría, S.; Garcia-sanz, J. A.; Pingarrón, J. M. 11PS04 Is a New Chemical Entity Identified by MicroRNA-Based Biosensing with Promising Therapeutic Potential against Cancer Stem Cells. *Sci. Rep.* **2019**, *9* (1), 1–22.
- (352) Aguado, T.; Romero-revilla, J. A.; Granados, R.; Campuzano, S.; Torrente-rodríguez, R. M.; Cuesta, Á. M.; Albiñana, V.; María, L.; Santamaría, S.; Garcia-sanz, J. A.; Pingarrón, J. M. A New Drug Discovery Paradigm Based on MicroRNA Biosensing to Identify Enhanced Cancer Therapies Associated with a Strong Chain of Translatability Contents : 1–22.
- (353) Qian, X.; Ren, Y.; Shi, Z.; Long, L.; Pu, P.; Sheng, J.; Yuan, X.; Kang, C. Sequence-Dependent Synergistic Inhibition of Human Glioma Cell Lines by Combined Temozolomide and MiR-21 Inhibitor Gene Therapy. *Mol. Pharm.* **2012**, *9* (9), 2636–2645.
- (354) Zhou, X.; Junxia, Z.; Qiang, J.; Ren, Y.; Yingyi, W.; Chunsheng, K. Reduction of MiR-21 Induces Glioma Cell Apoptosis via Activating Caspase 9 and 3. *Oncol. Rep.* **2010**, *24* (1), 195–201.
- (355) Chellgren, B. W.; Creamer, T. P. Short Sequences of Non-Proline Residues Can Adopt the Polyproline II Helical Conformation. *Biochemistry* **2004**, *43* (19), 5864–5869.
- (356) Lamfers, M. L. M.; Gianni, D.; Tung, C. H.; Idema, S.; Schagen, F. H. E.; Carette, J. E.; Quax, P. H. A.; Van Beusechem, V. W.; Vandertop, W. P.; Dirven, C. M. F.; Chiocca, E. A.; Gerritsen, W. R. Tissue Inhibitor of Metalloproteinase-3 Expression from an Oncolytic Adenovirus Inhibits Matrix Metalloproteinase Activity in Vivo without Affecting Antitumor Efficacy in Malignant Glioma. *Cancer Res.* **2005**, *65* (20), 9398–9405.
- (357) Chang, W.; White, P. Basic Procedures. In *Fmoc Solid Phase Peptide Synthesis*; Chan, W., White, P., Eds.; 1999.

SUPPLEMENTARY INFORMATION

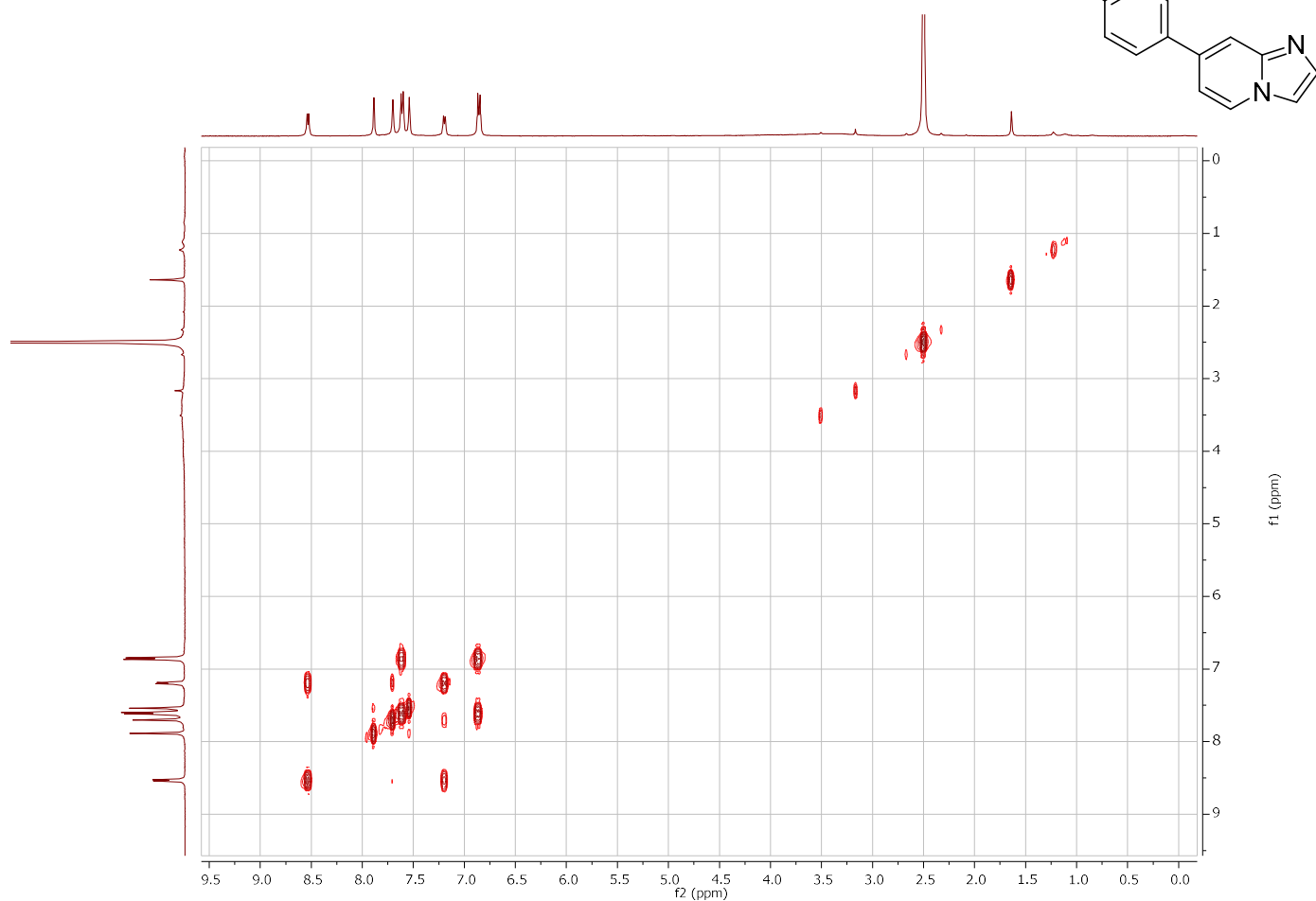
¹H NMR **1a** (500 MHz, DMSO-d₆)



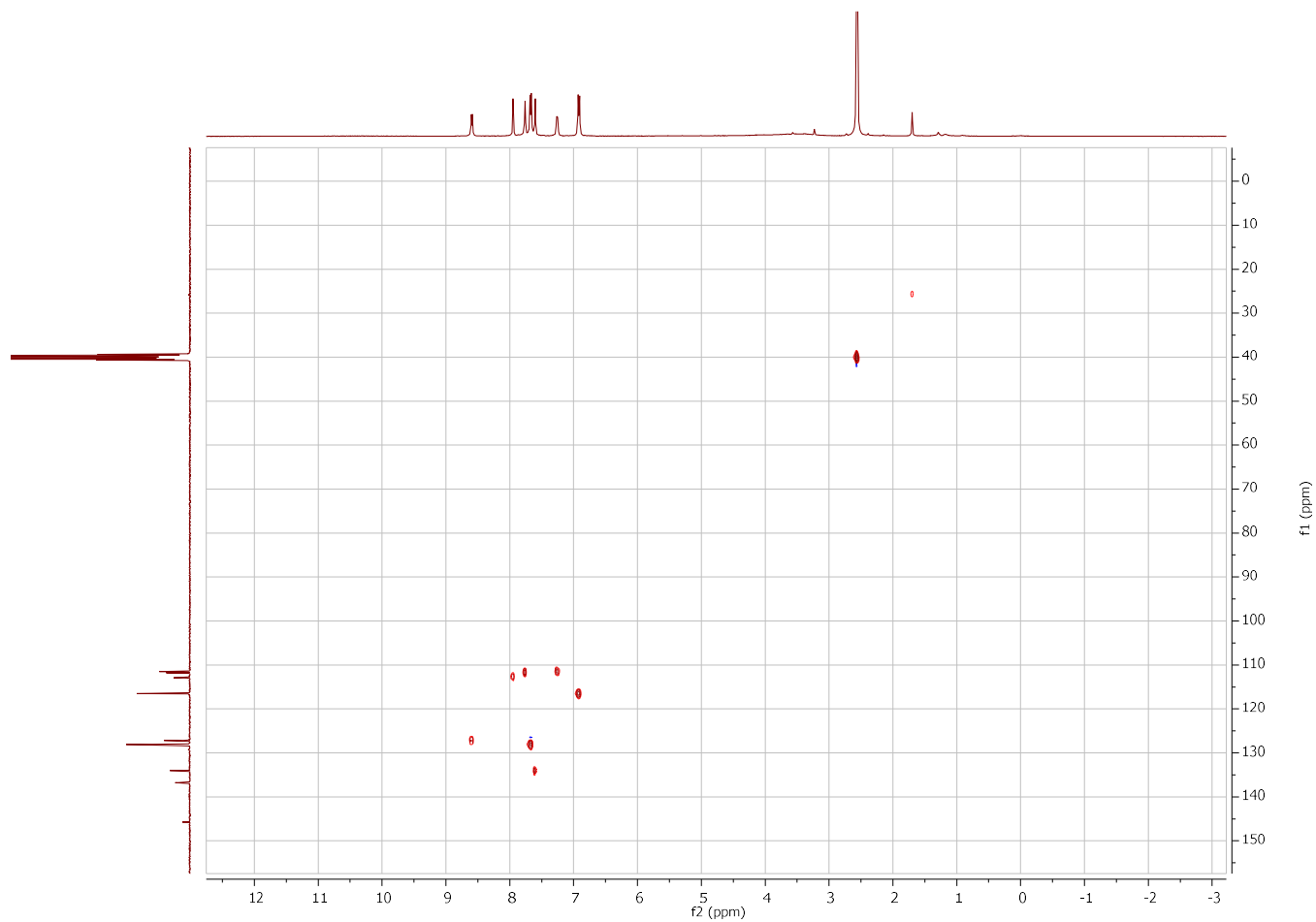
¹³C NMR **1a** (126 MHz, DMSO-d₆)



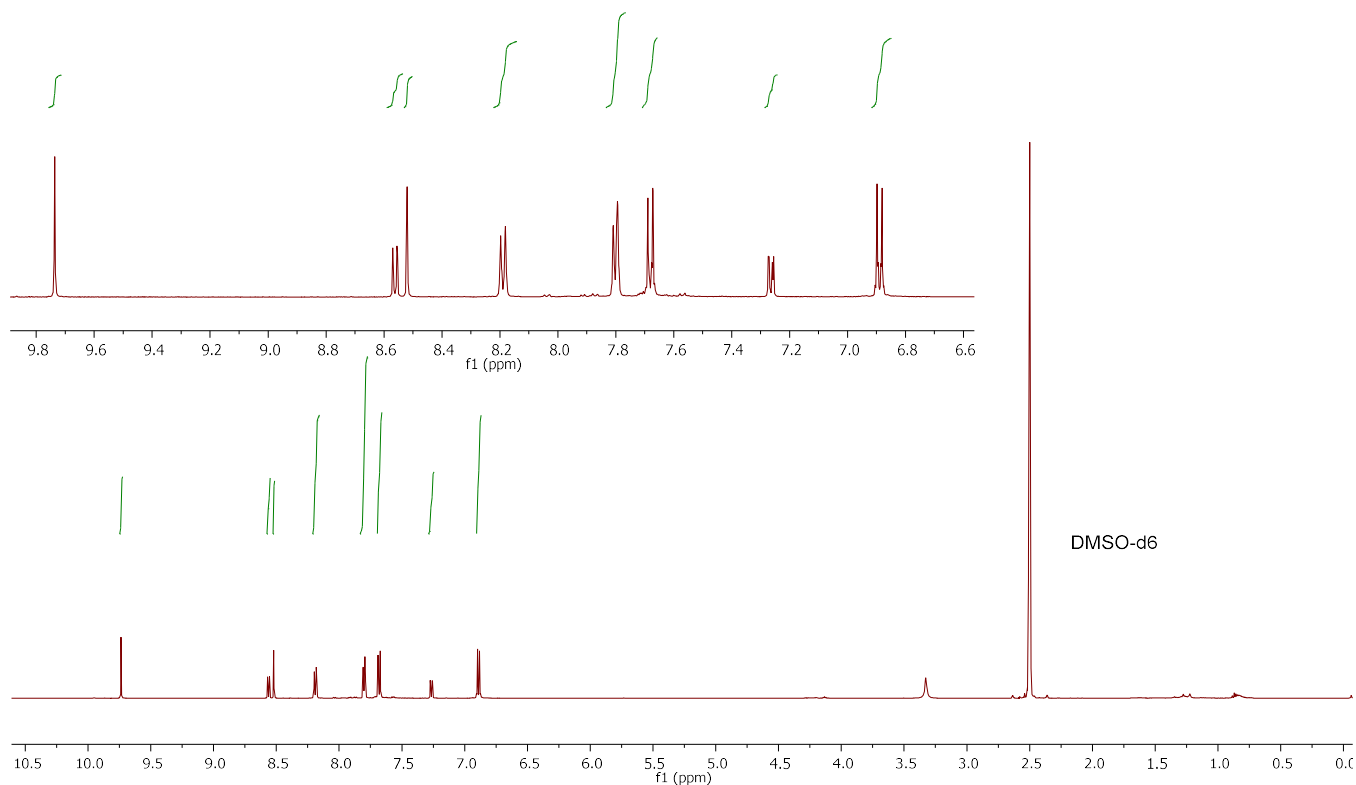
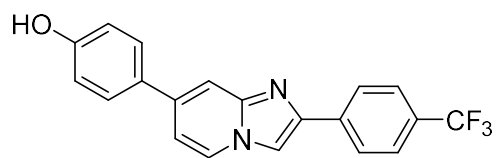
COSY **1a** (500 MHz, DMSO-d₆)



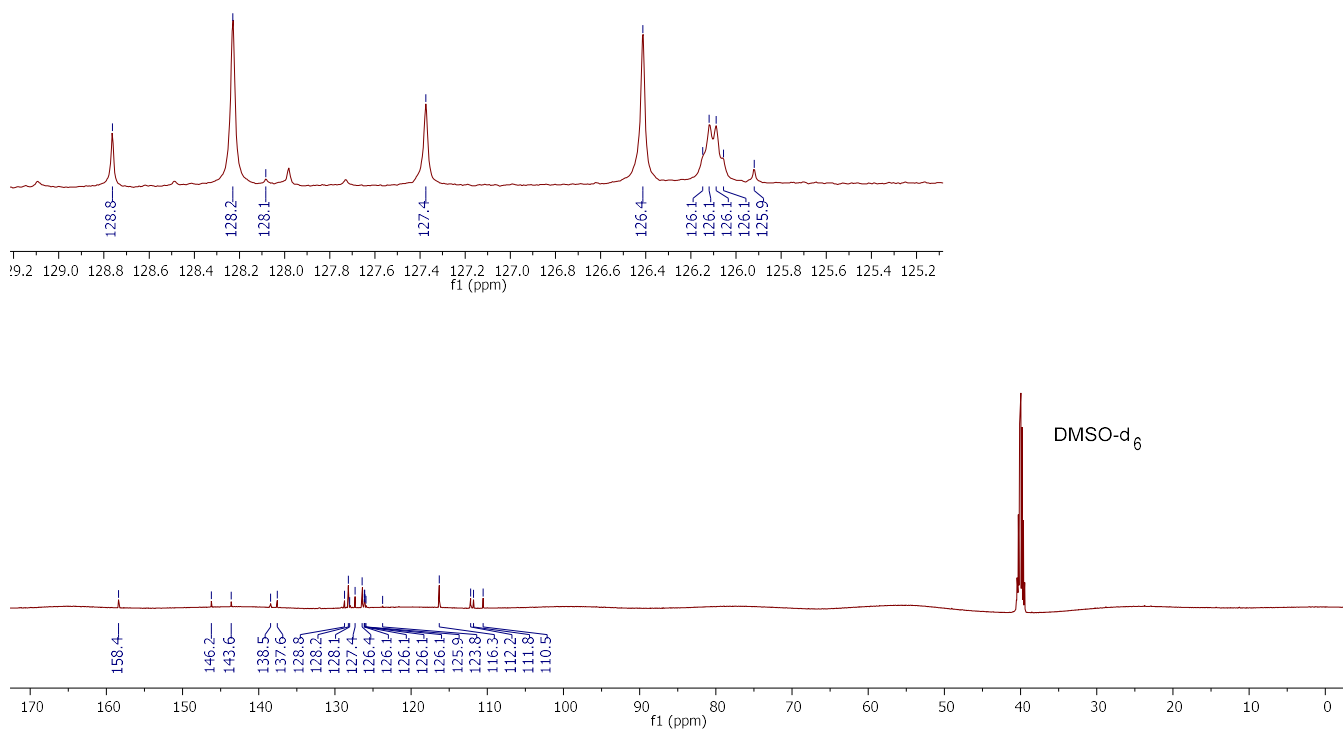
HSQC **1a** (500 MHz, DMSO-d₆)



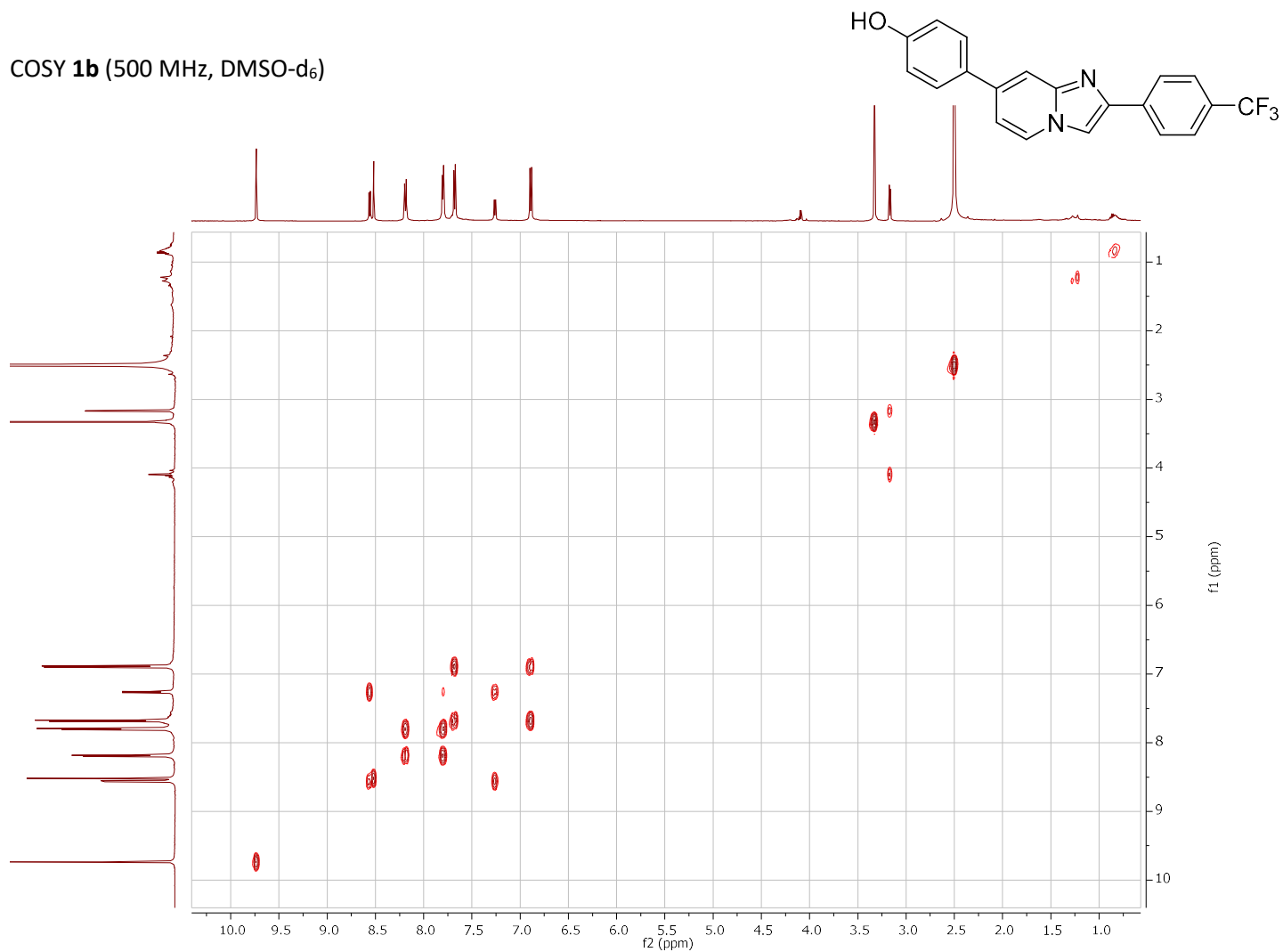
¹H NMR **1b** (500 MHz, DMSO-d₆)



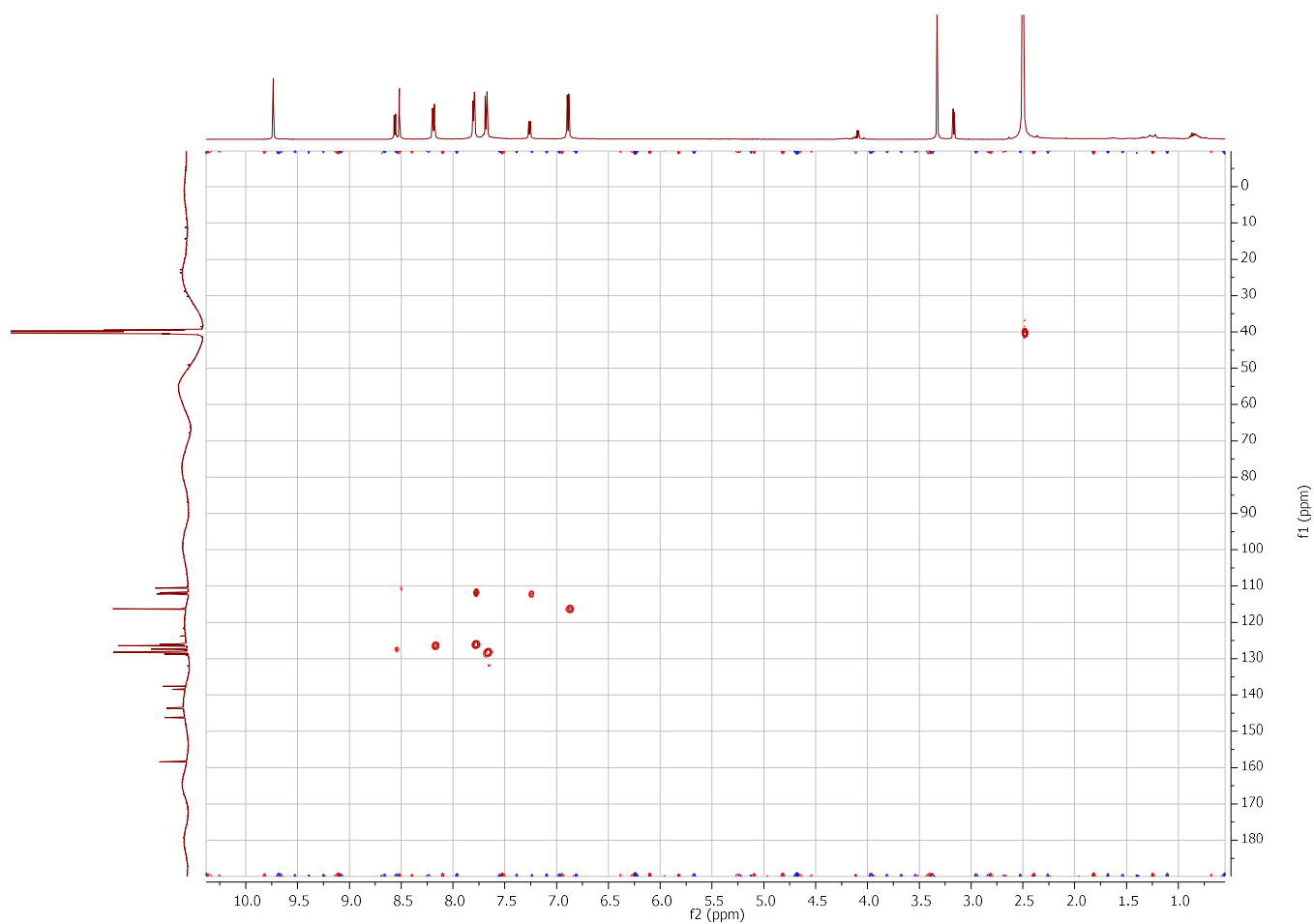
¹³C NMR GBG206 **1b** (126 MHz, DMSO-d₆)



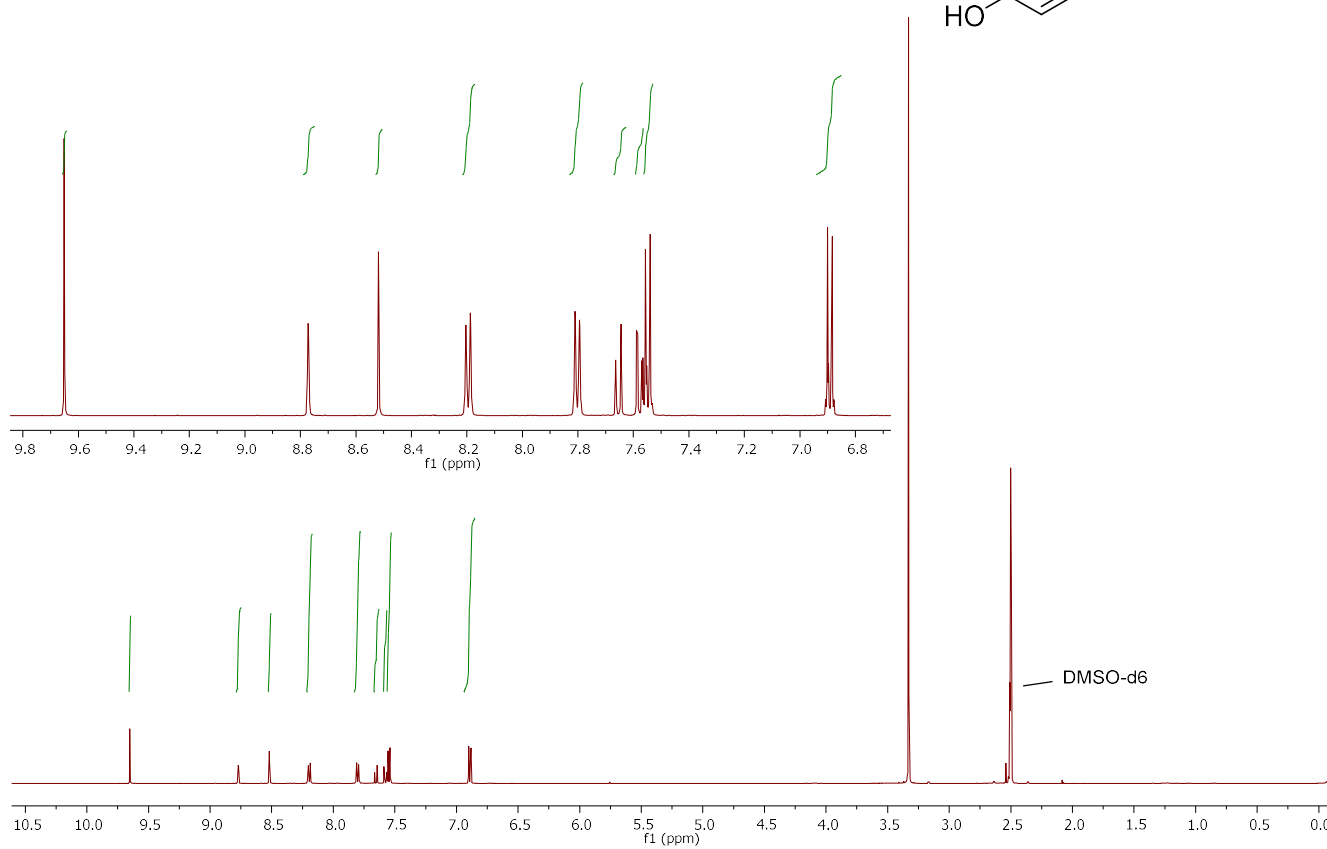
COSY **1b** (500 MHz, DMSO-d₆)



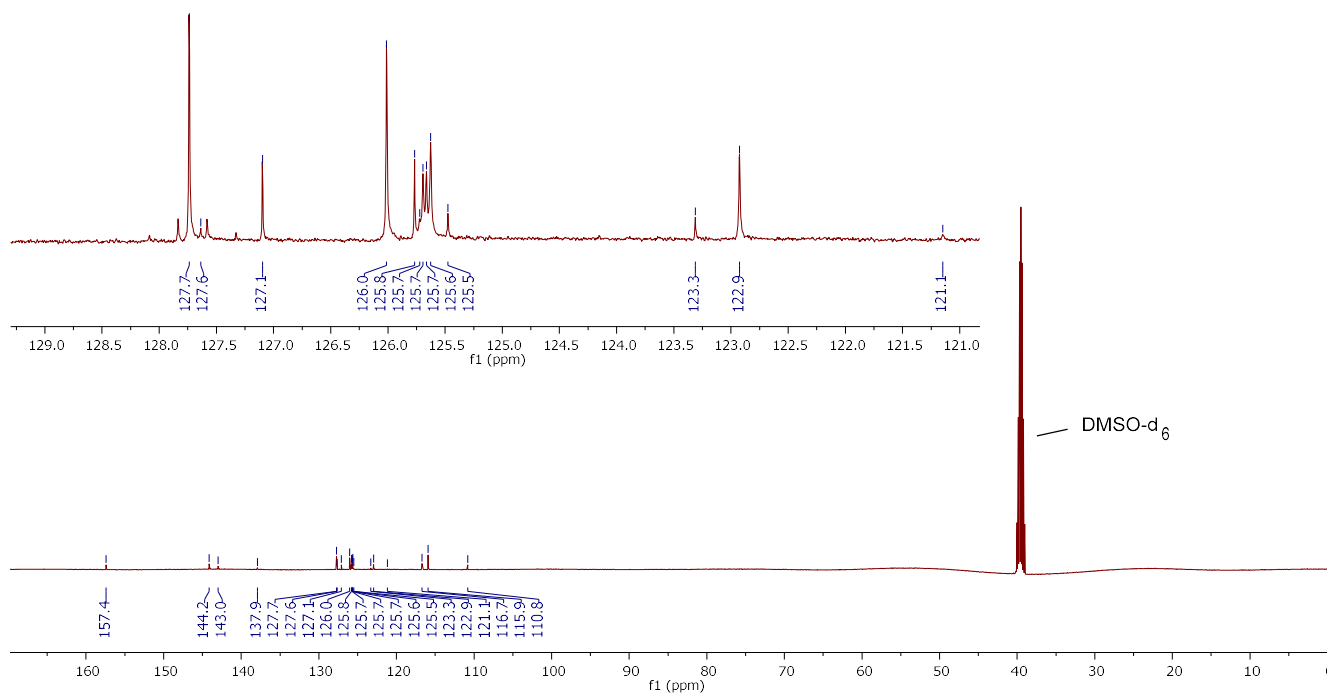
HSQC **1b** (500 MHz, DMSO-d₆)



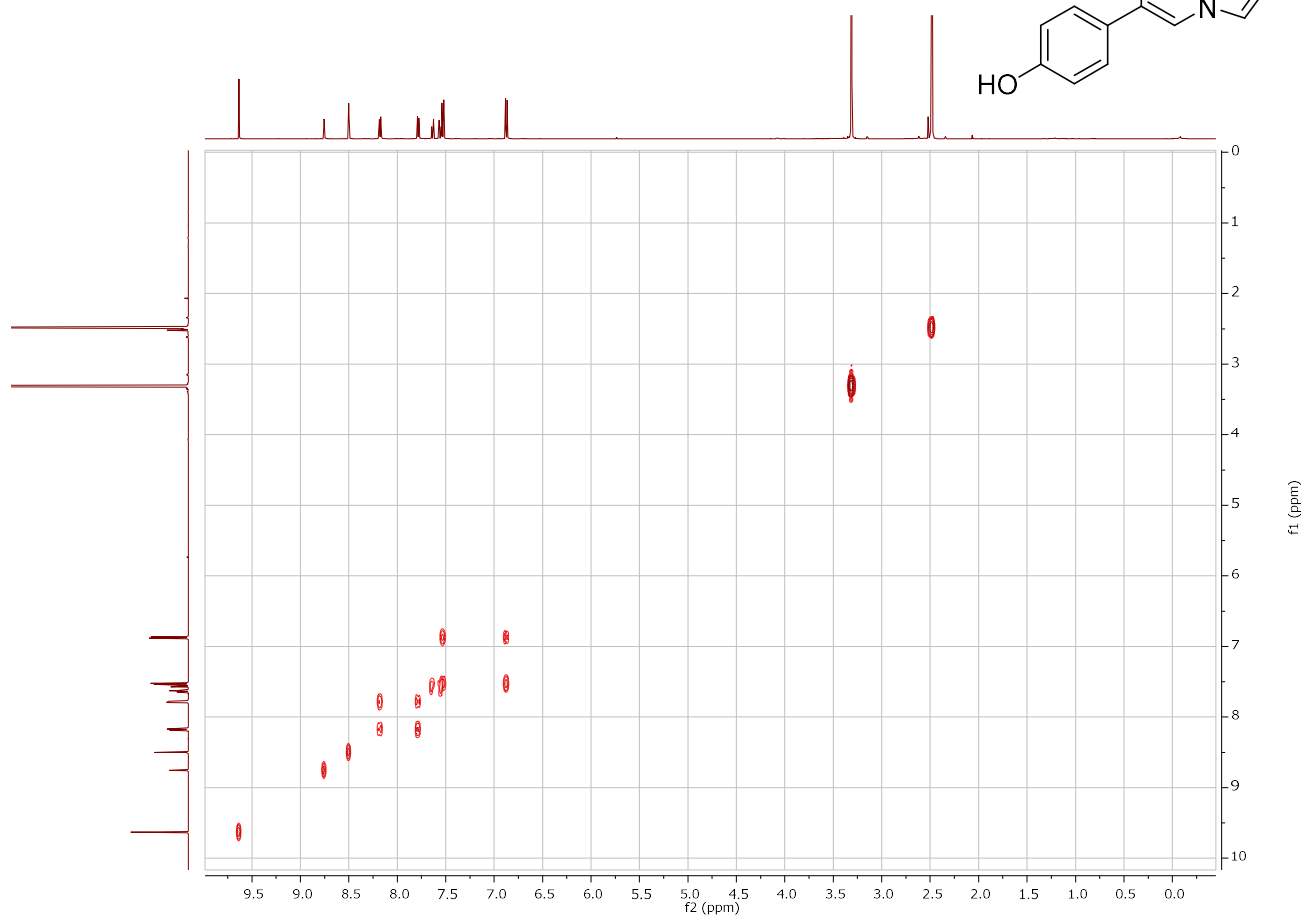
^1H NMR **1c** (500 MHz, DMSO- d_6)



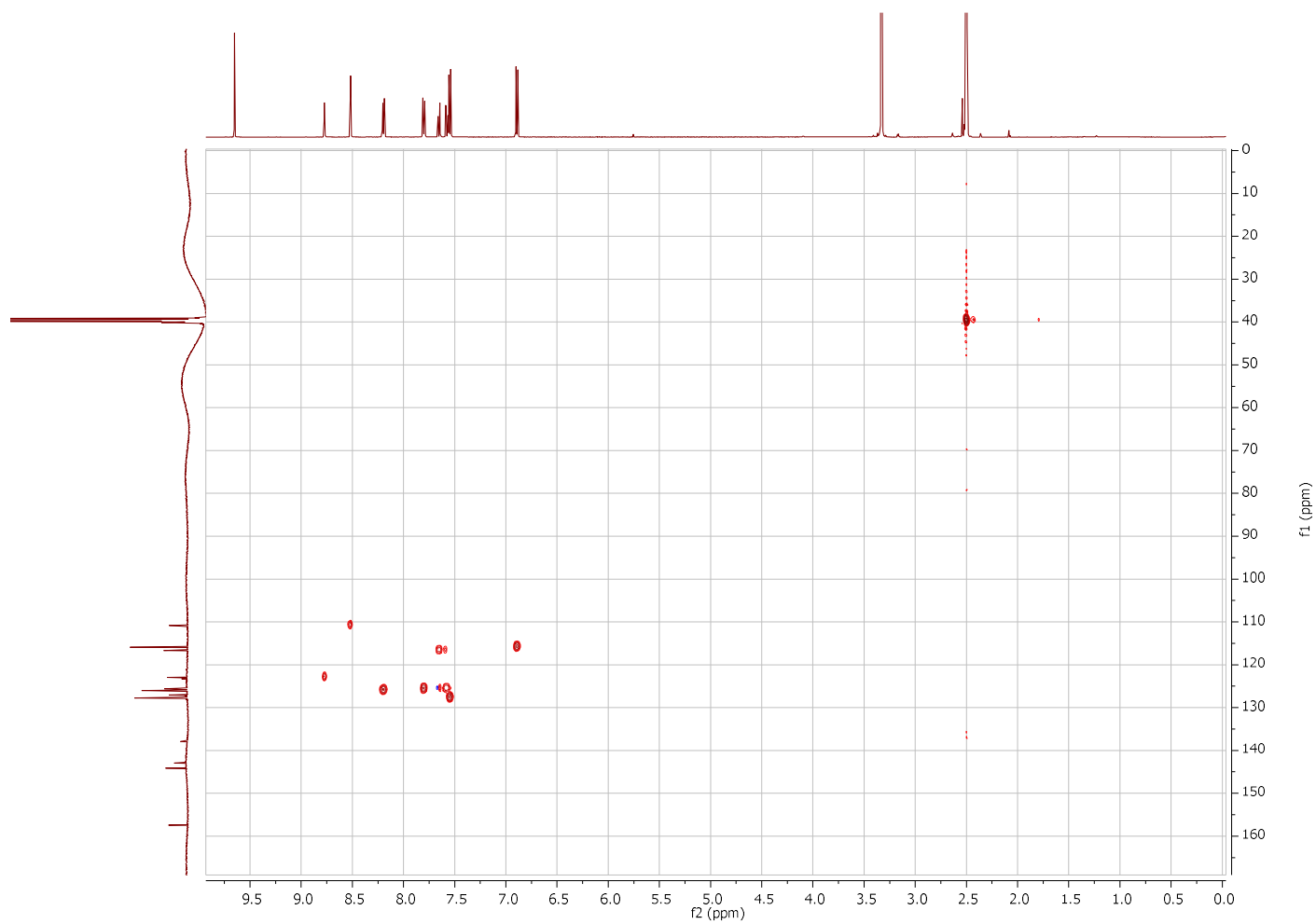
^{13}C NMR GBG210 **1c** (126 MHz, DMSO- d_6)

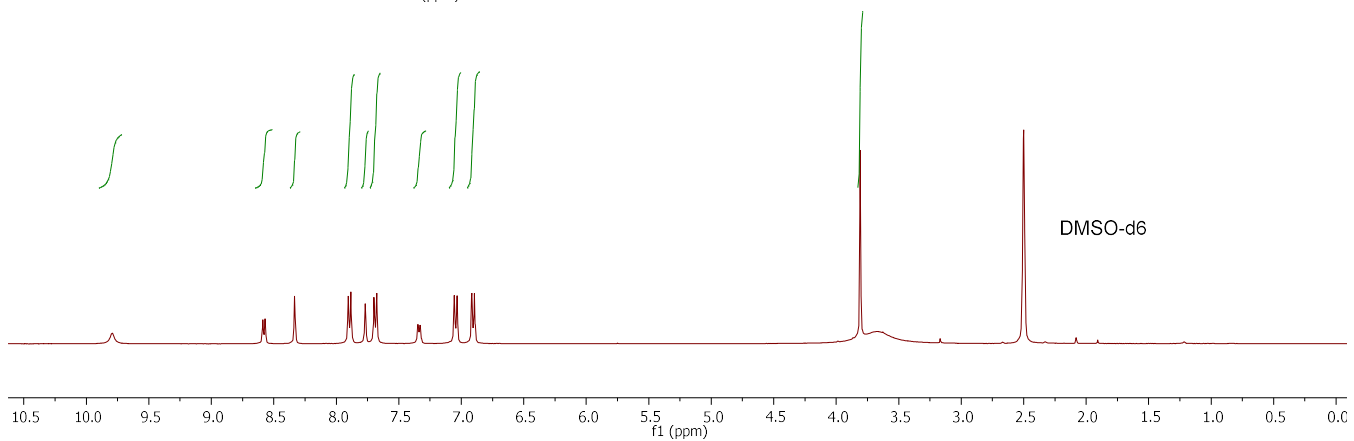
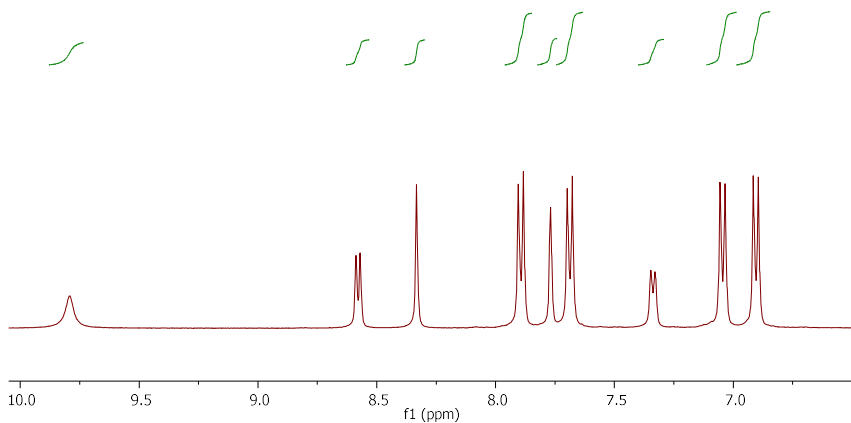
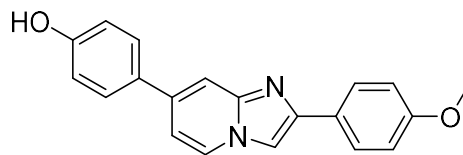
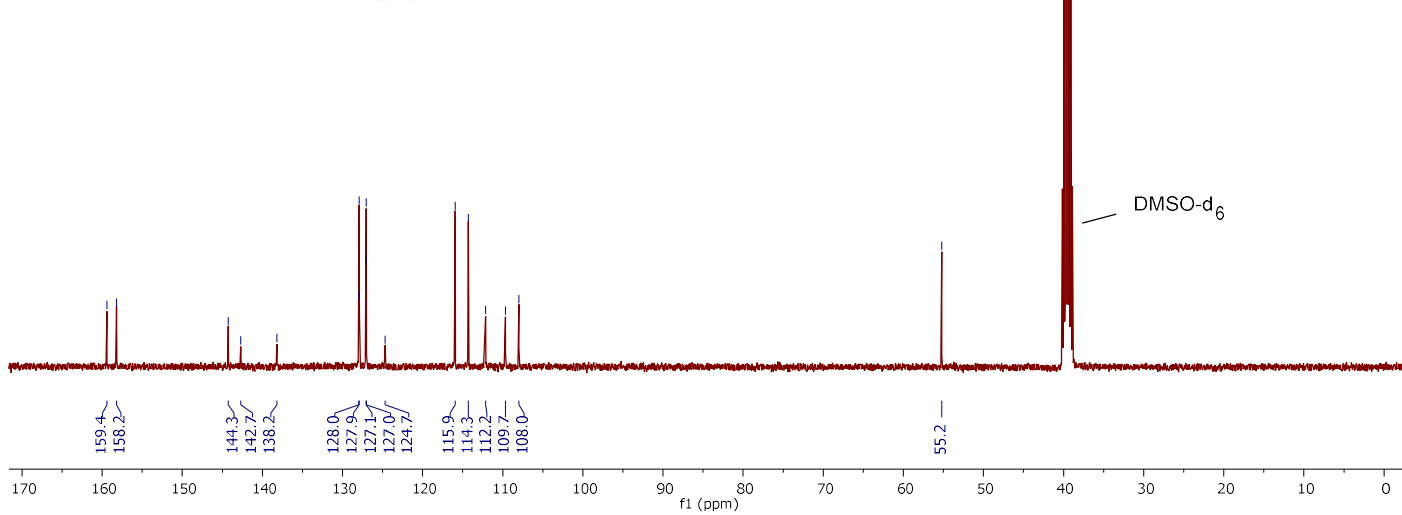
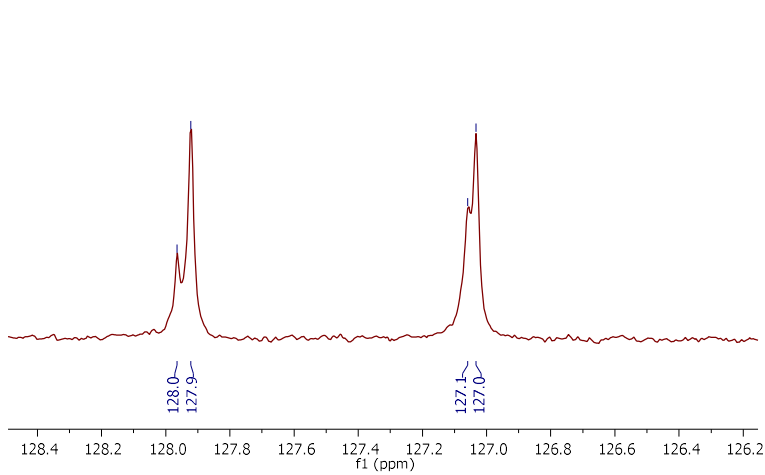


COSY **1c** (500 MHz, DMSO-d₆)

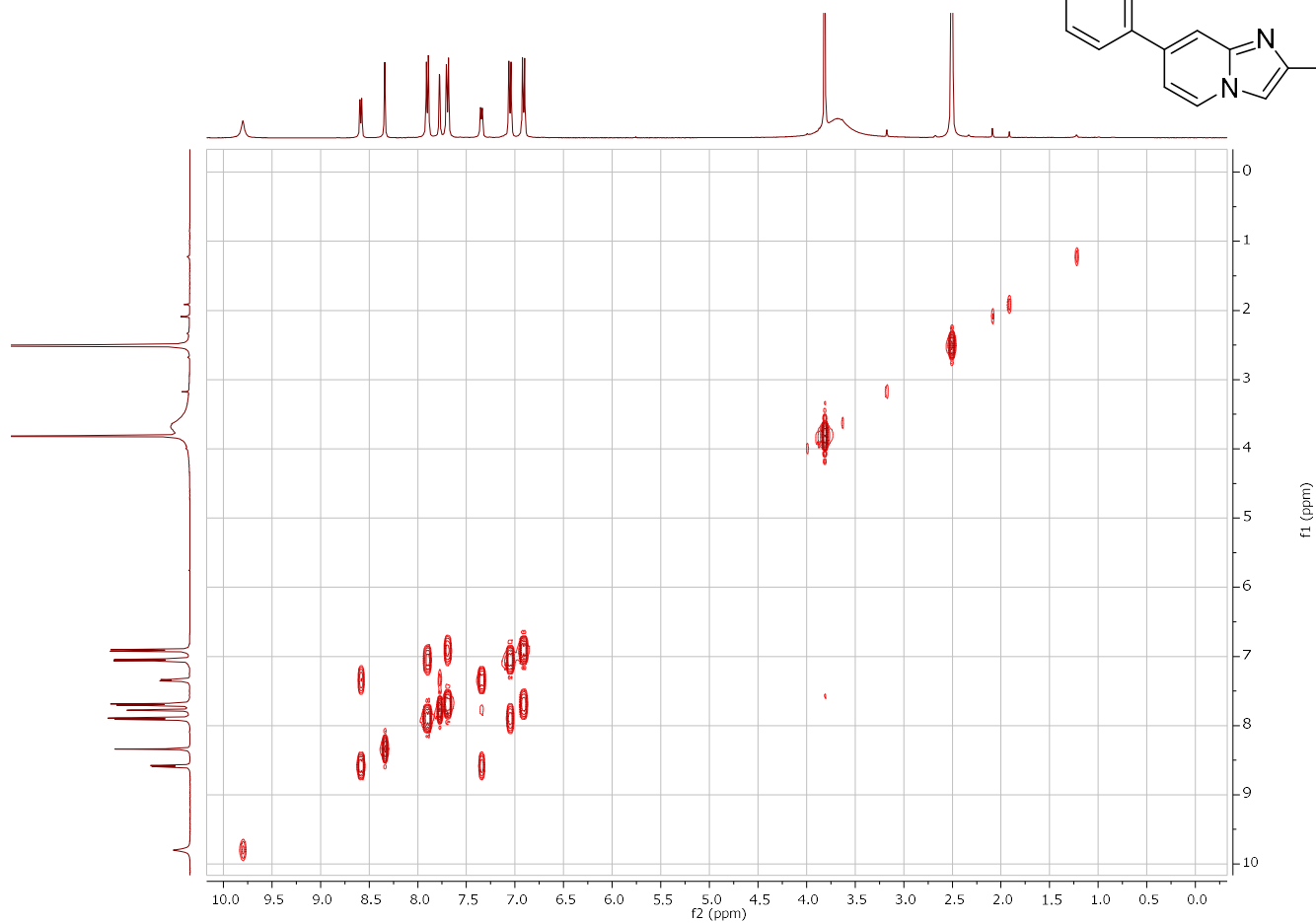


HSQC **1c** (500 MHz, DMSO-d₆)

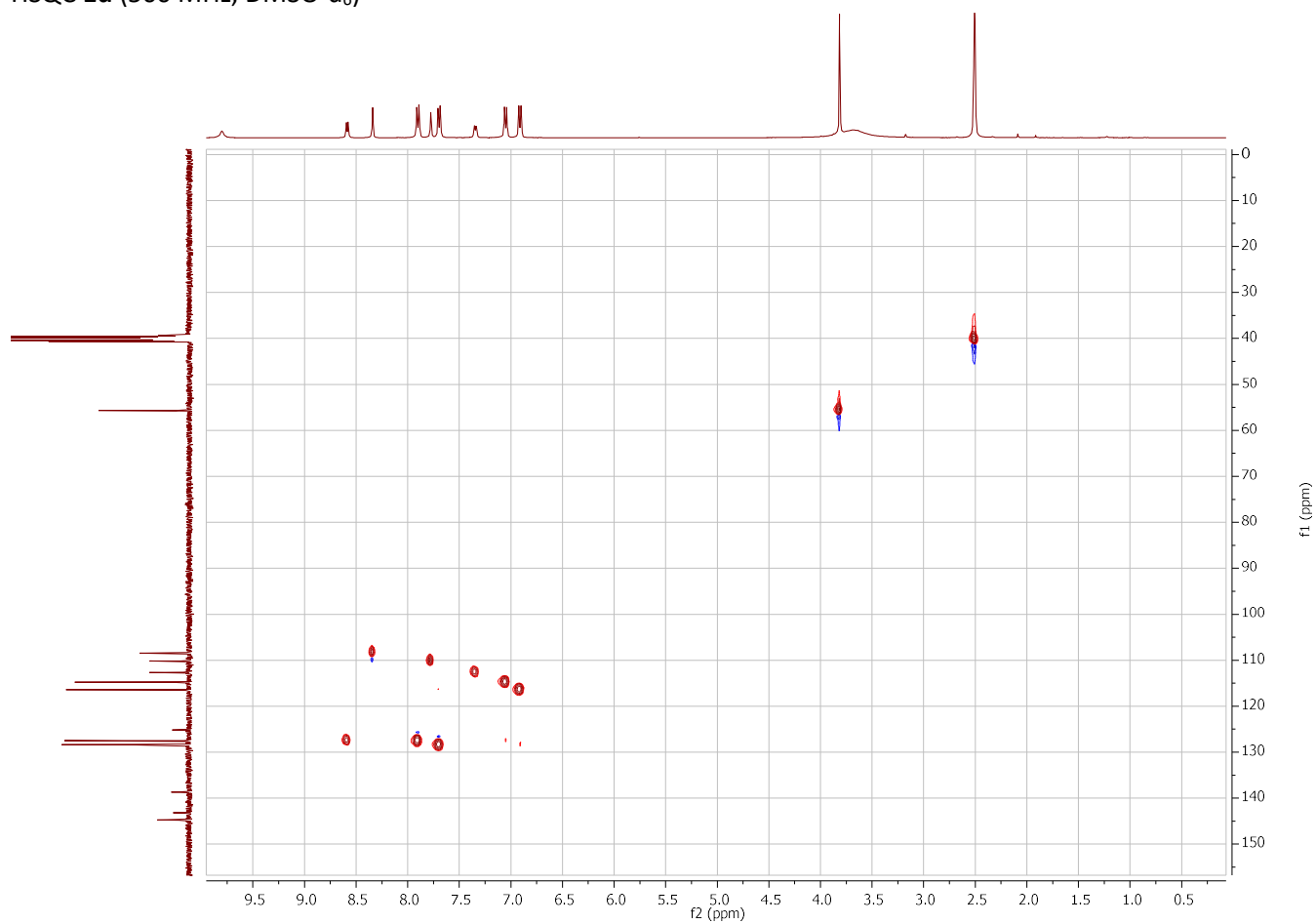


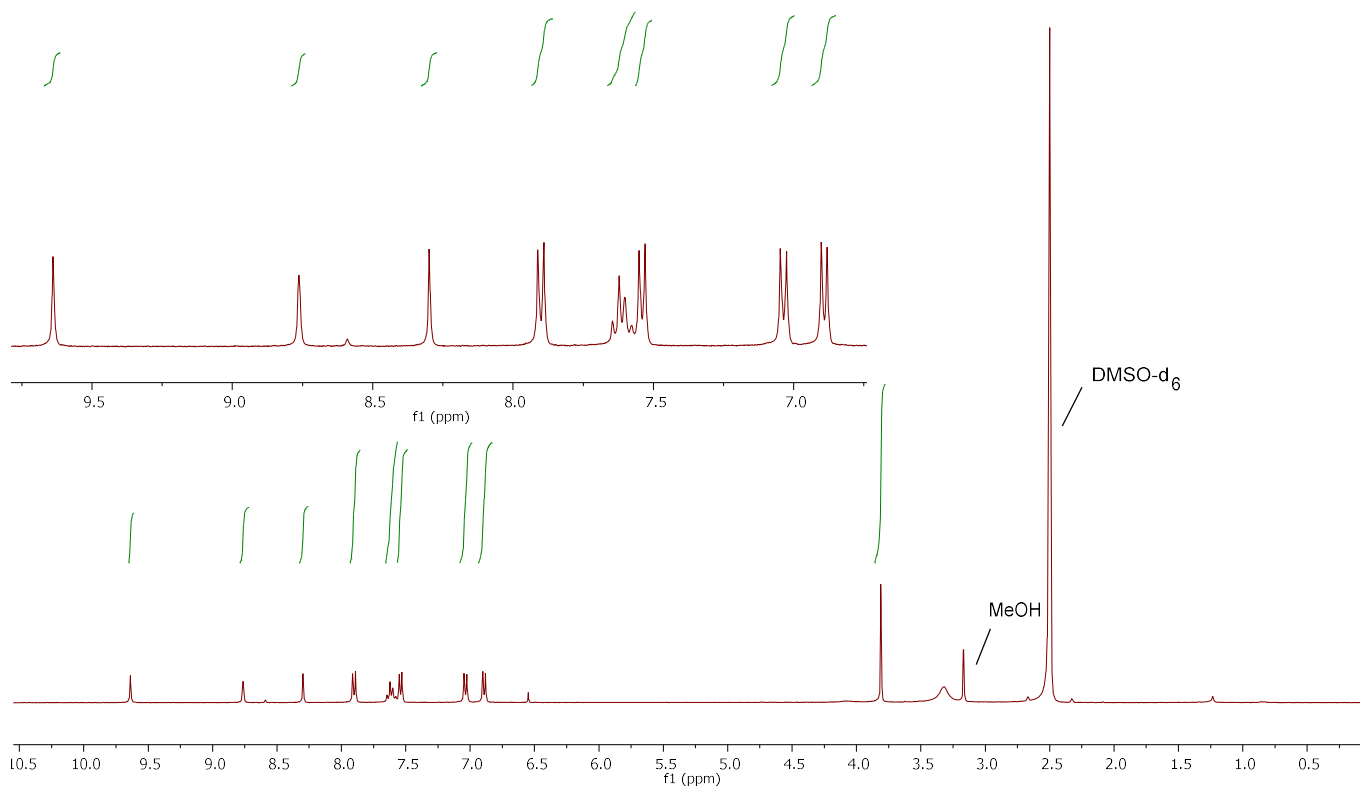
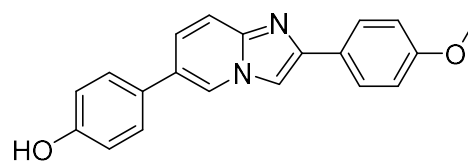
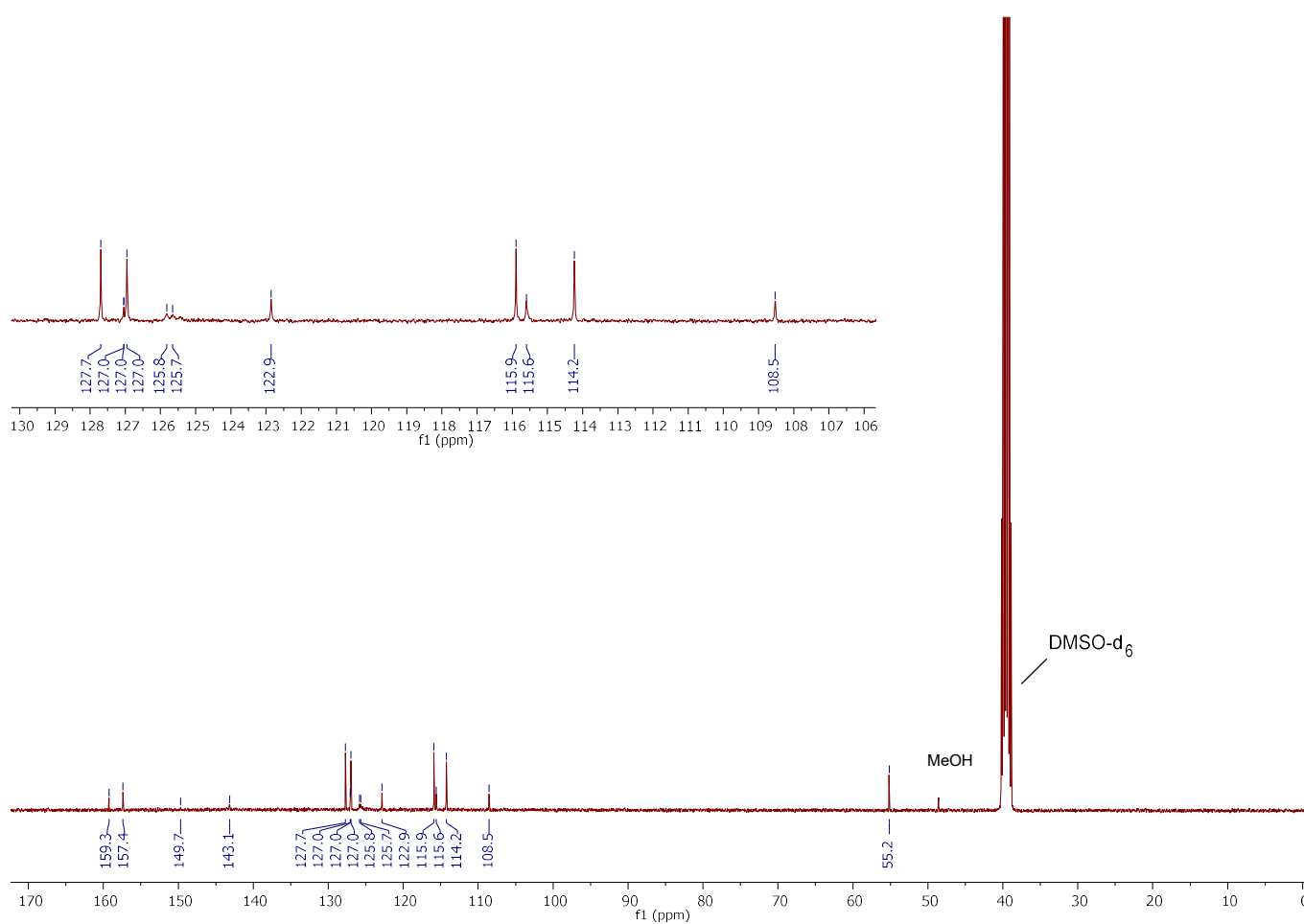
^1H NMR **1d** (400 MHz, DMSO- d_6) ^{13}C NMR **1d** (126MHz, DMSO- d_6)

COSY **1d** (500 MHz, DMSO-d₆)

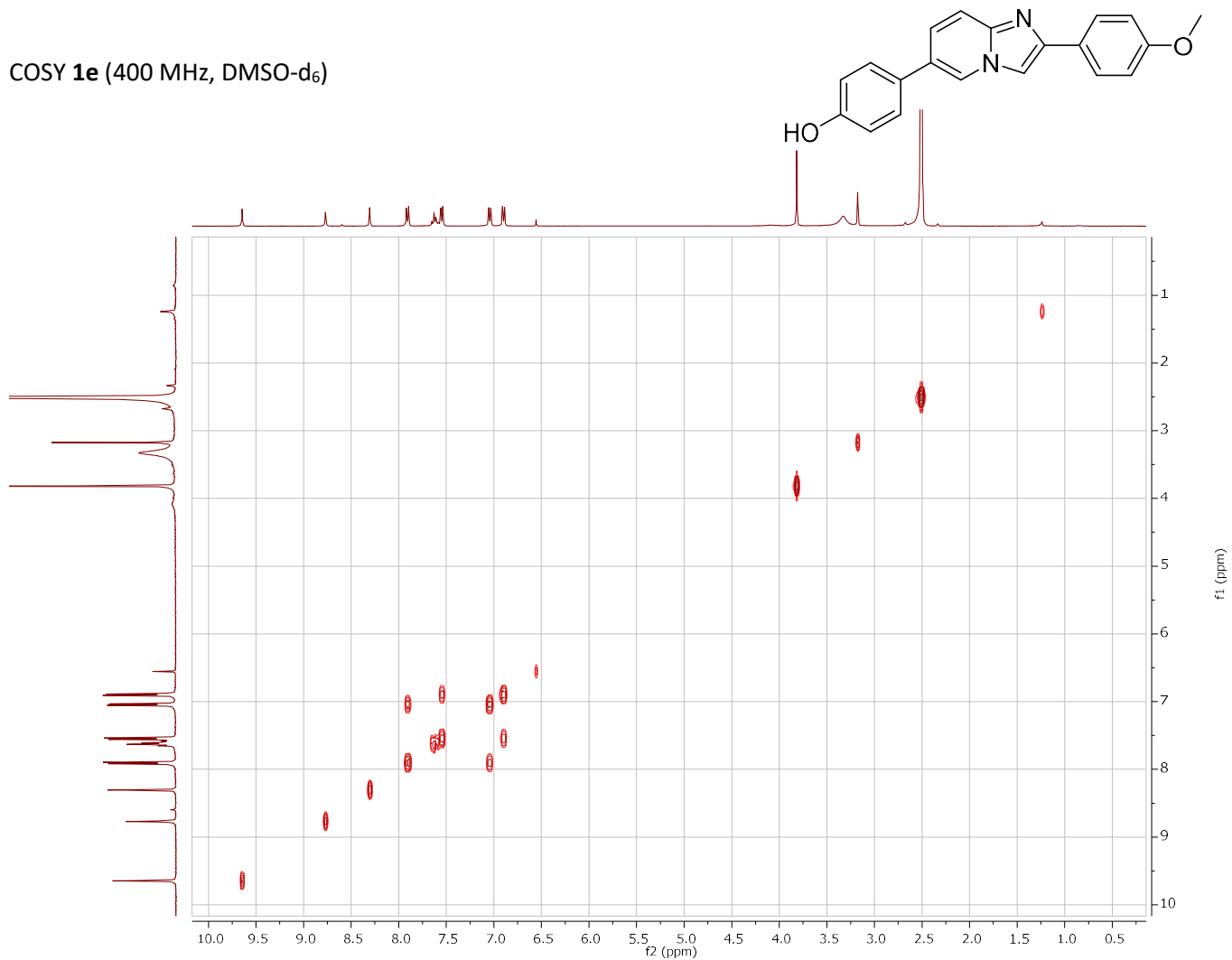


HSQC **1d** (500 MHz, DMSO-d₆)

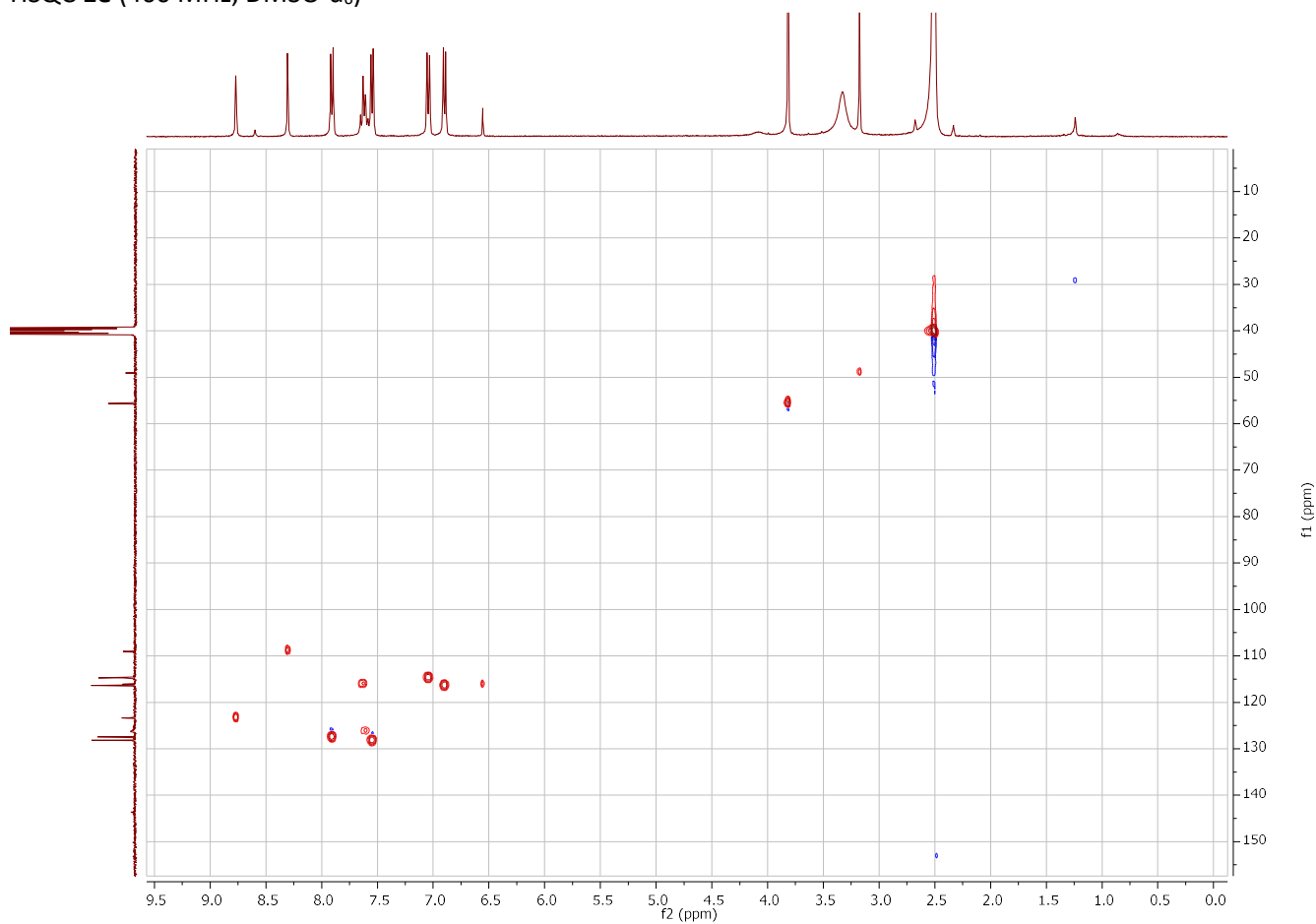


^1H NMR **1e** (400 MHz, DMSO- d_6) ^{13}C NMR **1e** (126 MHz, DMSO- d_6)

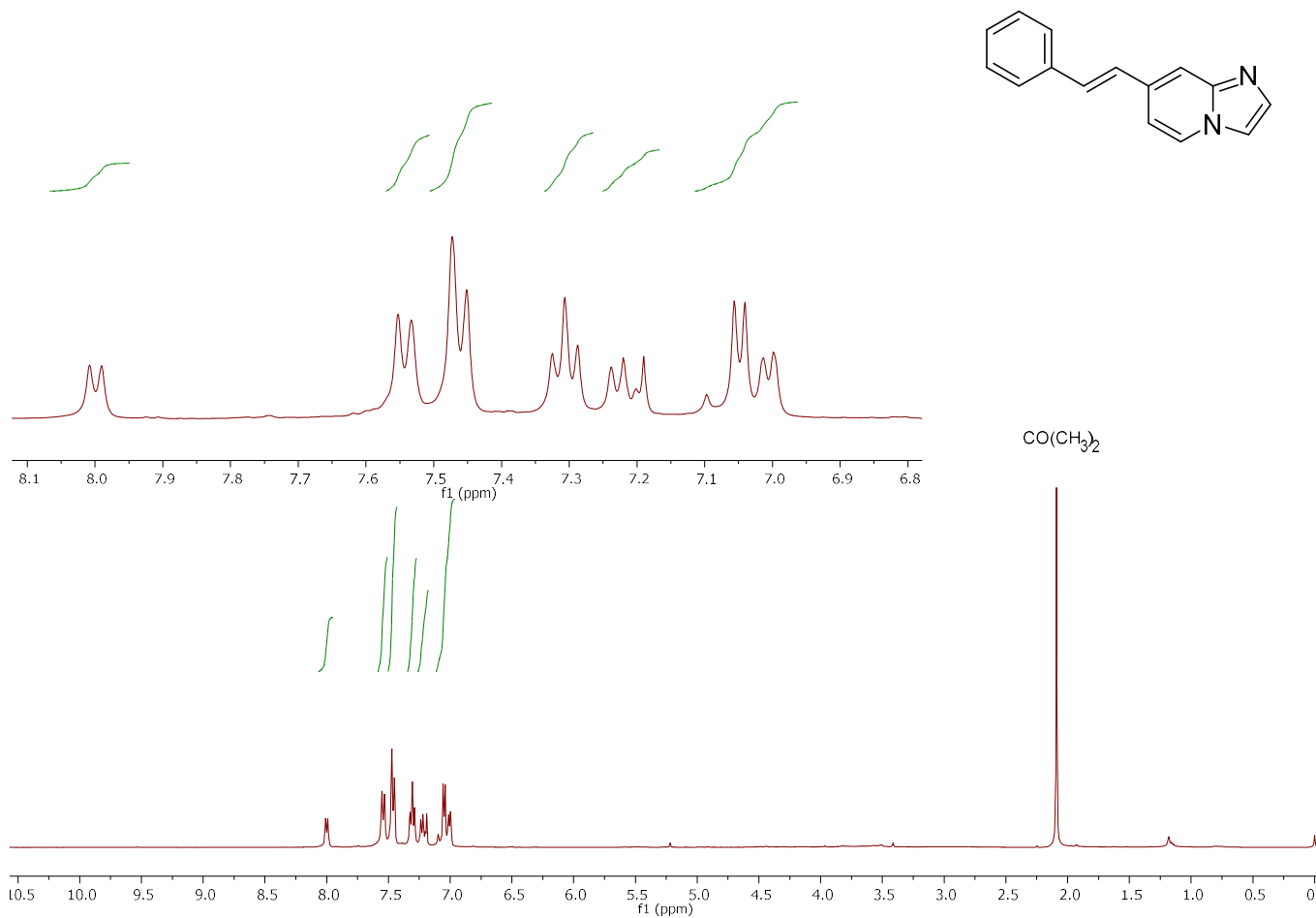
COSY **1e** (400 MHz, DMSO-d₆)



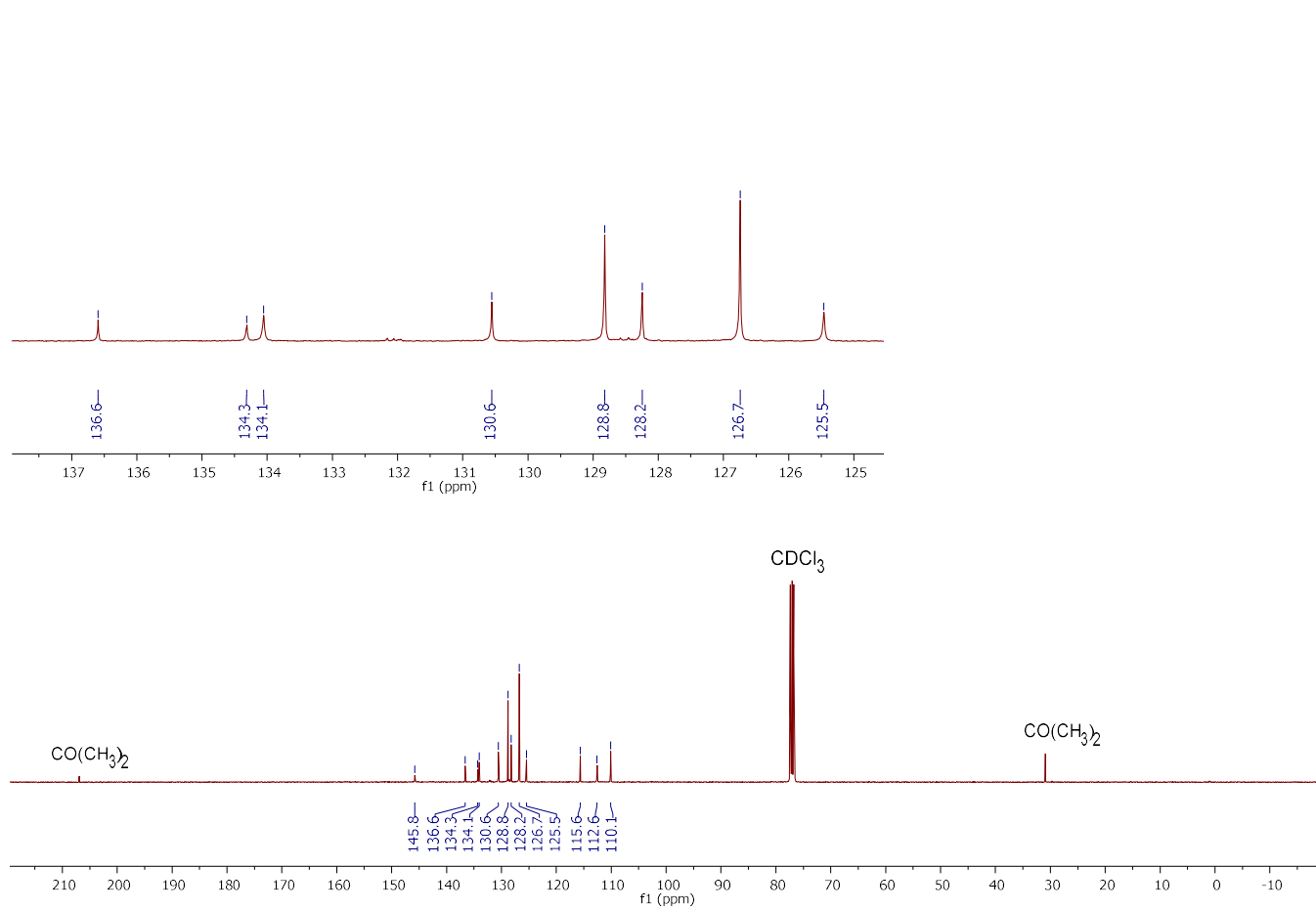
HSQC **1e** (400 MHz, DMSO-d₆)



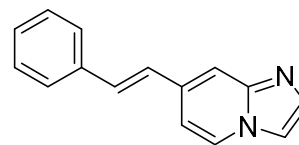
¹H NMR **1f** (400 MHz, CDCl₃)



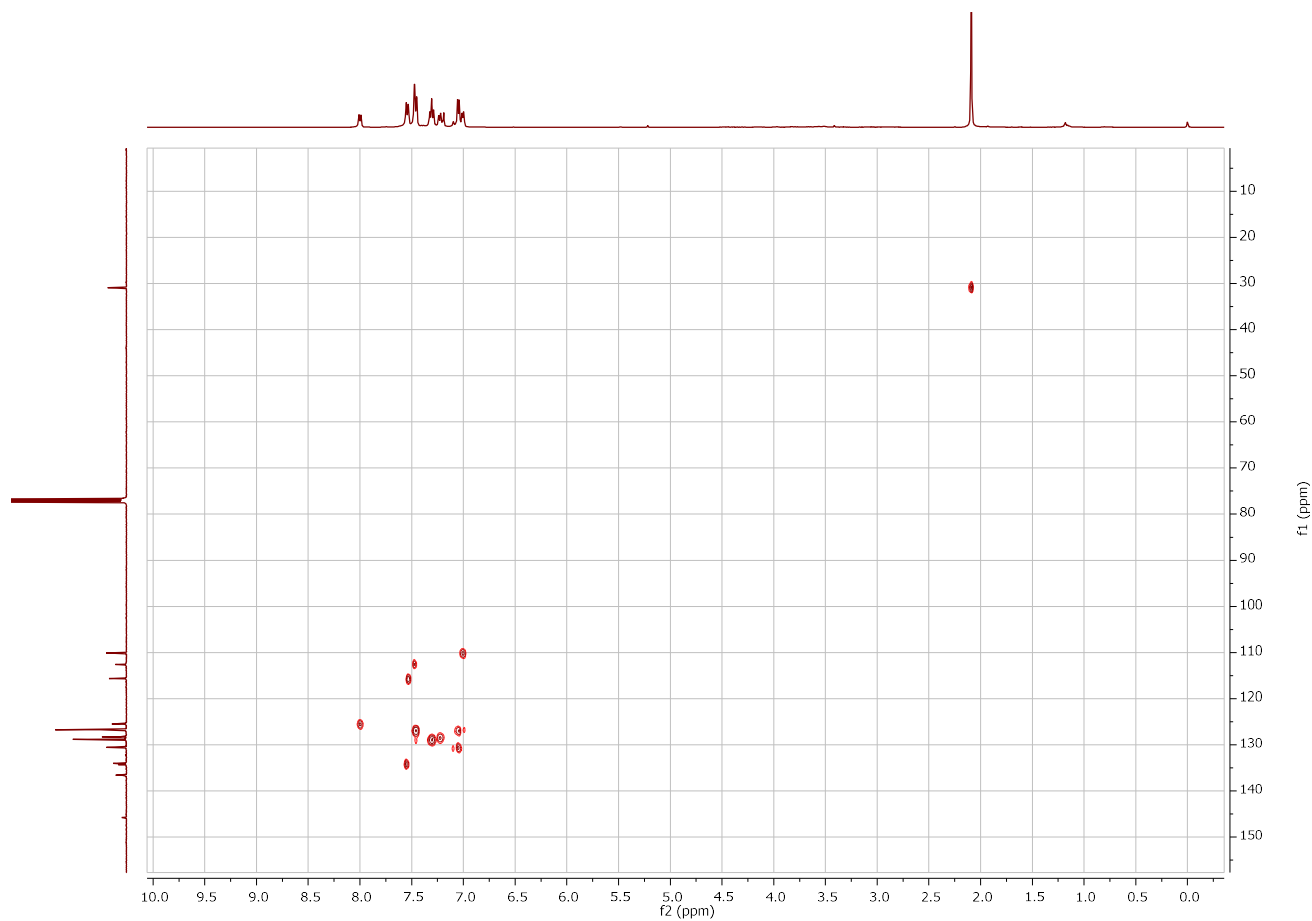
¹³C NMR **1f** (101 MHz, CDCl₃)



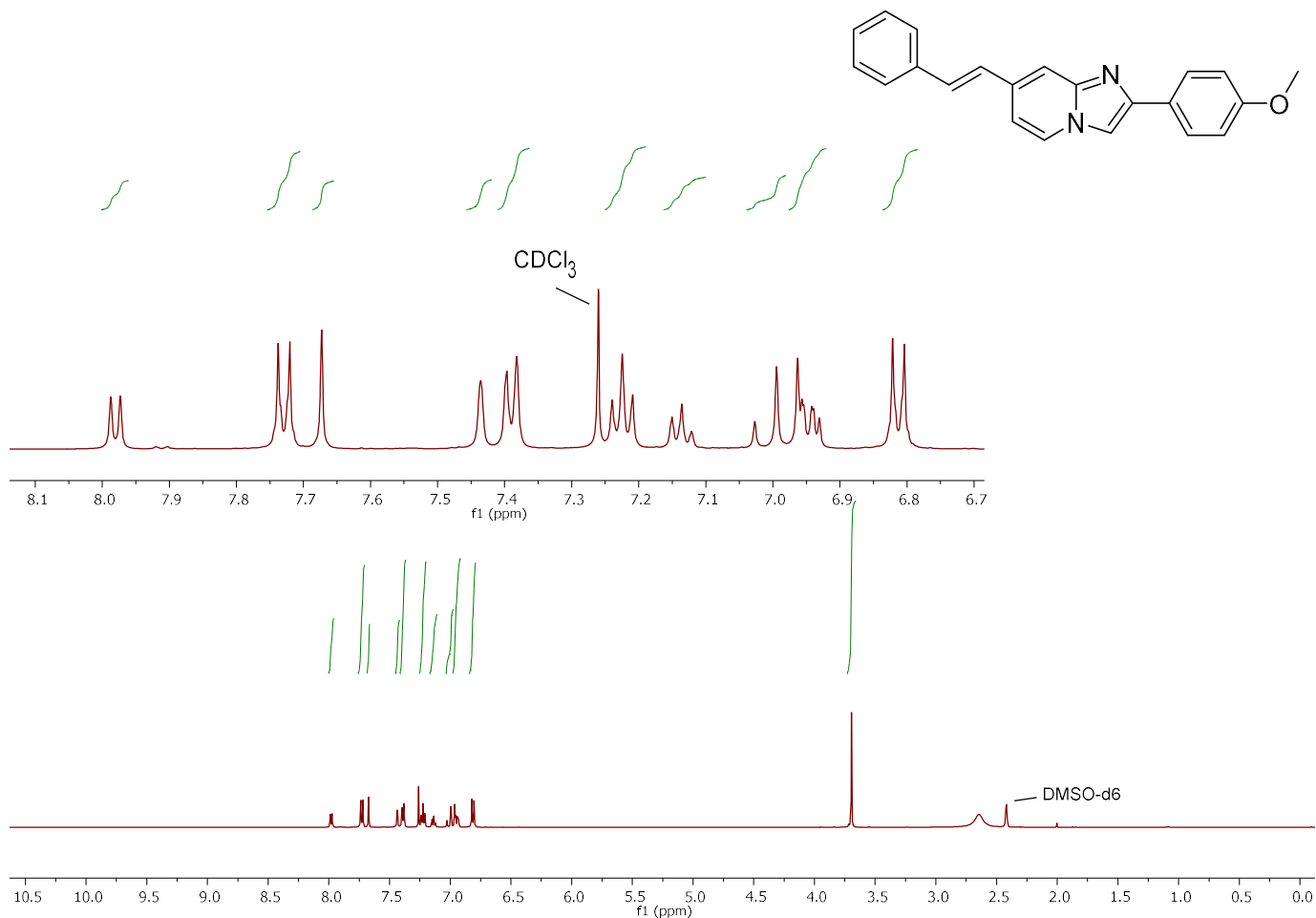
COSY **1f** (101 MHz, CDCl₃)



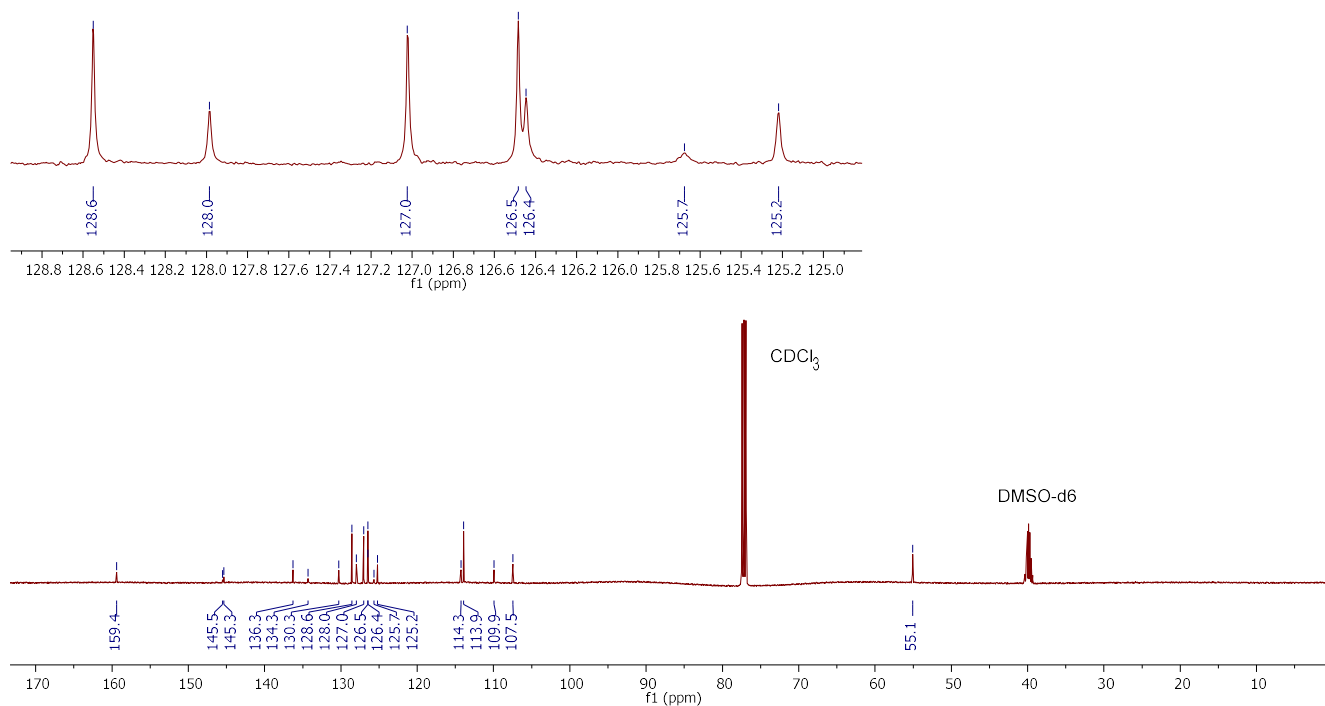
COSY **1f** (101 MHz, CDCl₃)



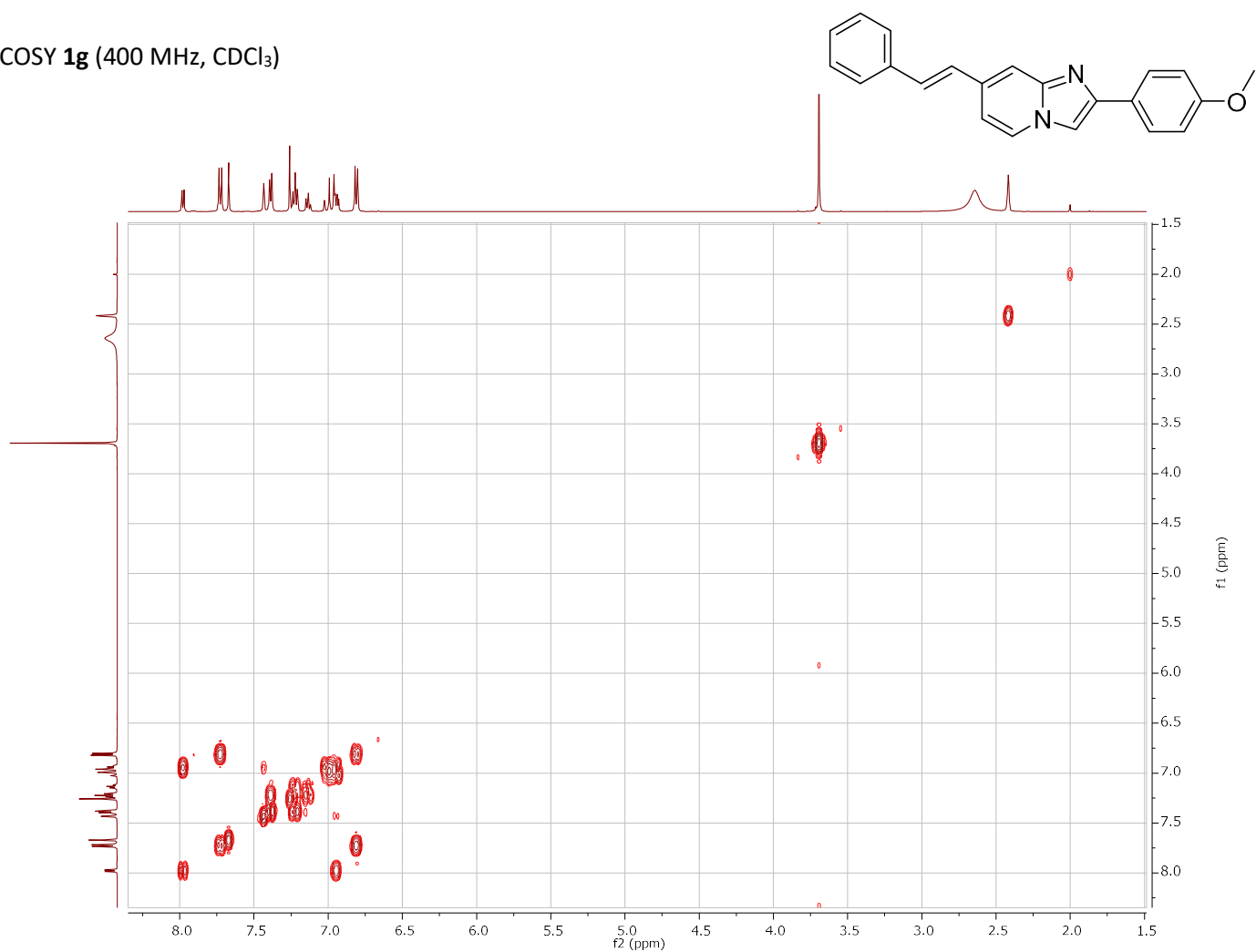
^1H NMR **1g** (500 MHz, CDCl_3) – Note: 0.2 mL of deuterated DMSO were added to increase solubility



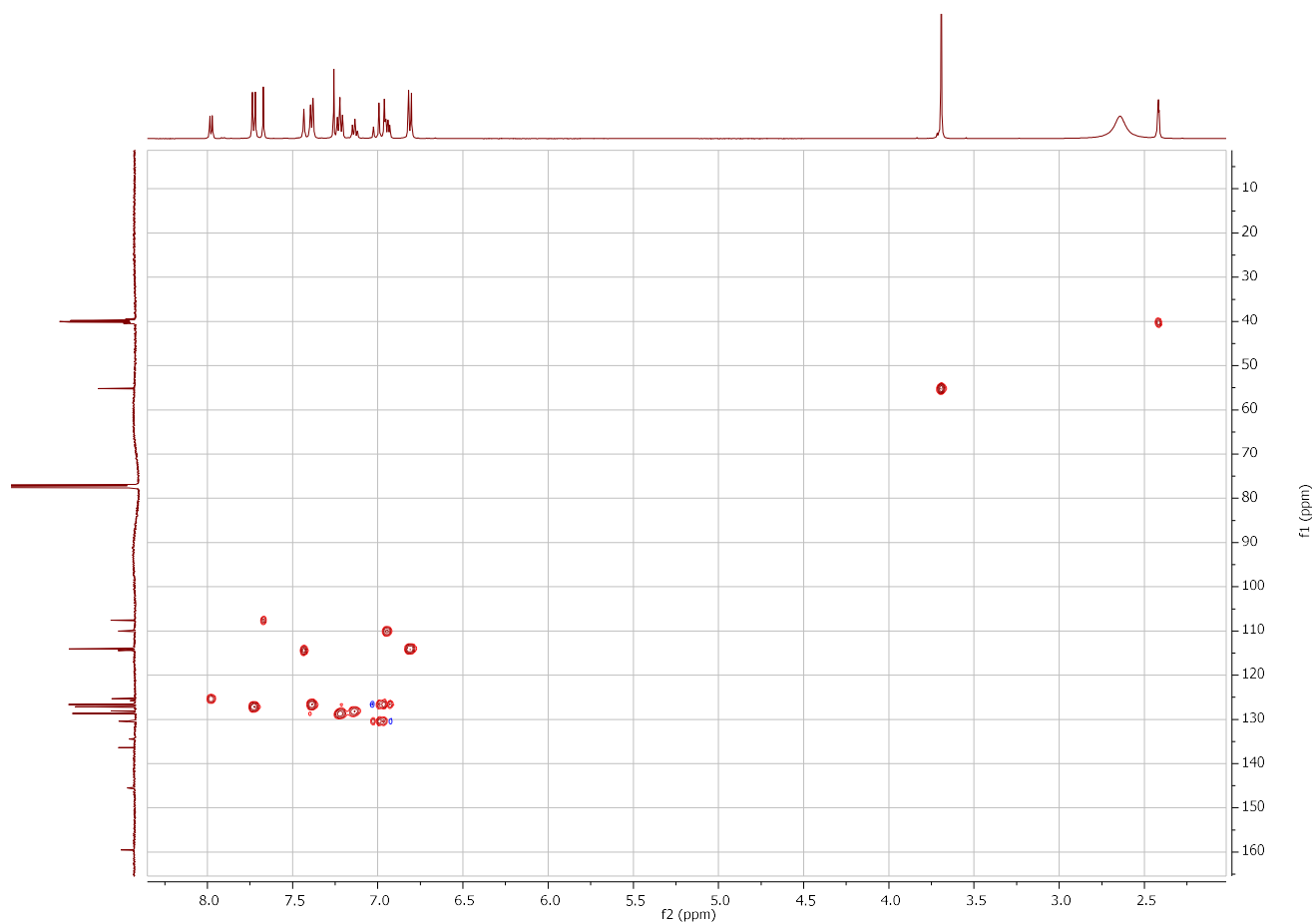
^{13}C NMR **1g** (125 MHz, CDCl_3)

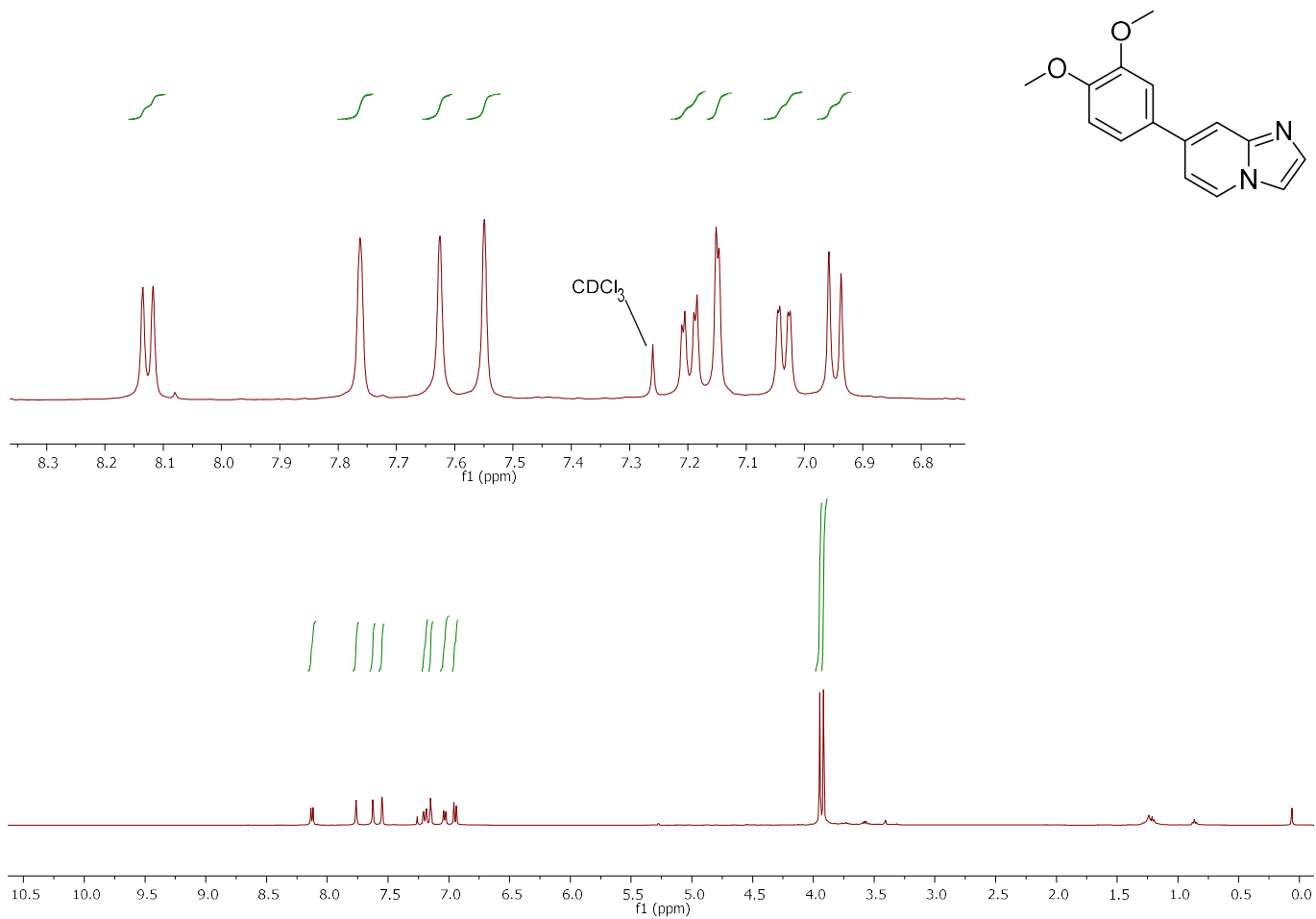
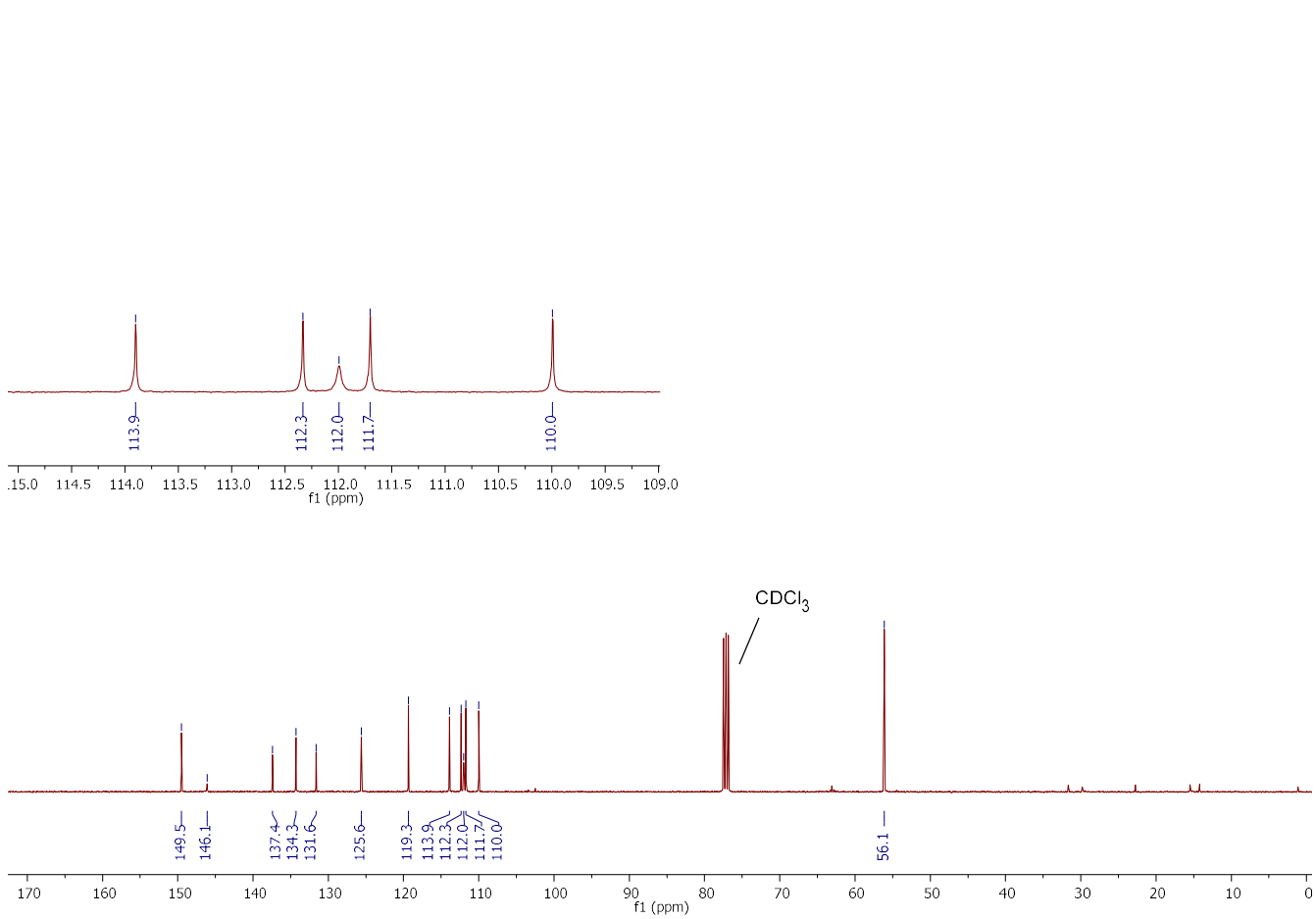


COSY **1g** (400 MHz, CDCl₃)

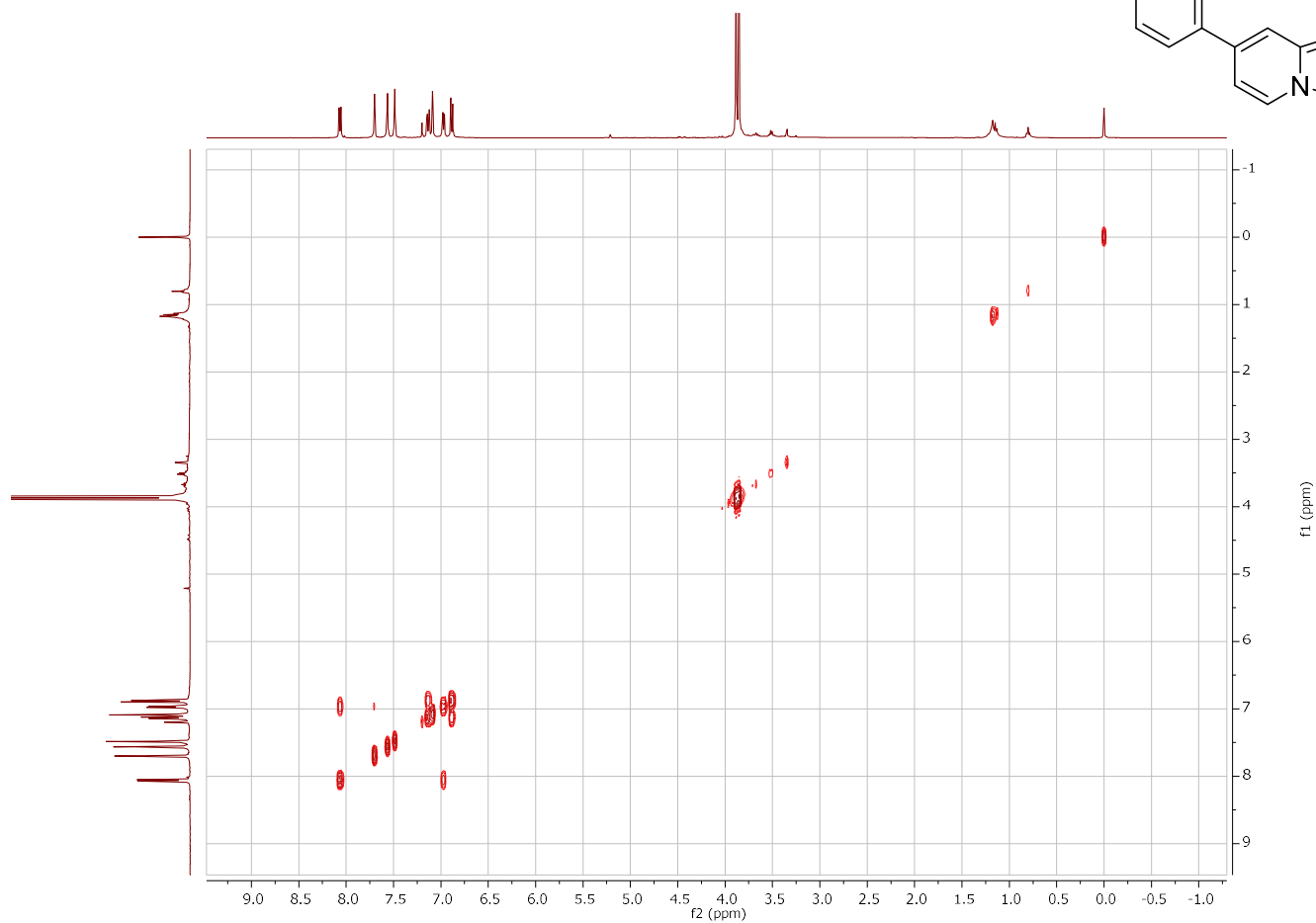


HSQC **1g** (400 MHz, CDCl₃)

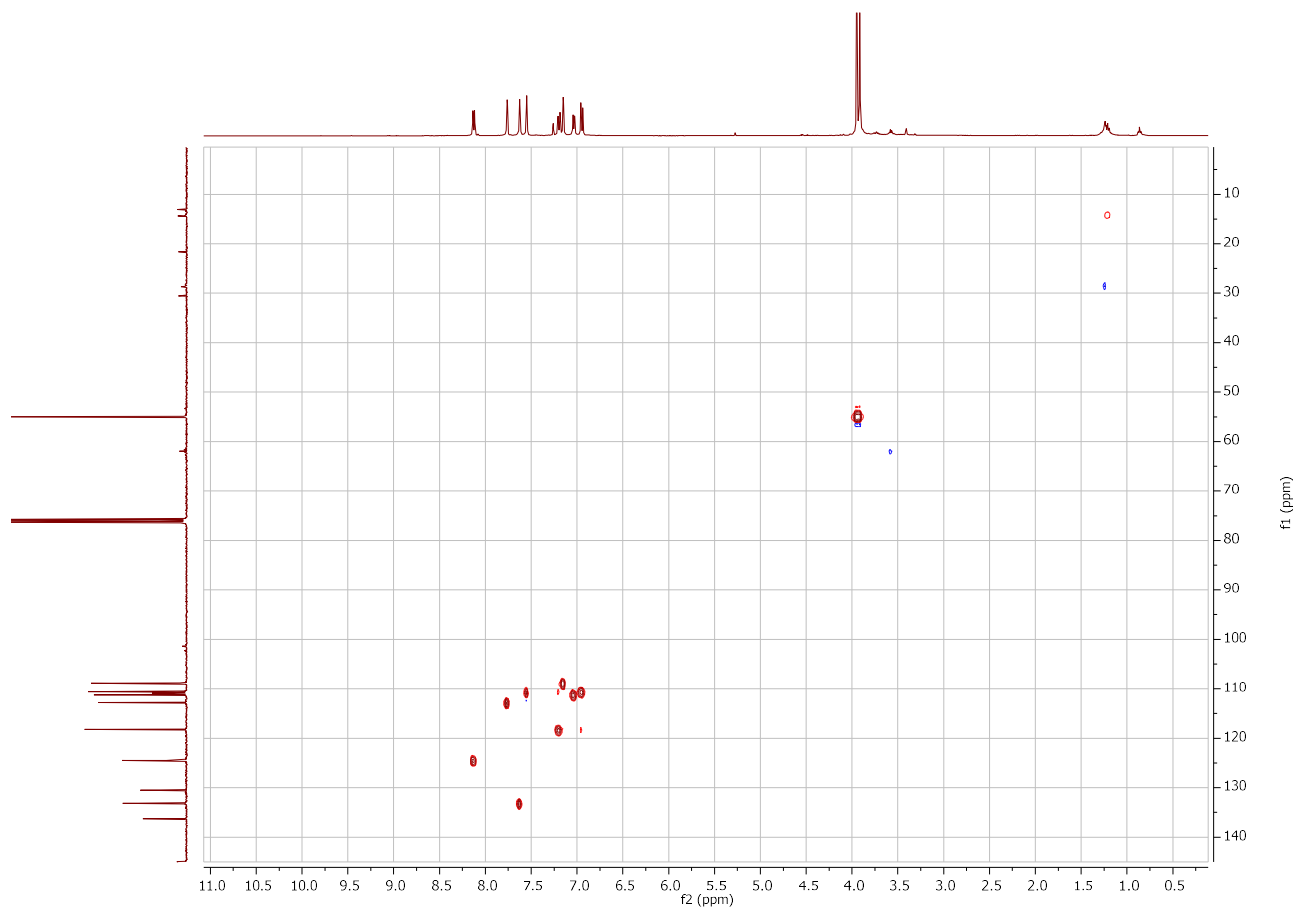


^1H NMR **1h** (400 MHz, CDCl_3) ^{13}C NMR **1h** (101 MHz, CDI_3)

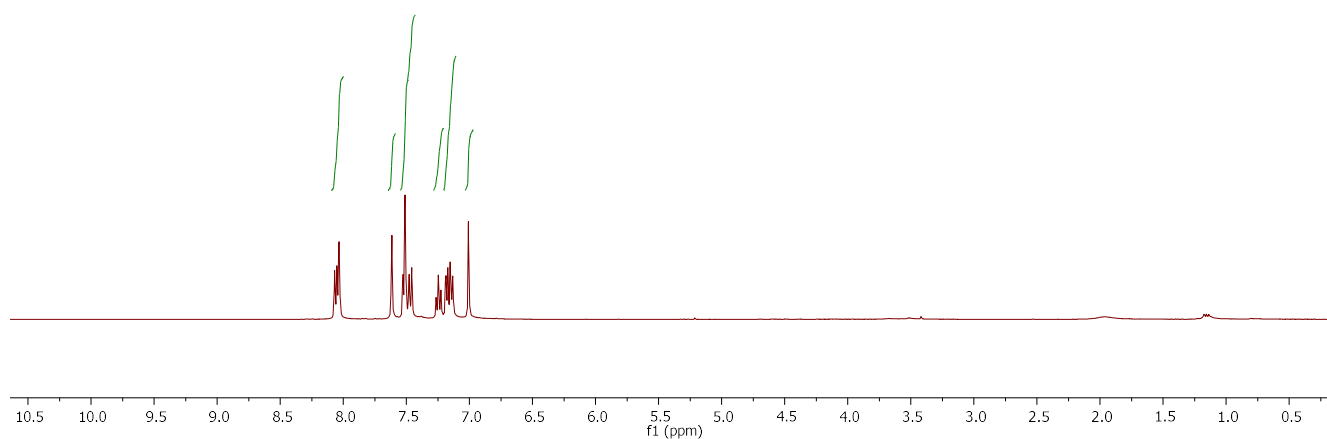
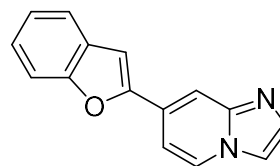
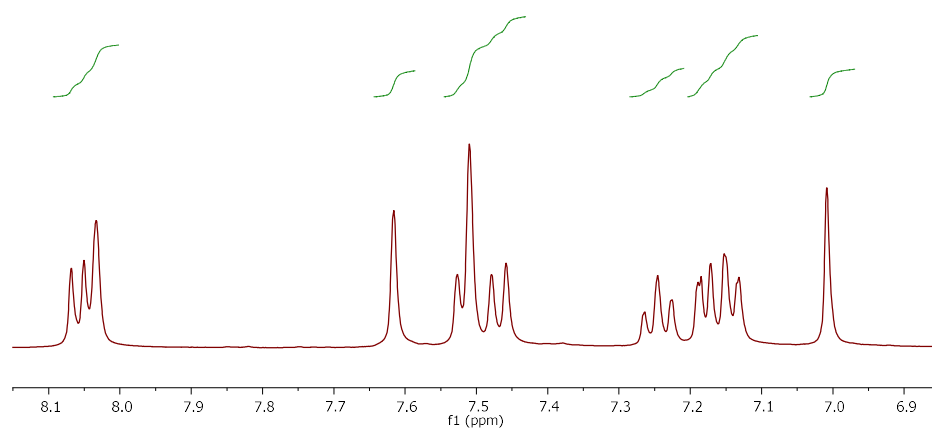
COSY 1h (400 MHz, CDCl₃)



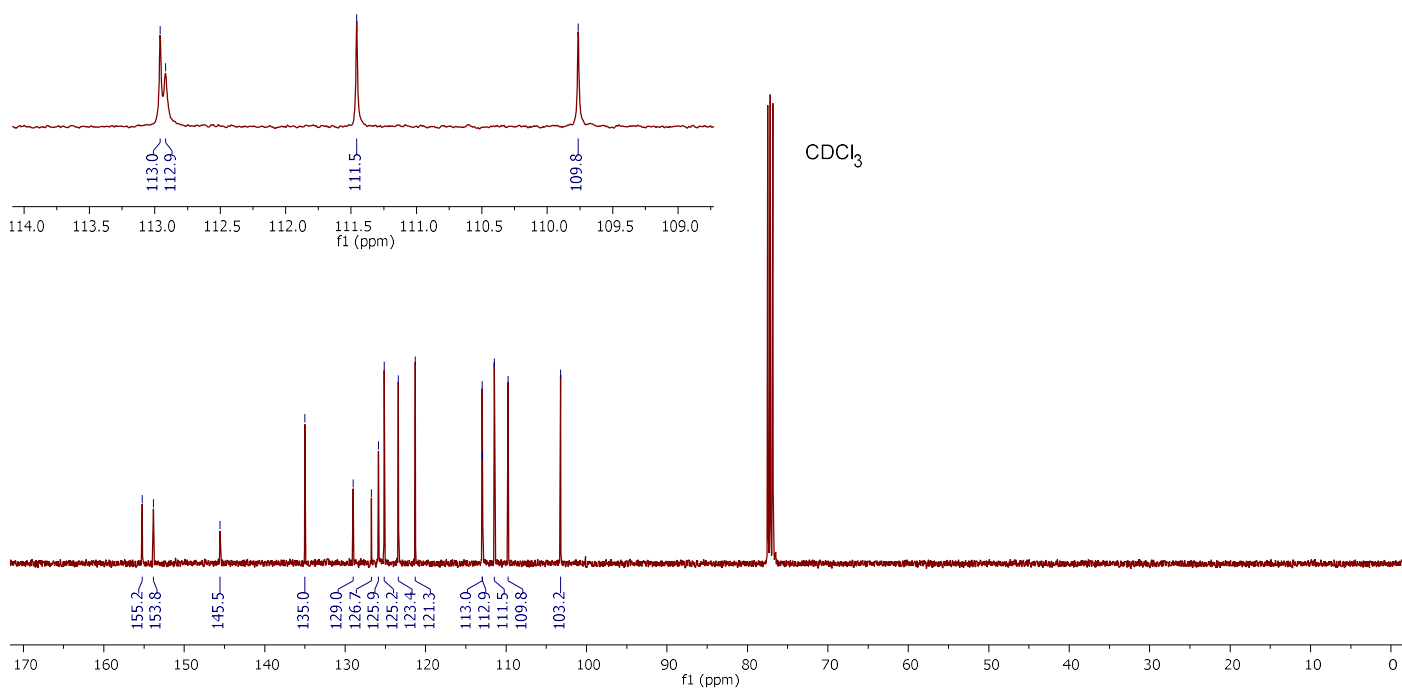
HSQC 1h (400 MHz, CDCl₃)



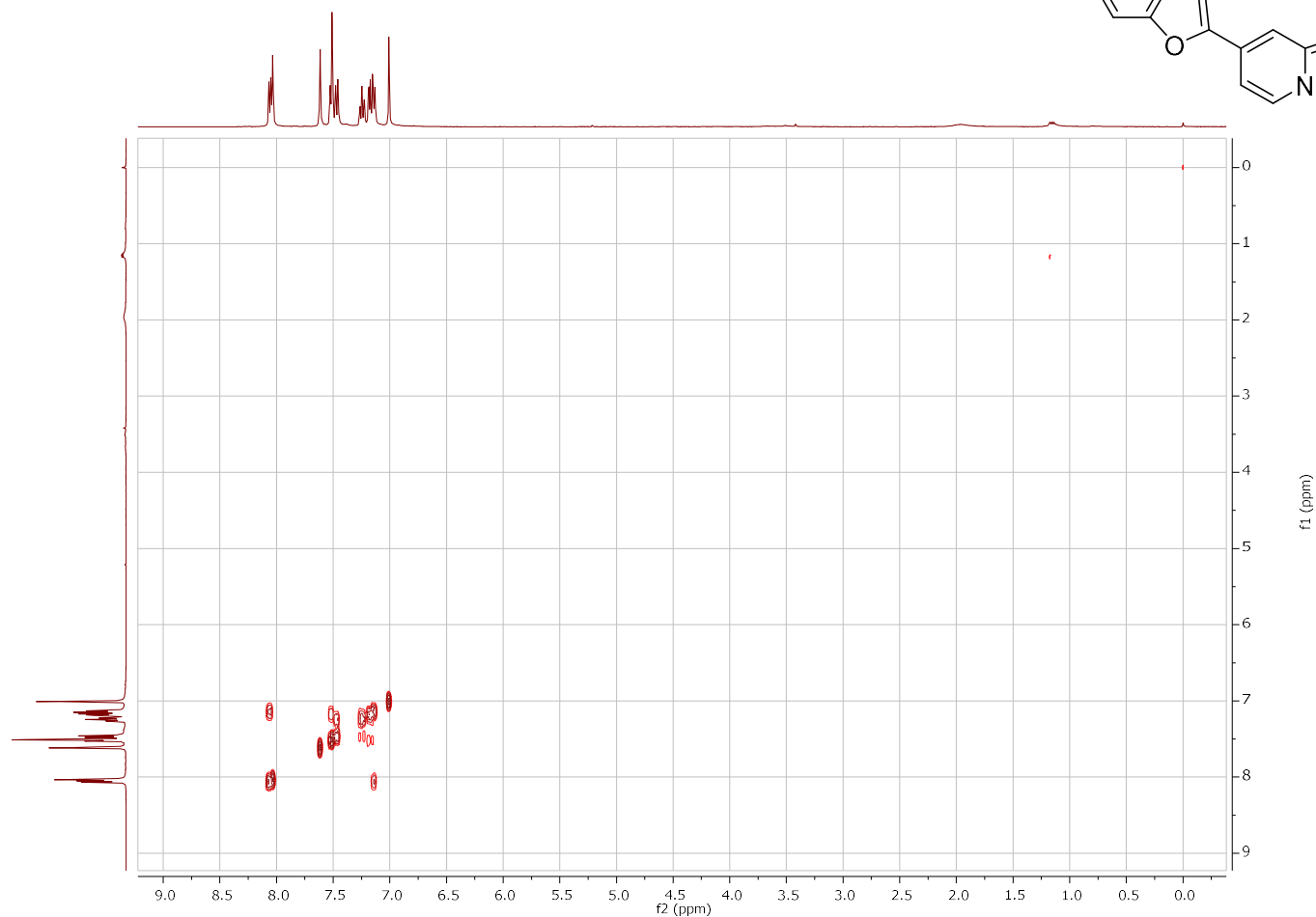
¹H NMR **1i** (400 MHz, CDCl₃)



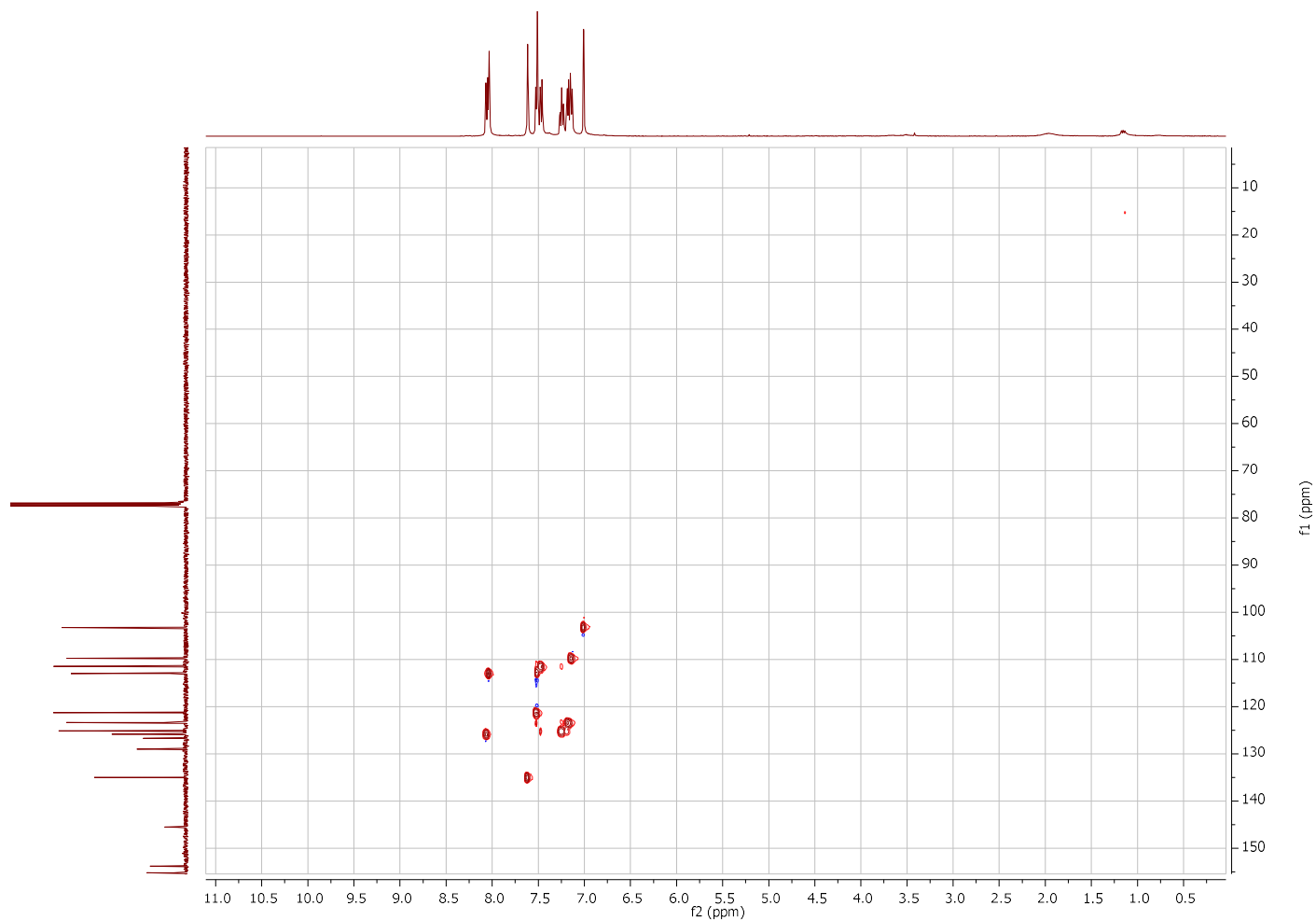
¹³C NMR **1i** (400 MHz, CDCl₃)



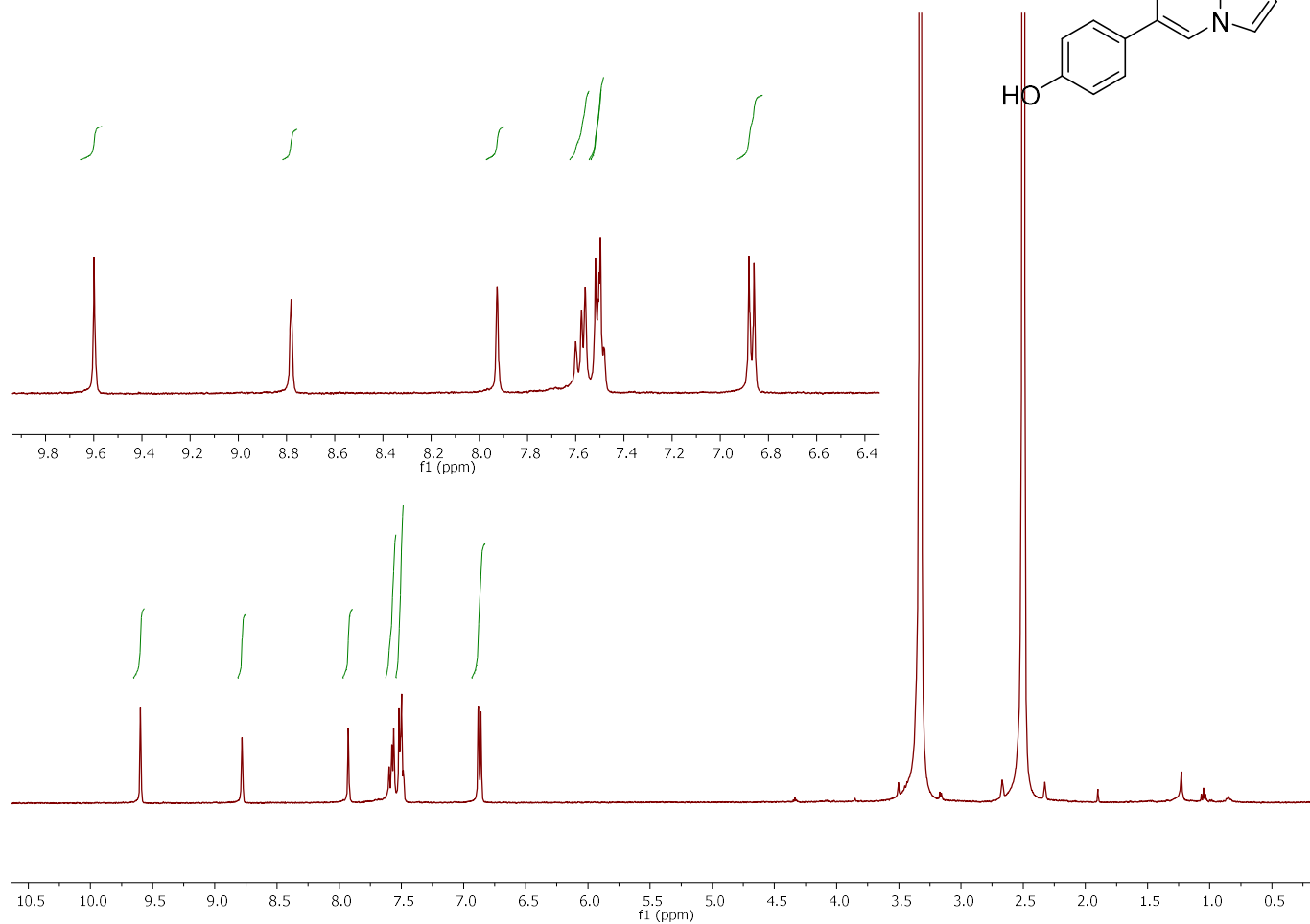
COSY **1i** (400 MHz, CDCl₃)



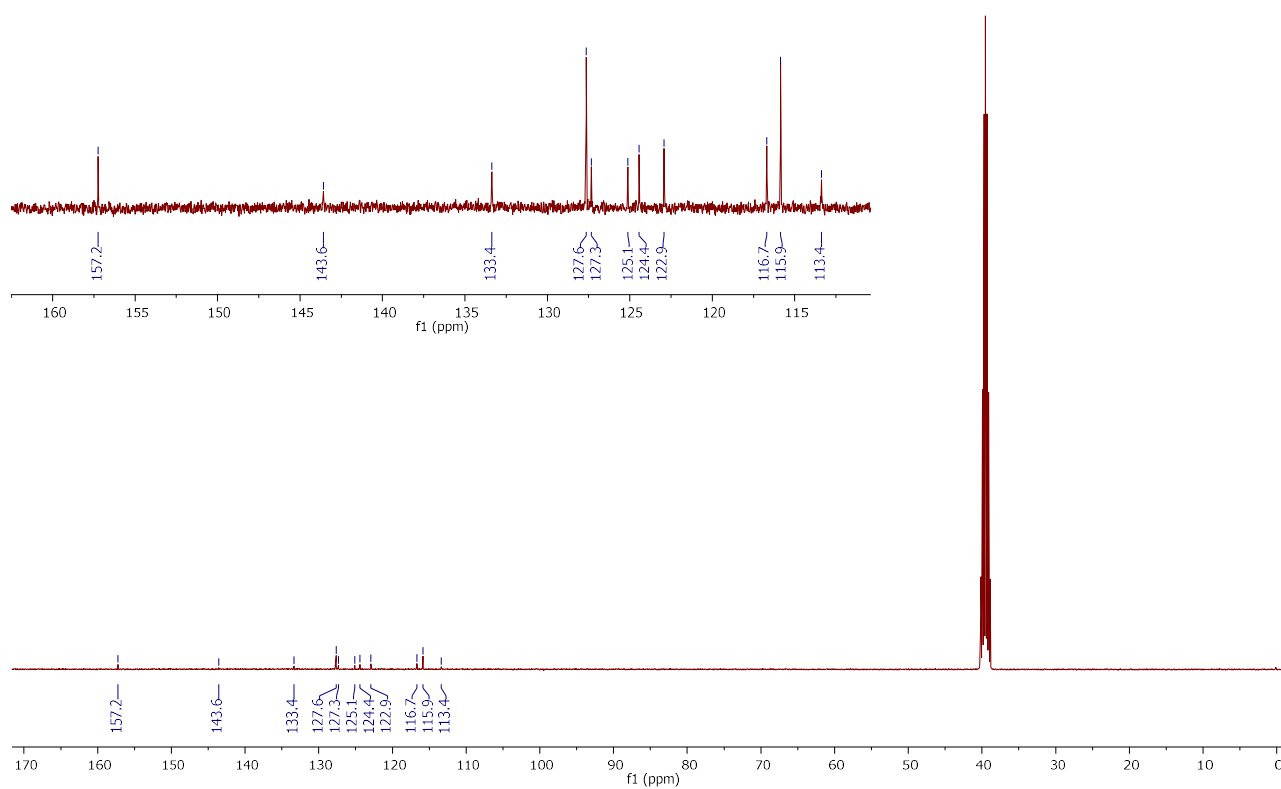
HSQC **1i** (400 MHz, CDCl₃)



^1H NMR **1j** (400 MHz, DMSO- d_6)



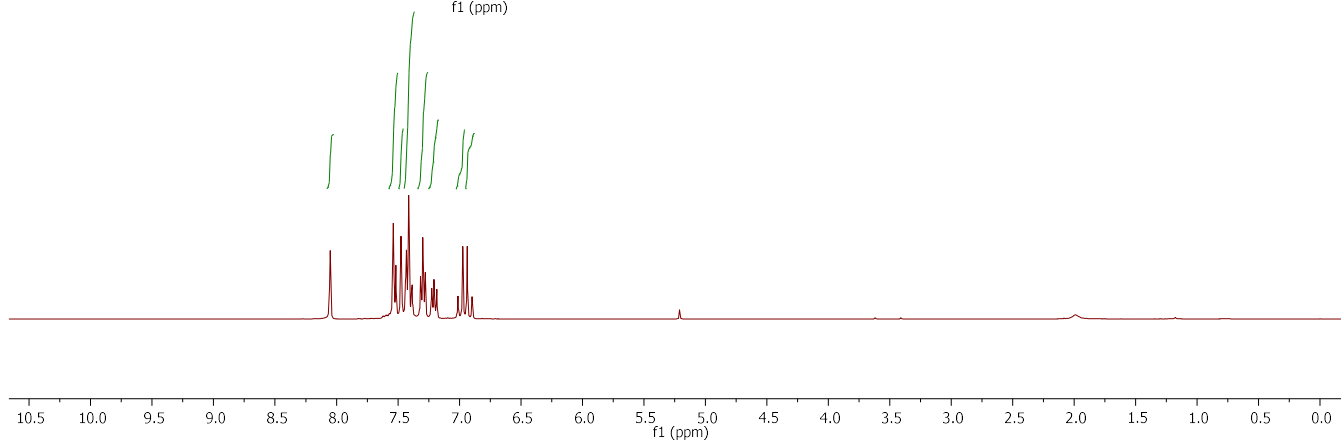
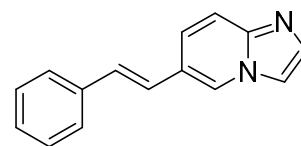
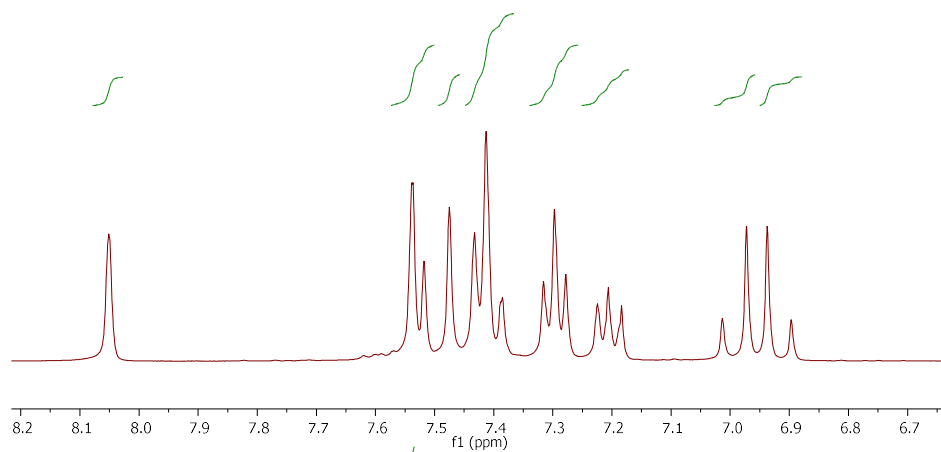
^{13}C NMR **1j** (101 MHz, DMSO- d_6)



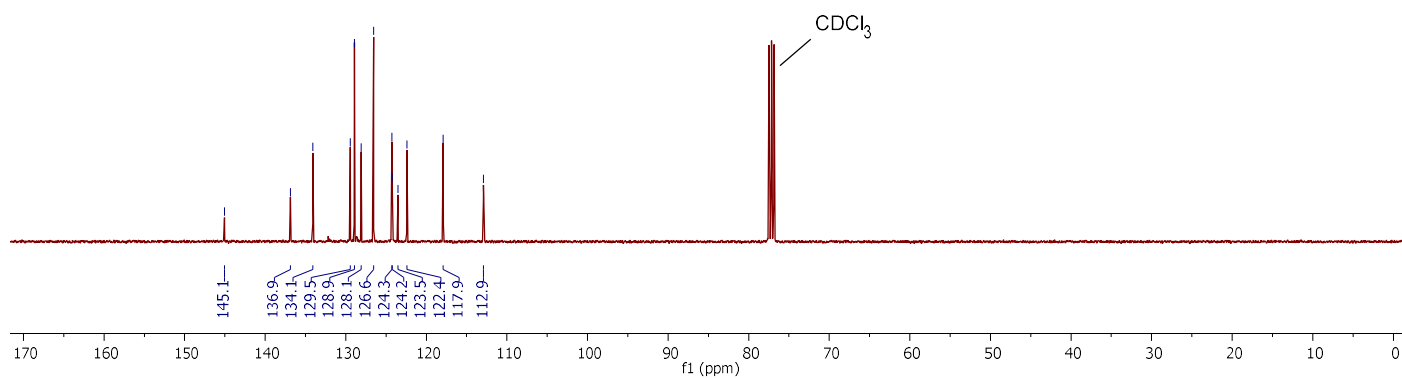
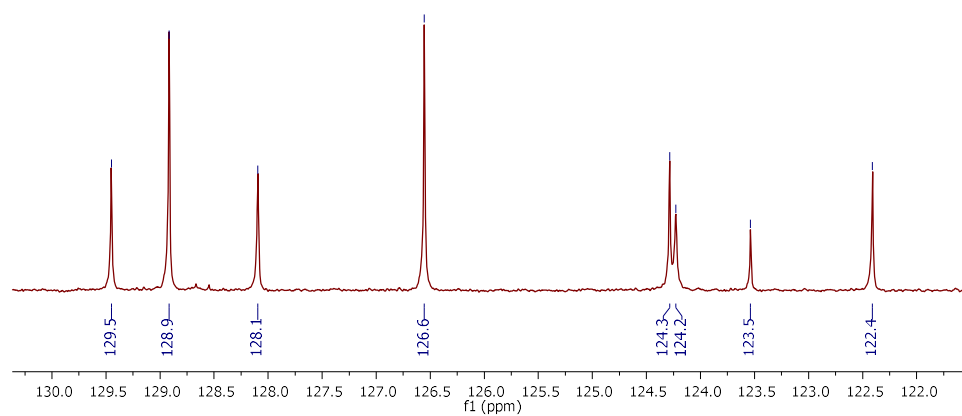
COSY **1j** (400 MHz, DMSO-d₆)

Chemical structure of **1j** (4-(4-hydroxyphenyl)quinoline) is shown in the top right corner.

¹H NMR **1k** (400 MHz, CDCl₃)



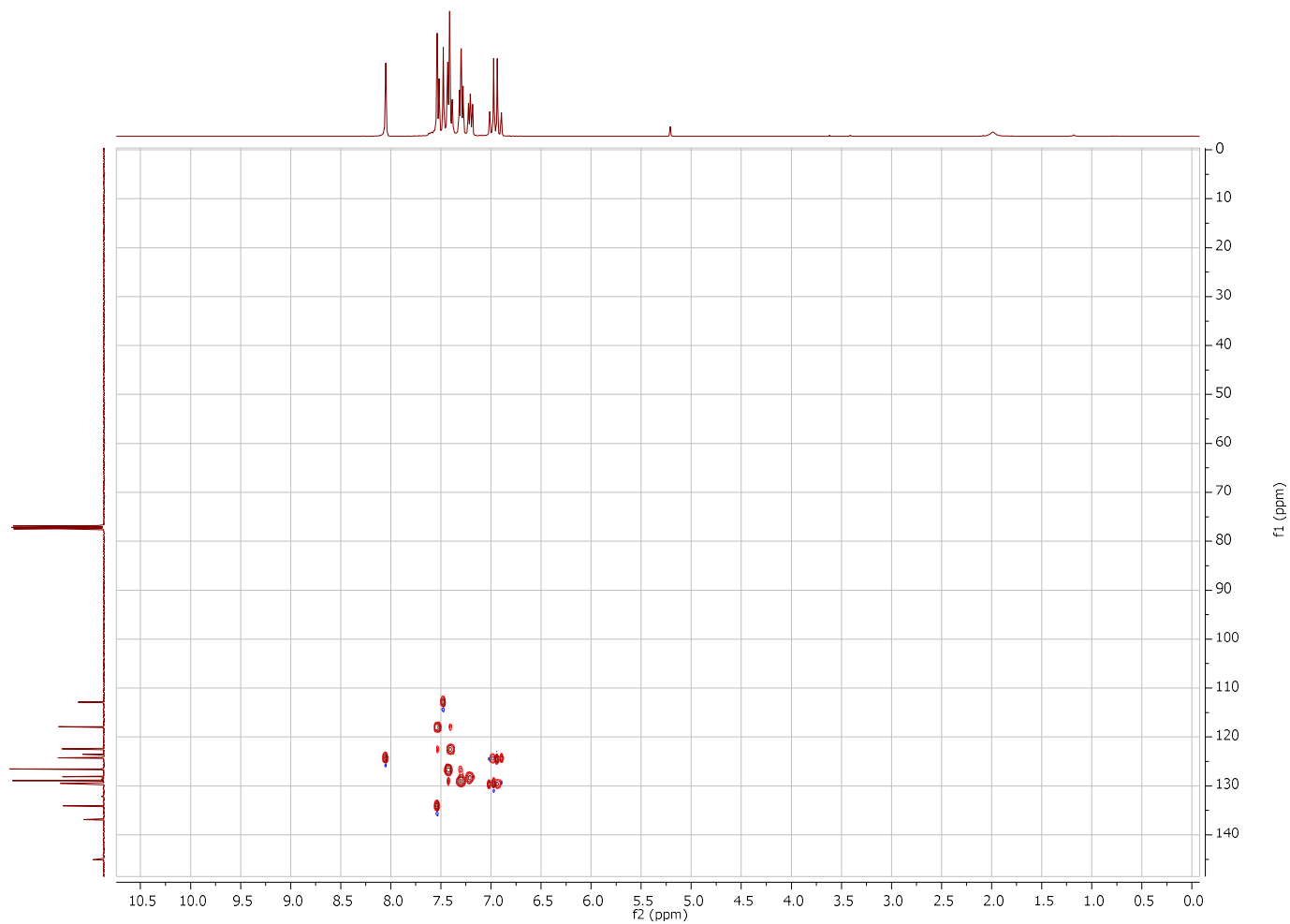
¹³C NMR **1k** (126 MHz, CDCl₃)



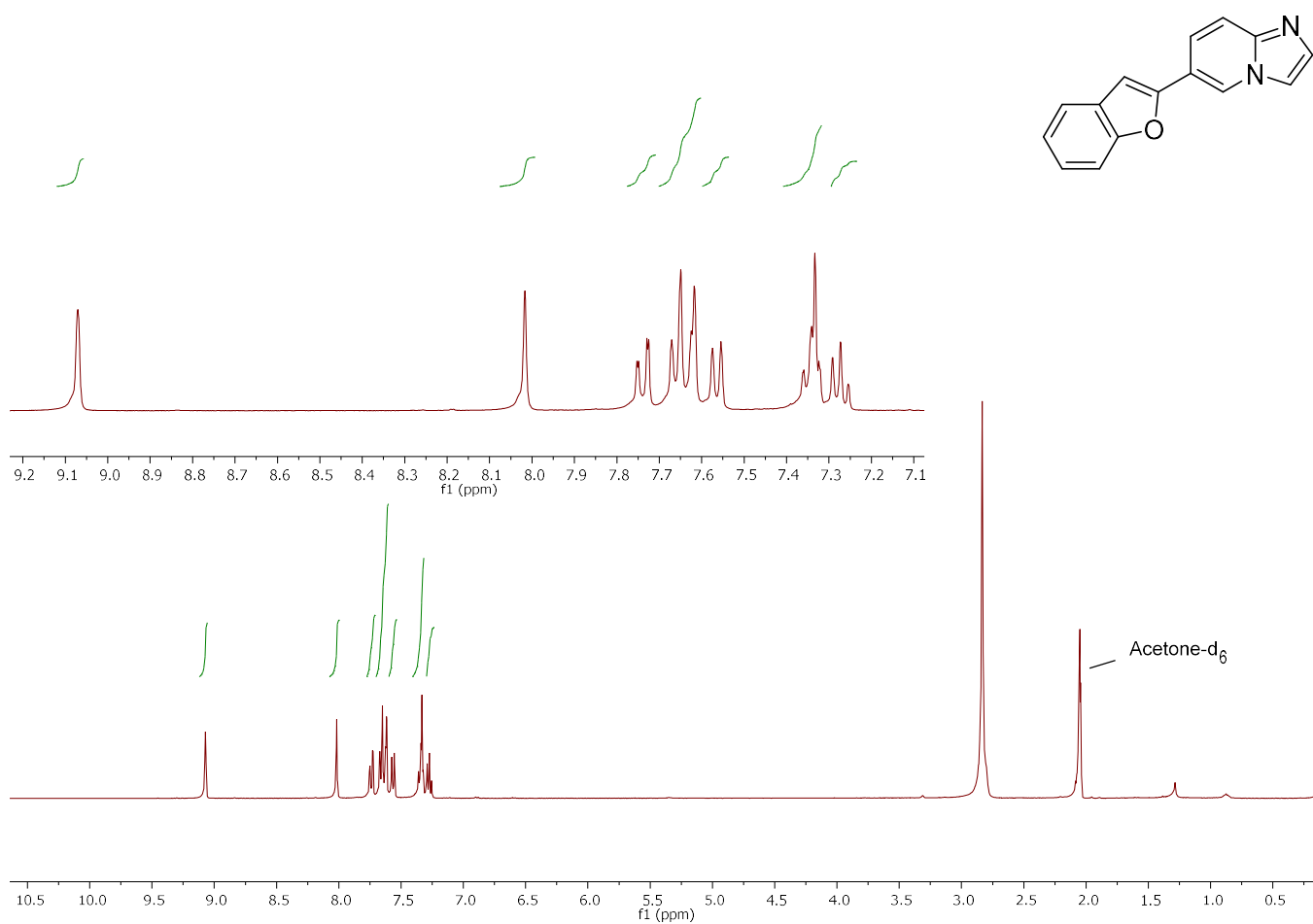
COSY **1k** (400 MHz, CDCl₃)



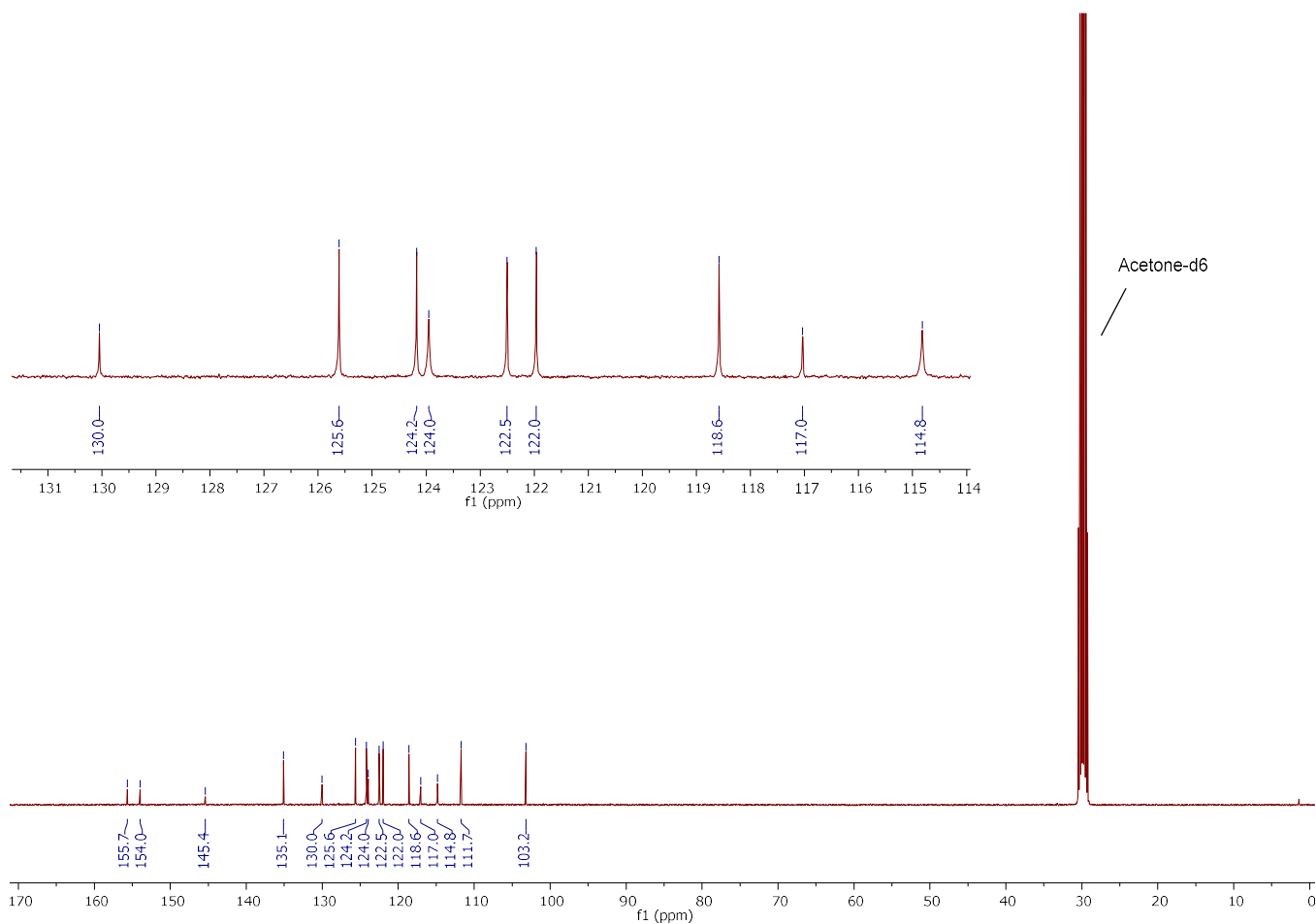
HSQC **1k** (400 MHz, CDCl₃)



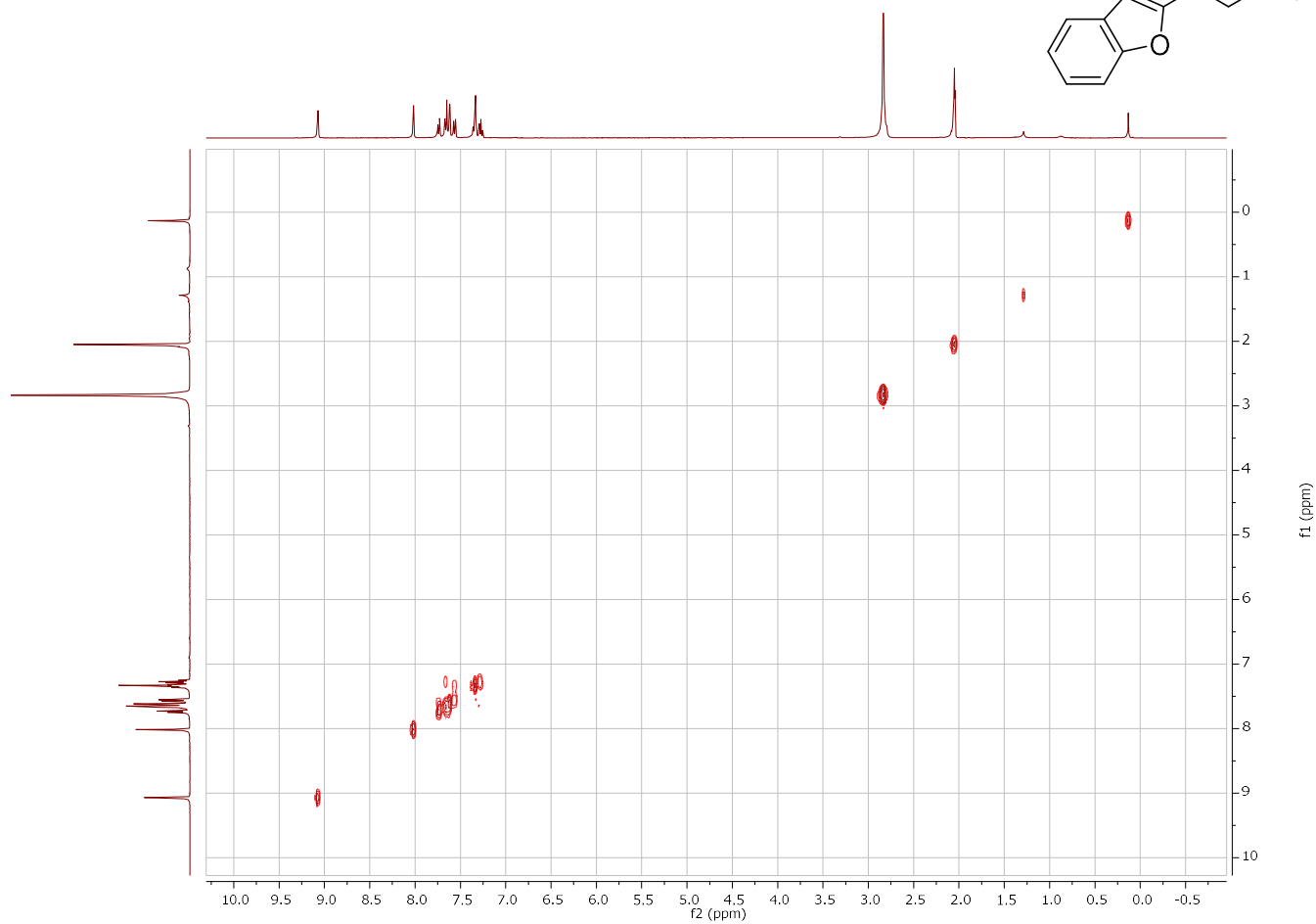
¹H NMR **1l** (400 MHz, Acetone-d₆)



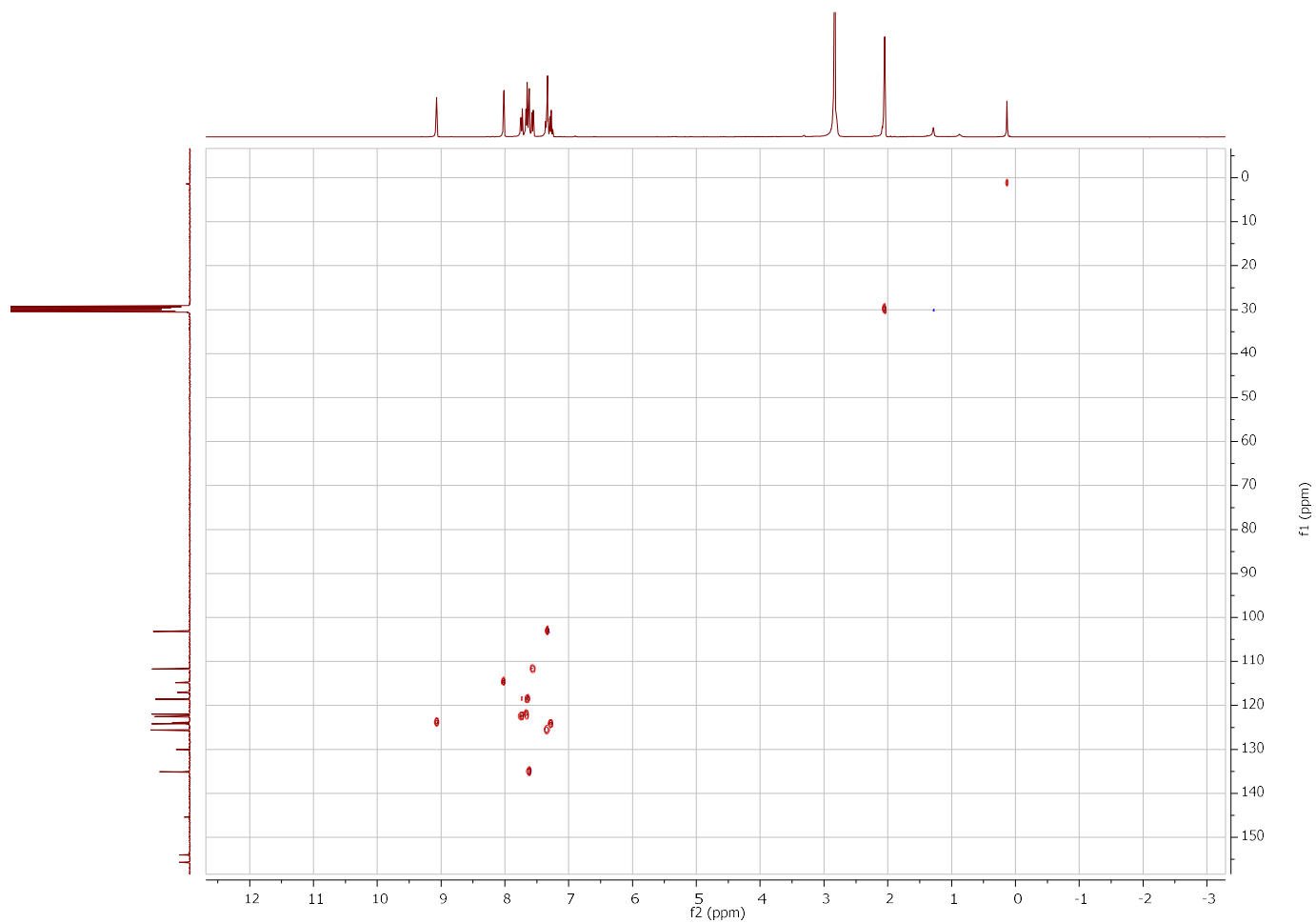
¹³C NMR **1l** (101 MHz, Acetone-d₆)



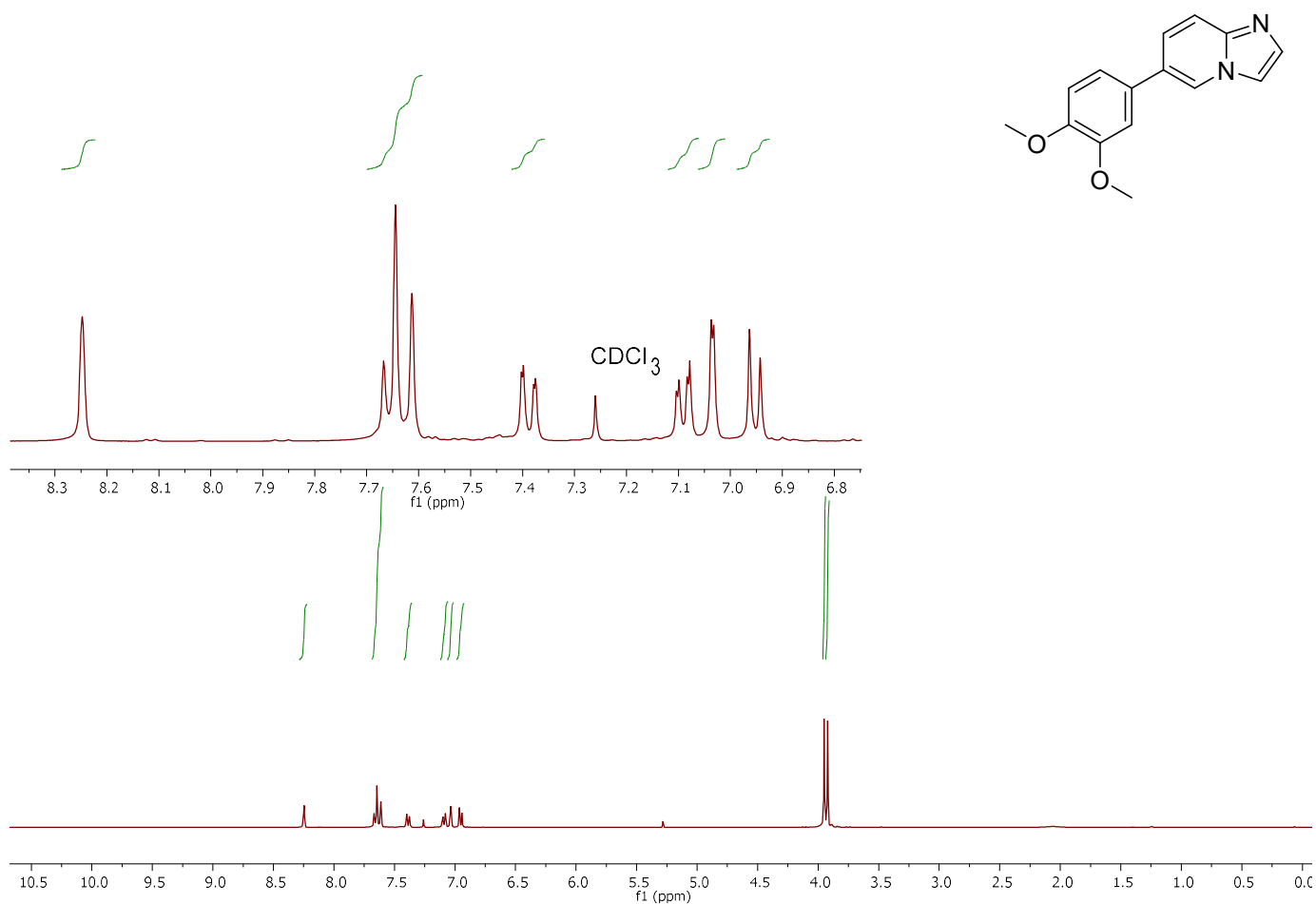
COSY **1l** (101 MHz, Acetone-d₆)



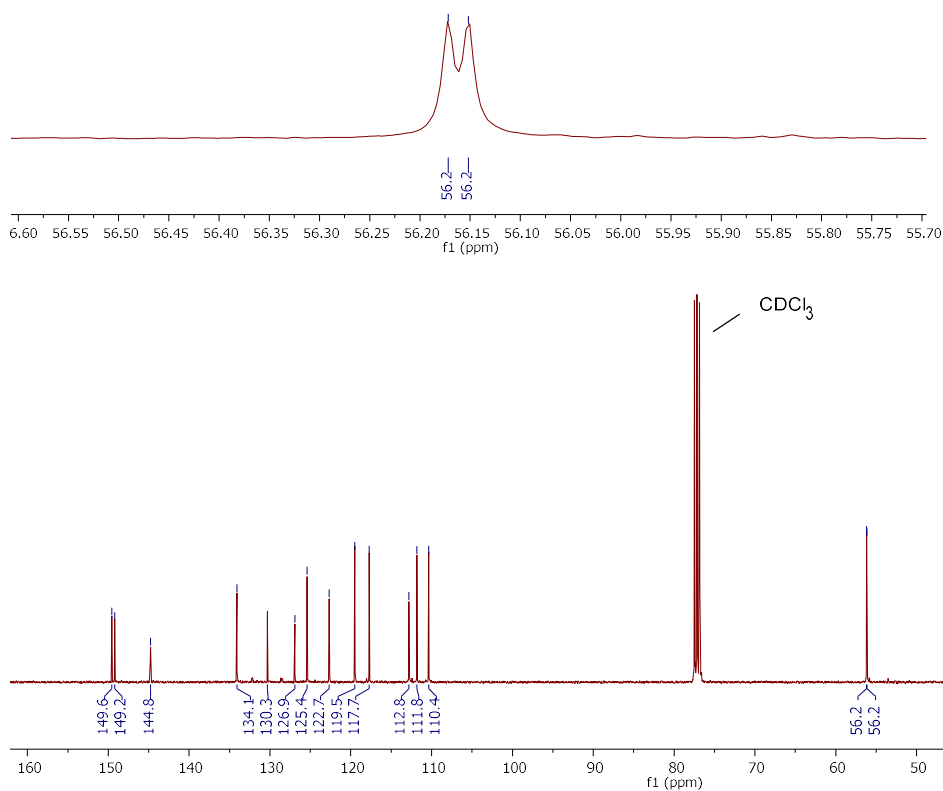
HSQC **1l** (400 MHz, Acetone-d₆)



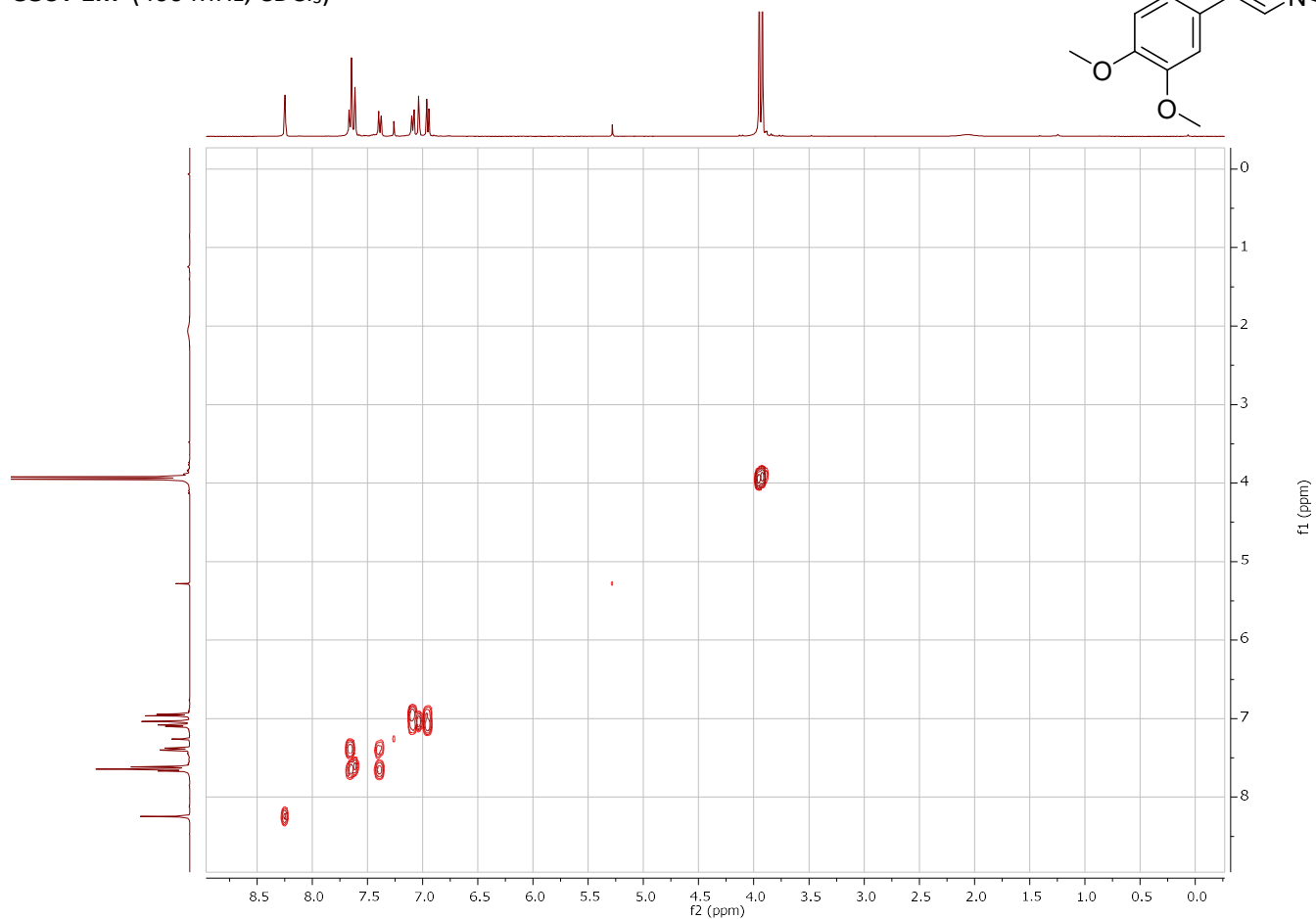
¹H NMR **1m** (400 MHz, CDCl₃)



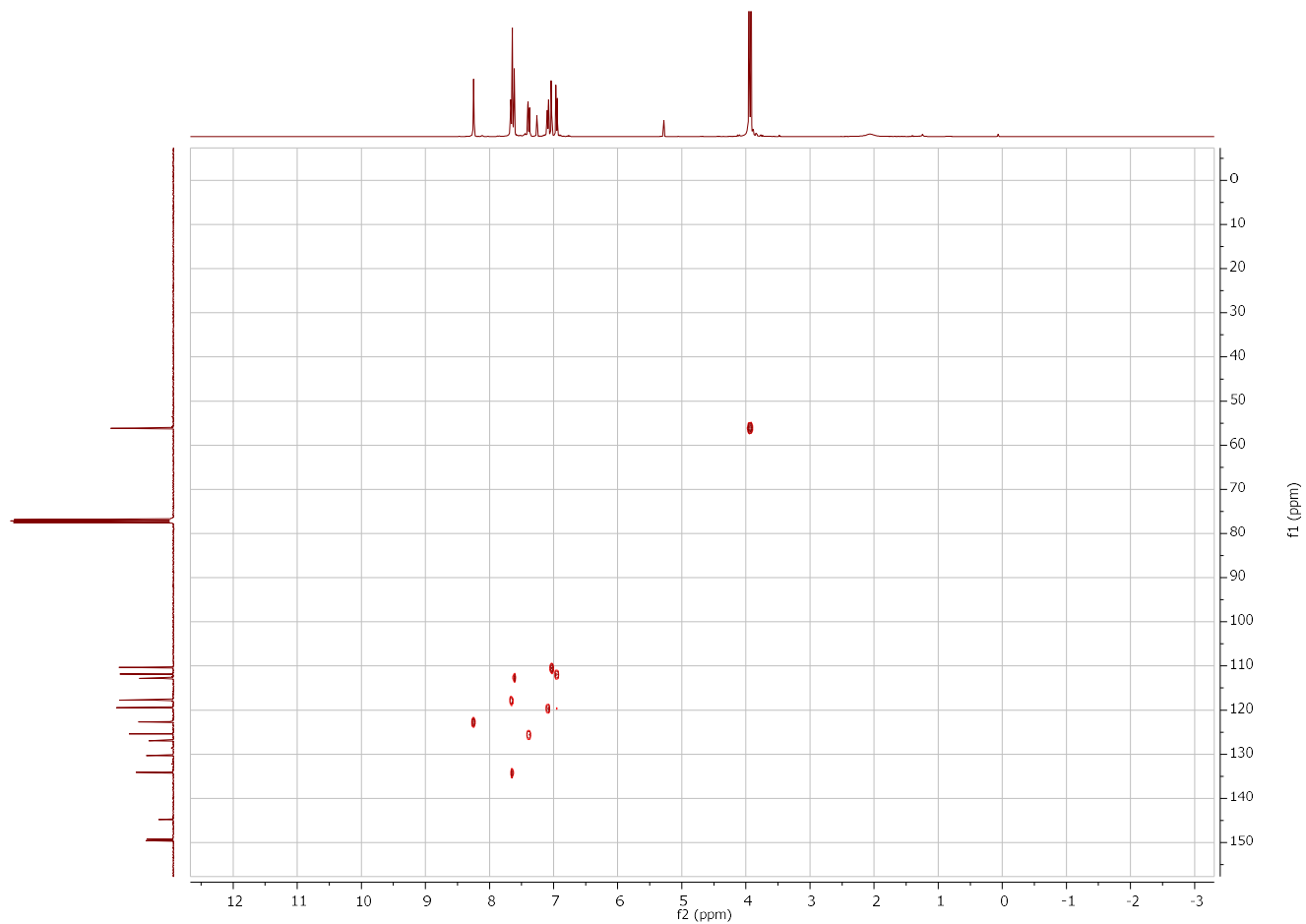
¹³C NMR **1m** (101 MHz, CDCl₃)



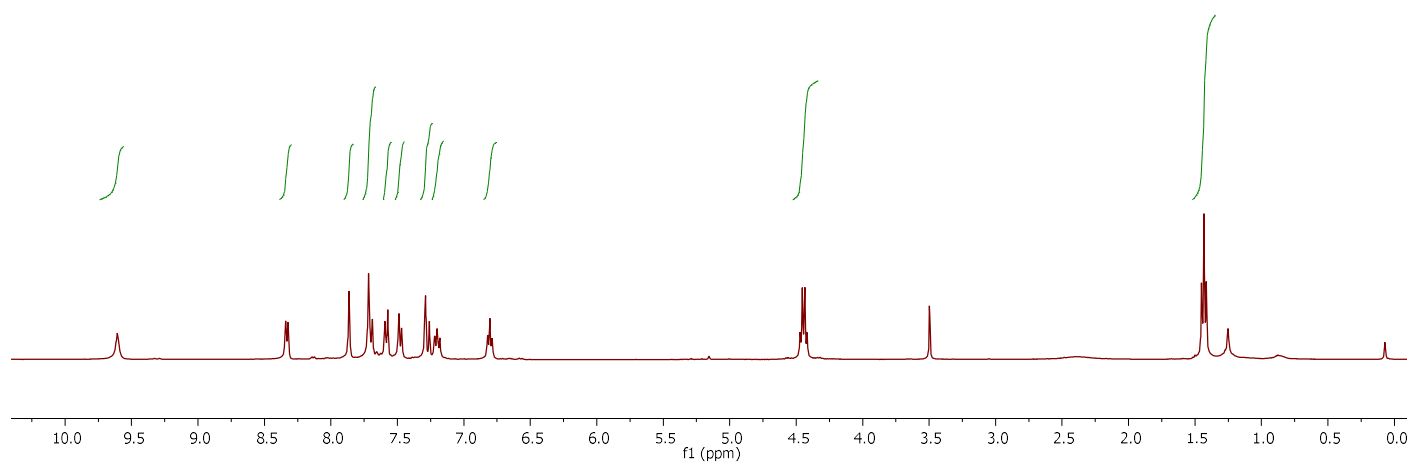
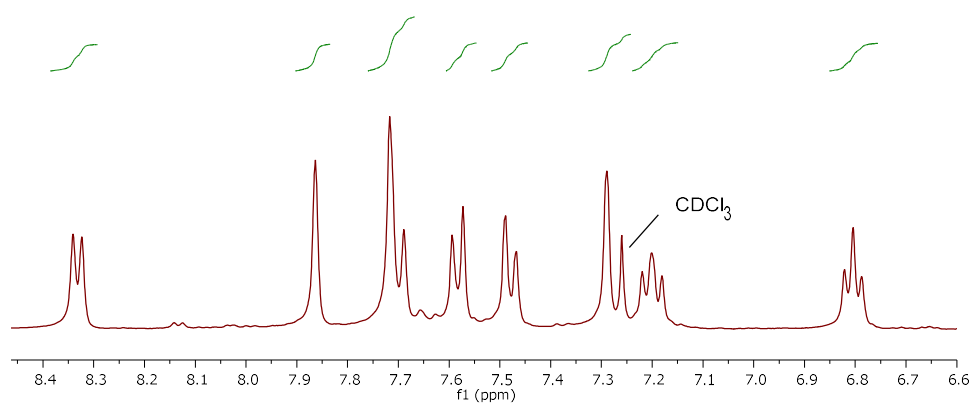
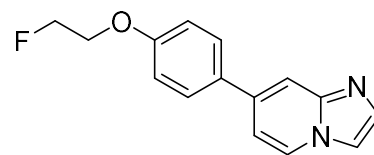
COSY 1m (400 MHz, CDCl₃)



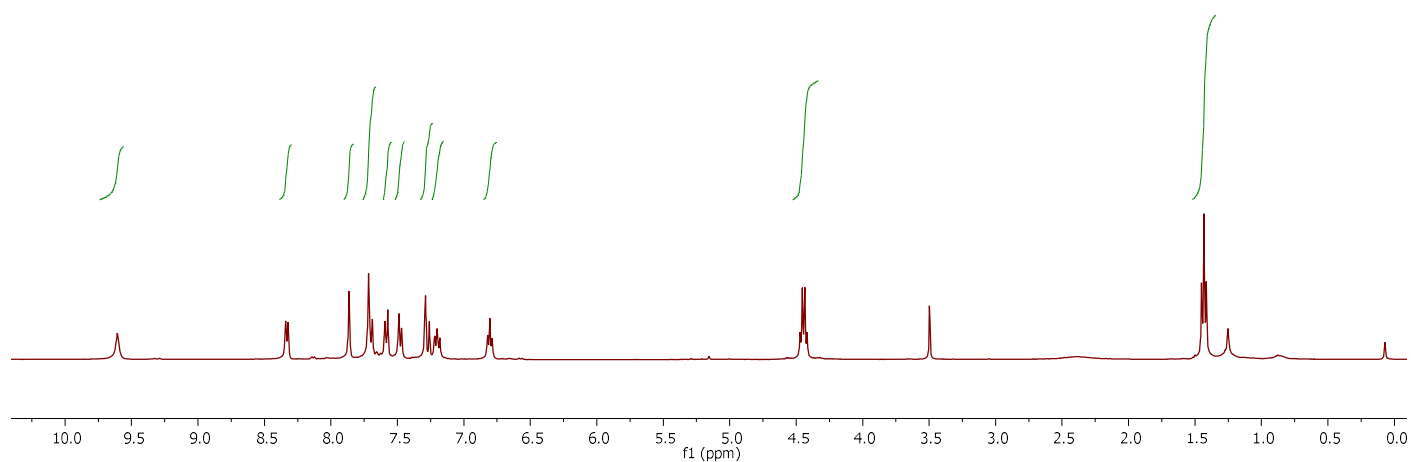
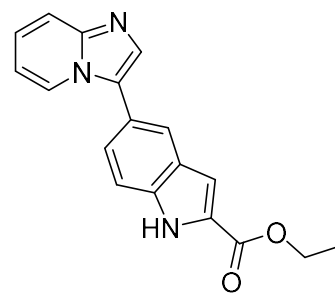
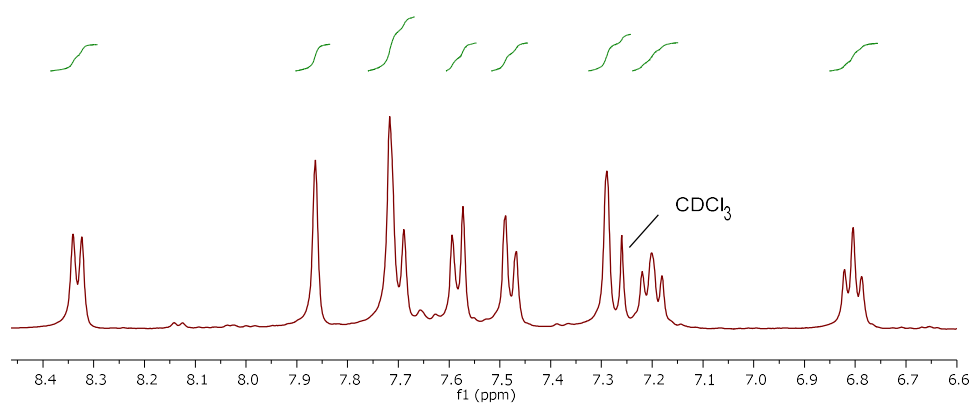
HSQC 1m (400 MHz, CDCl₃)



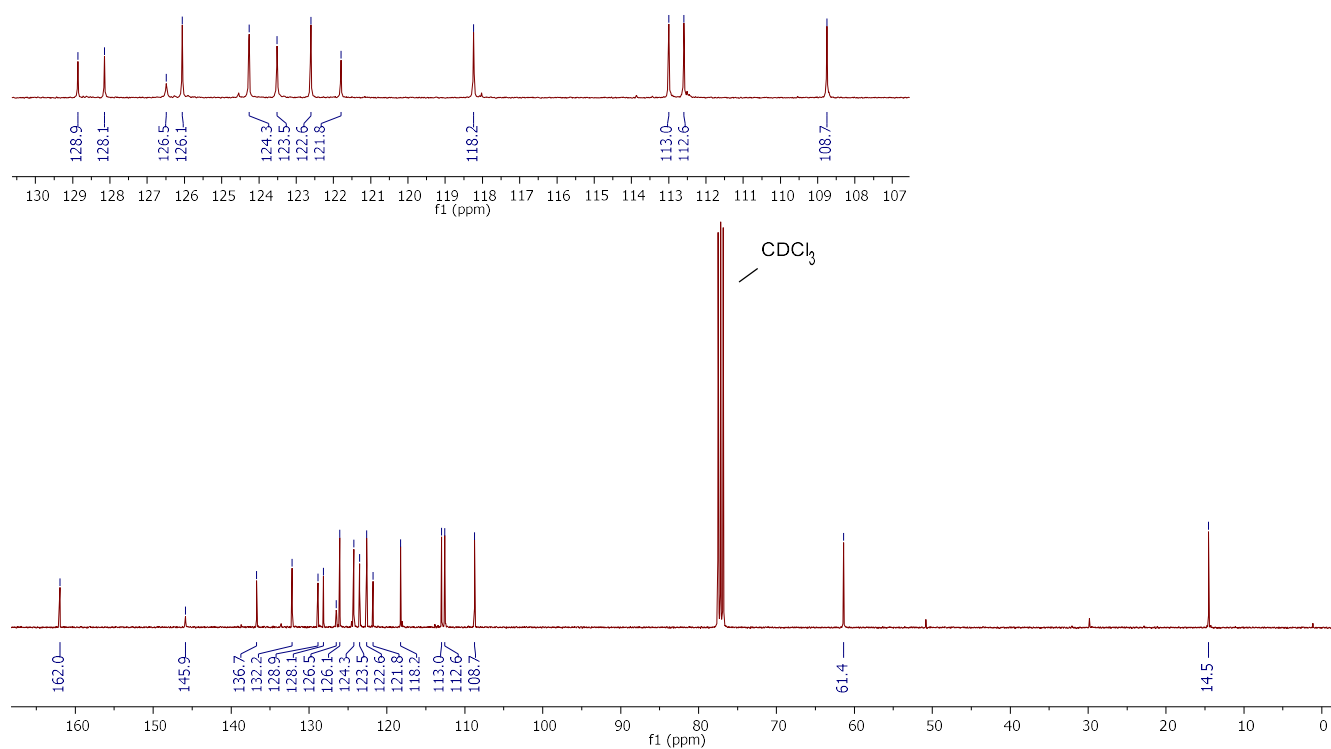
^1H NMR **1n** (400 MHz, CDCl_3)



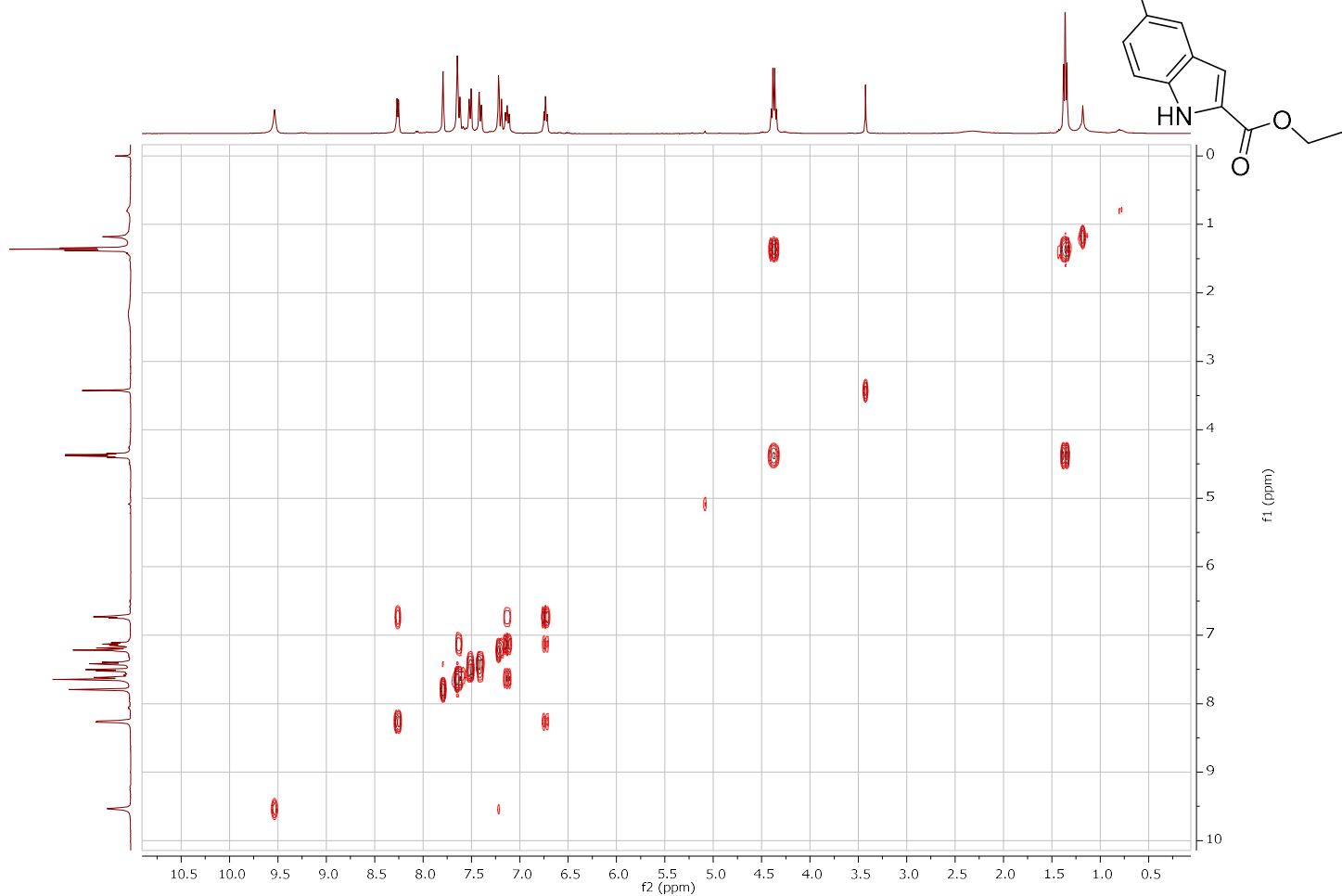
¹H NMR **9** (400 MHz, CDCl₃)



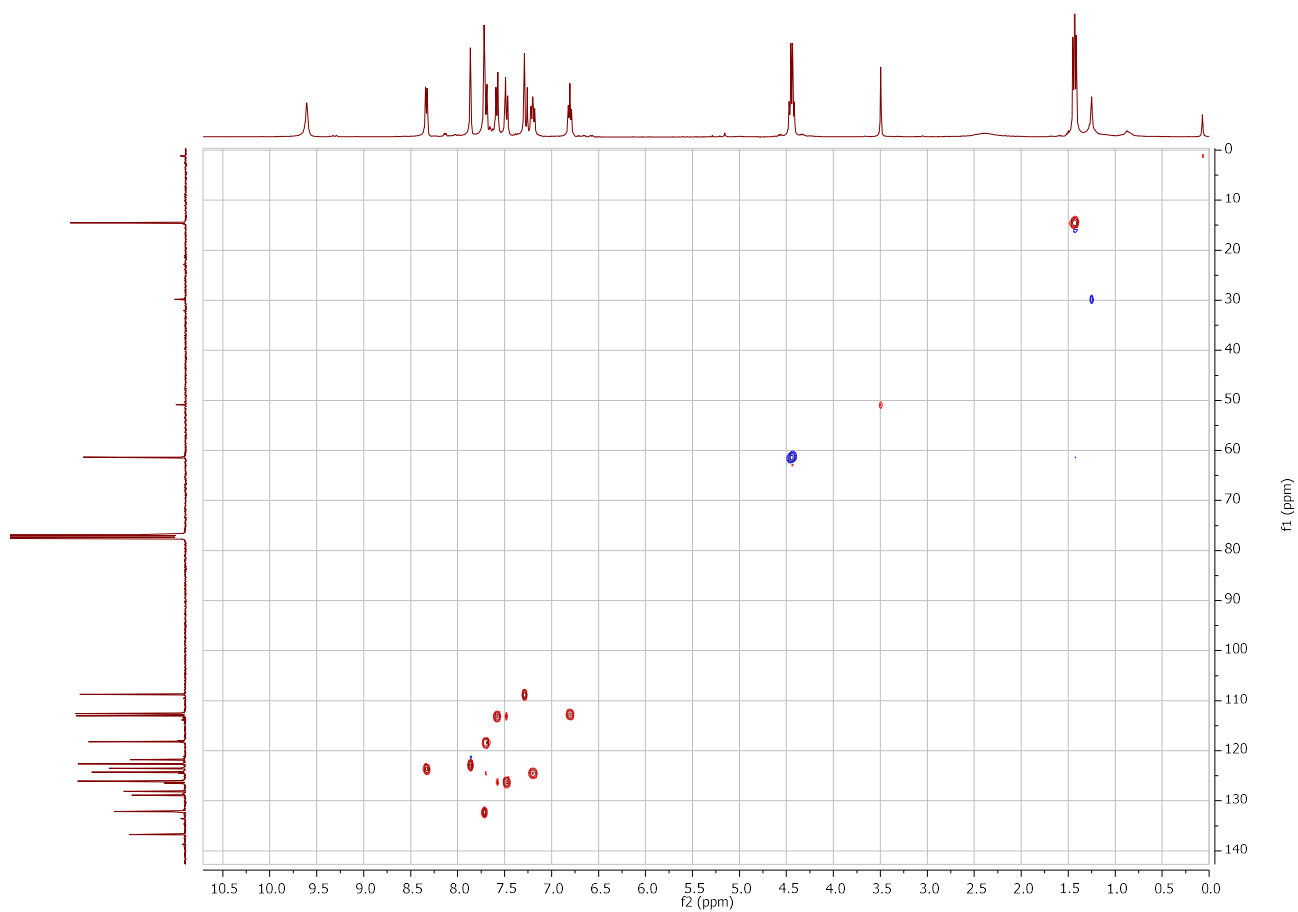
¹³C NMR **9** (101 MHz, CDCl₃)



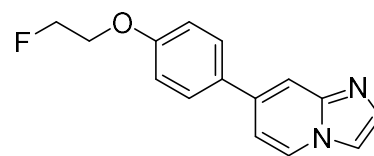
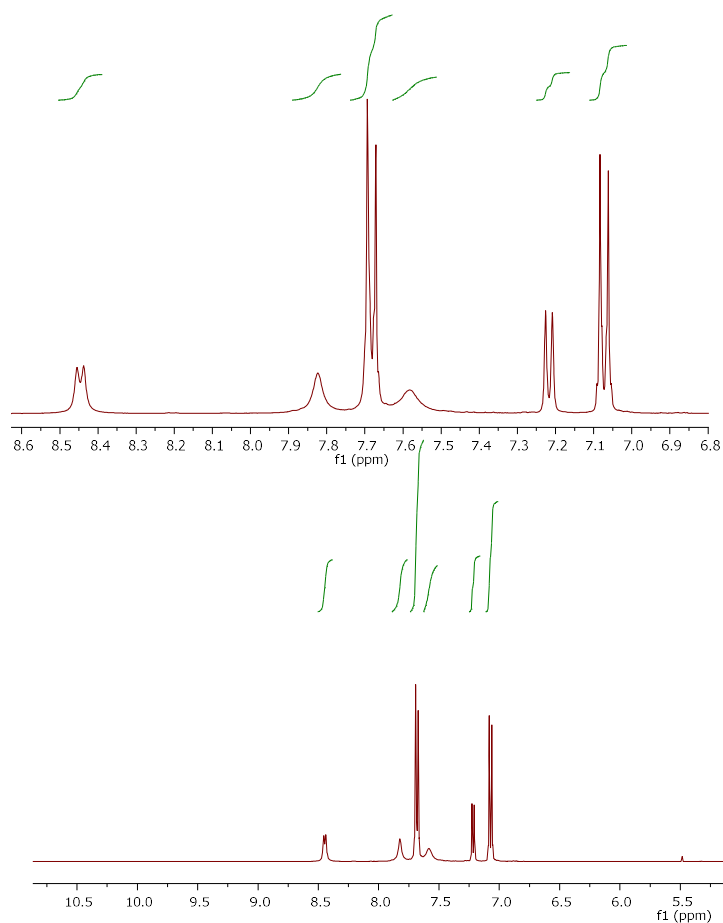
COSY 9 (400 MHz, CDCl₃)



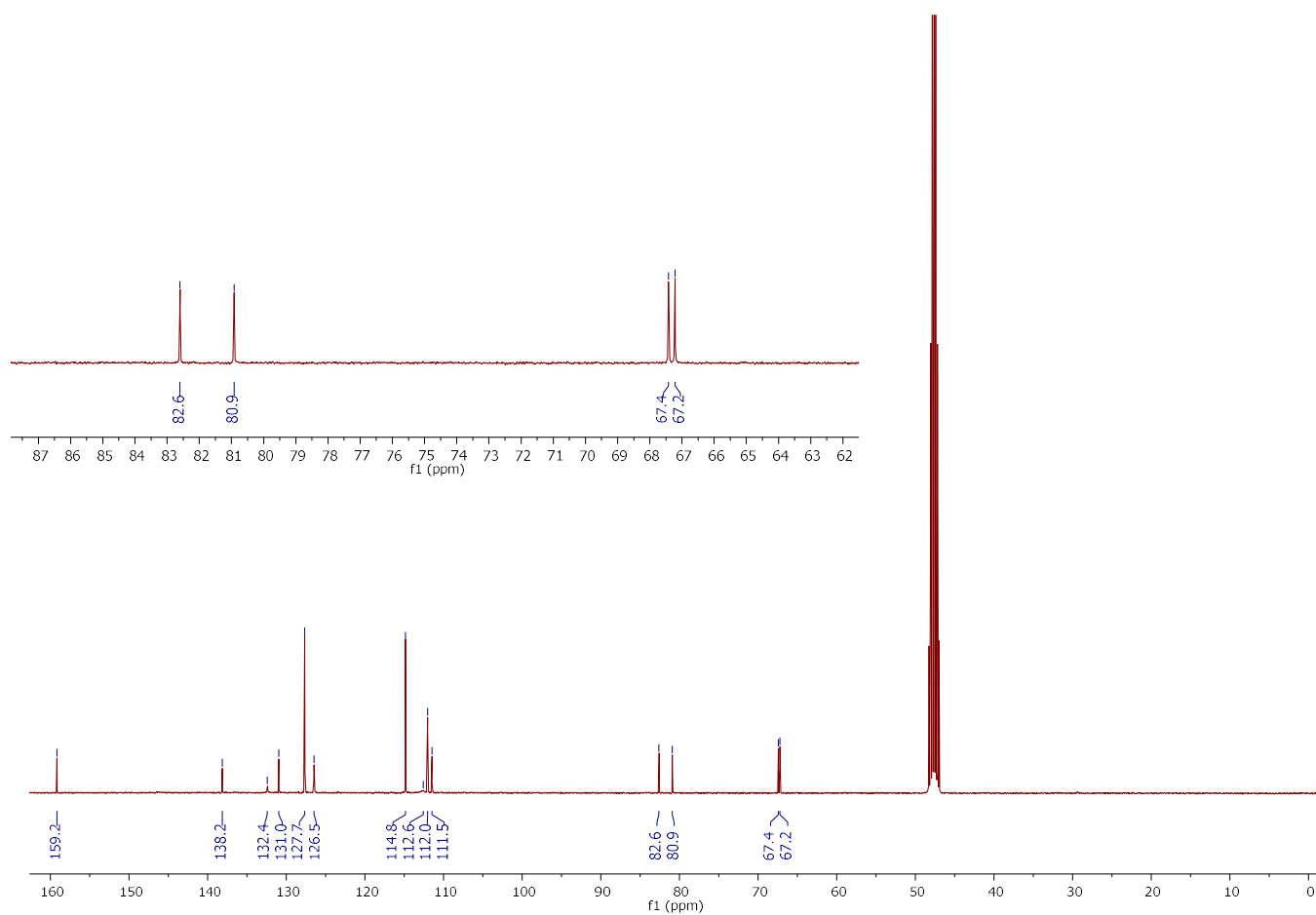
HSQC 9 (400 MHz, CDCl₃)



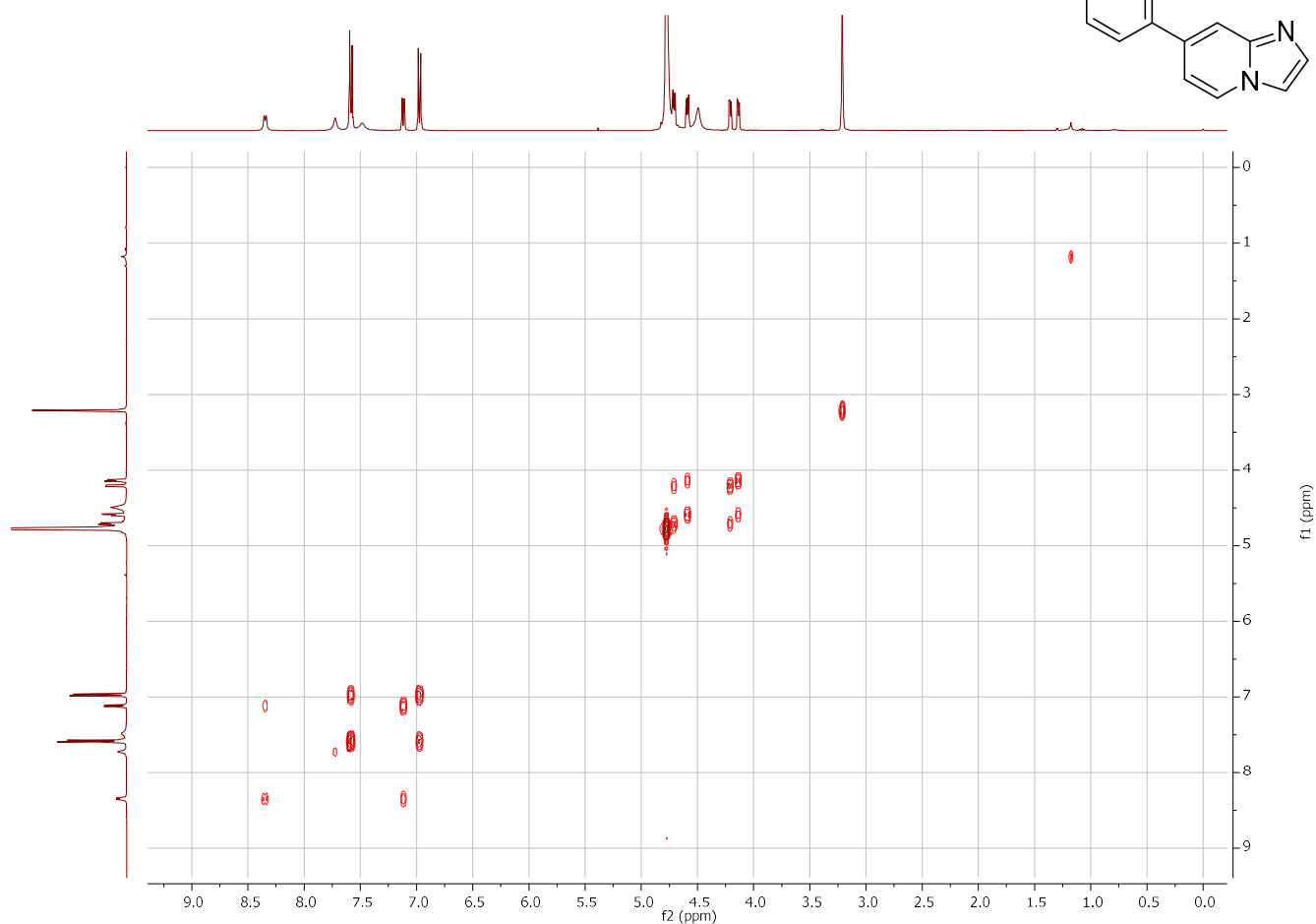
¹H NMR **1n** (400 MHz, MeOD)



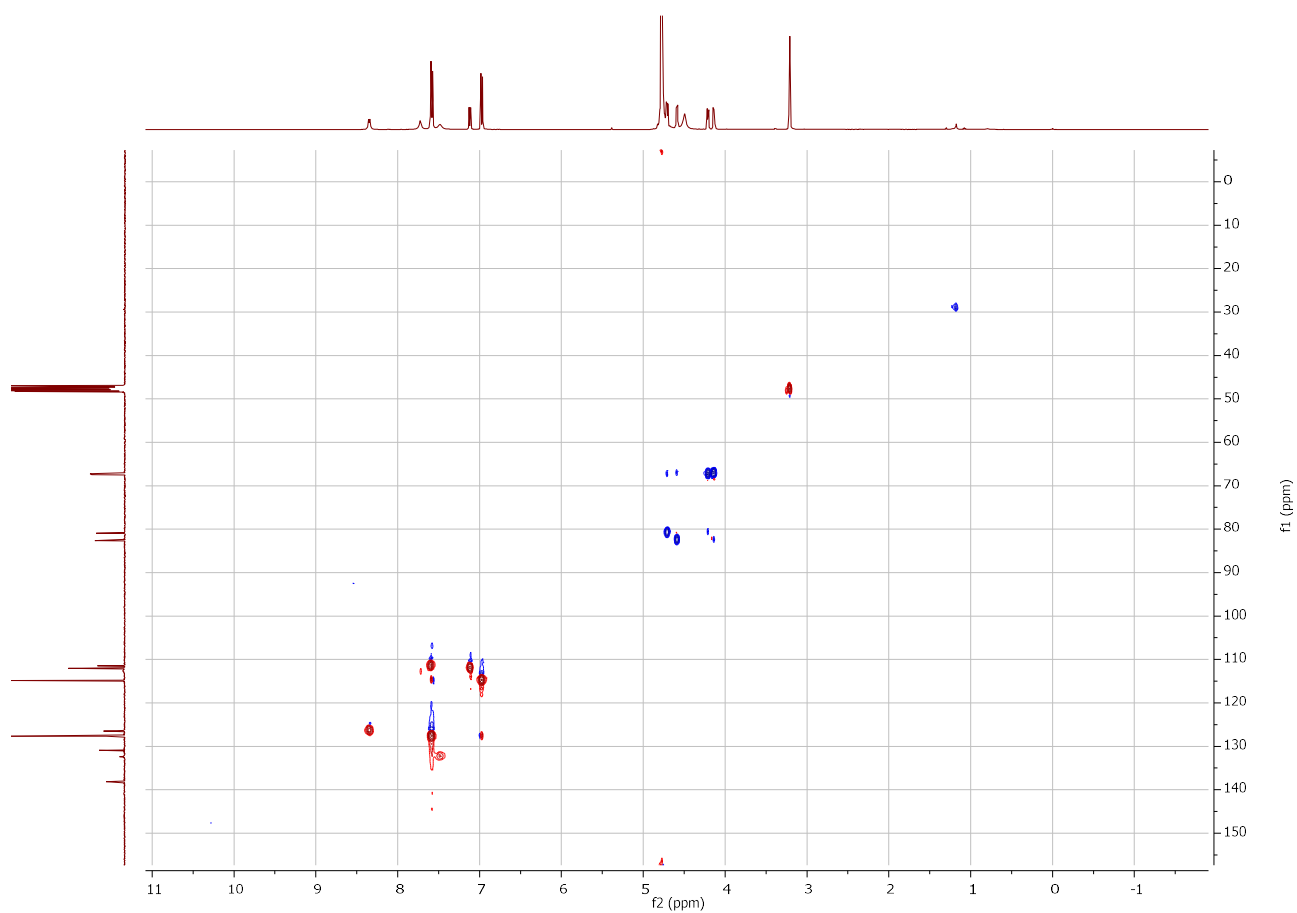
¹³C NMR **1n** (101 MHz, MeOD)



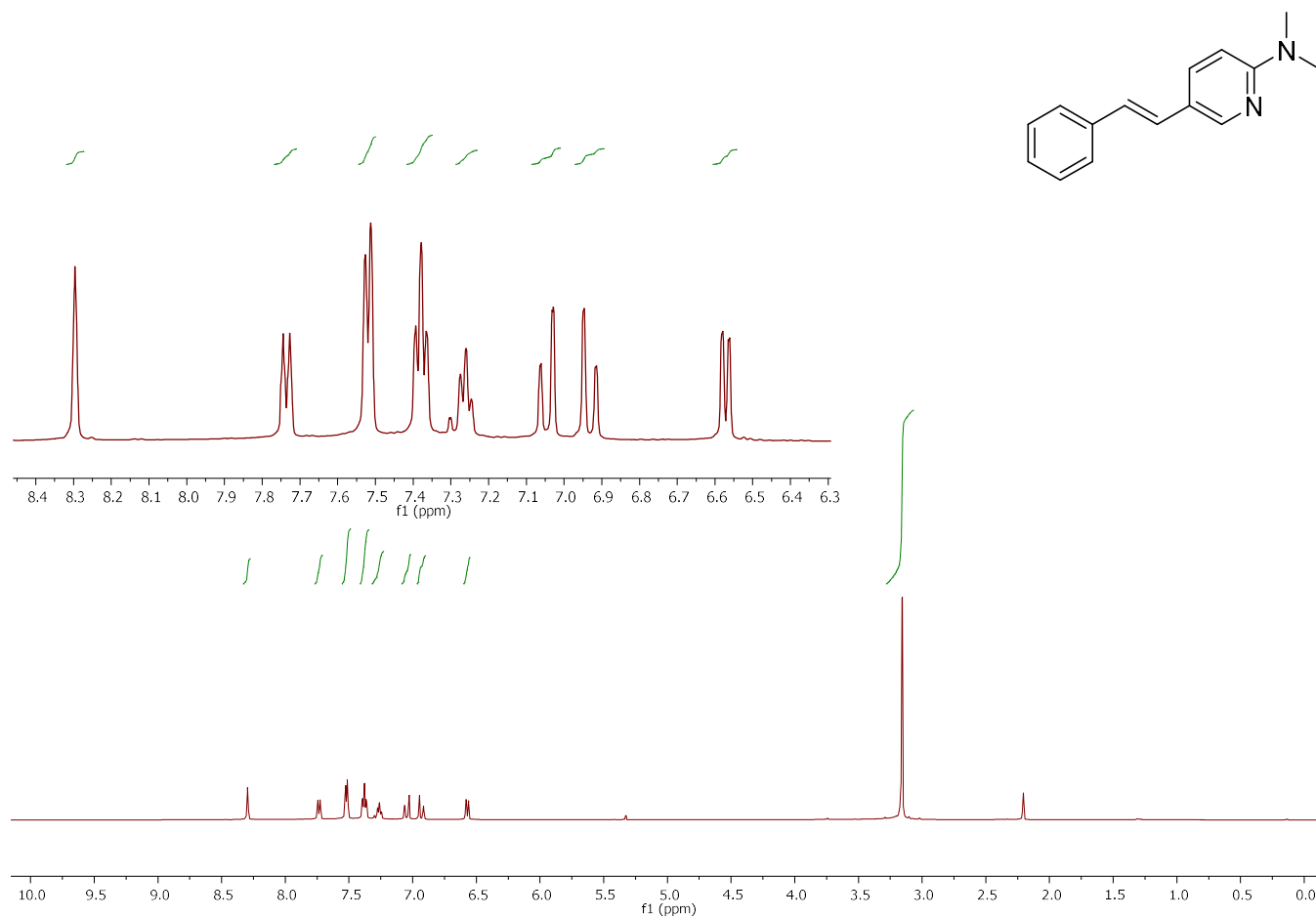
COSY **1n** (400 MHz, MeOD)



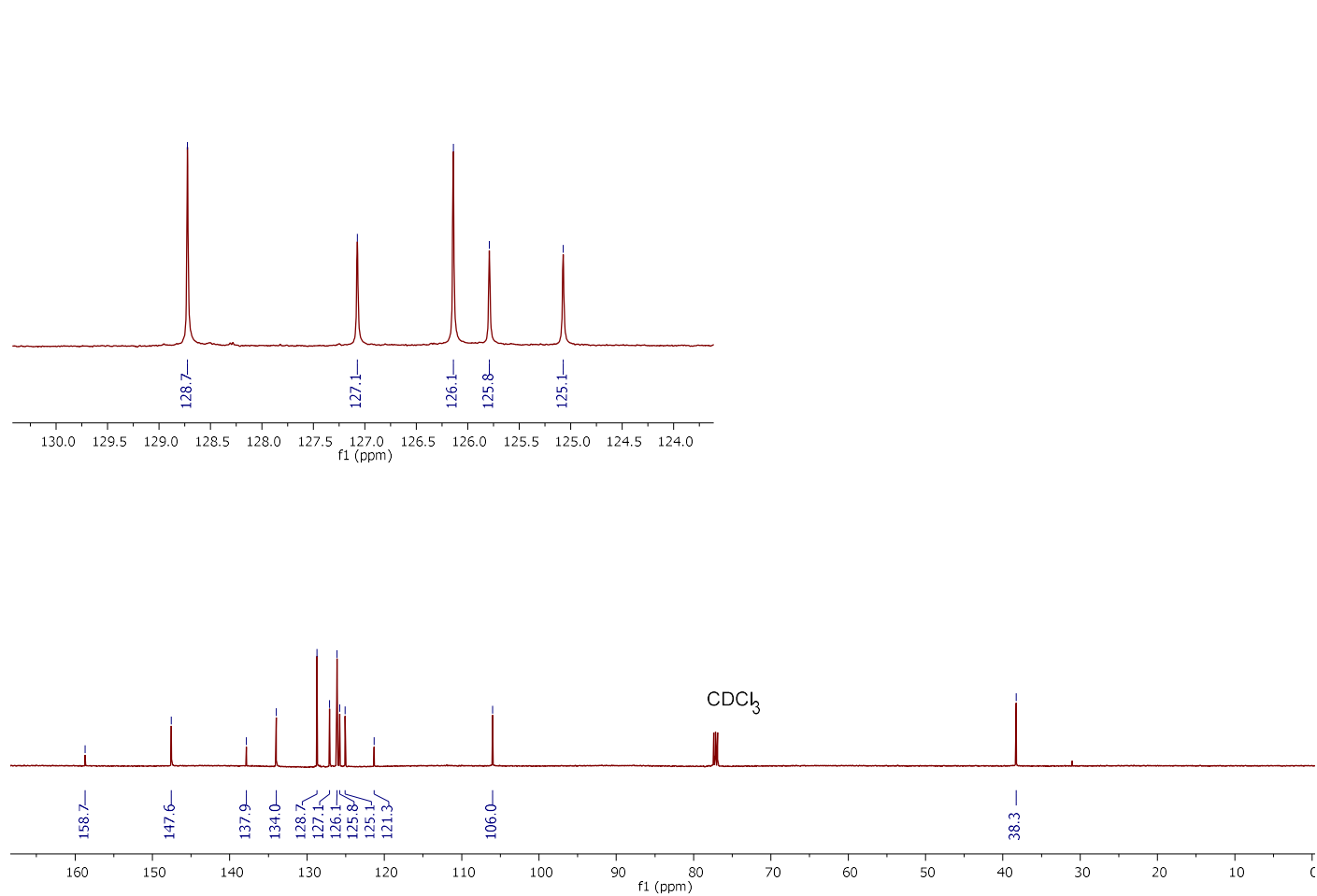
COSY **1n** (400 MHz, MeOD)



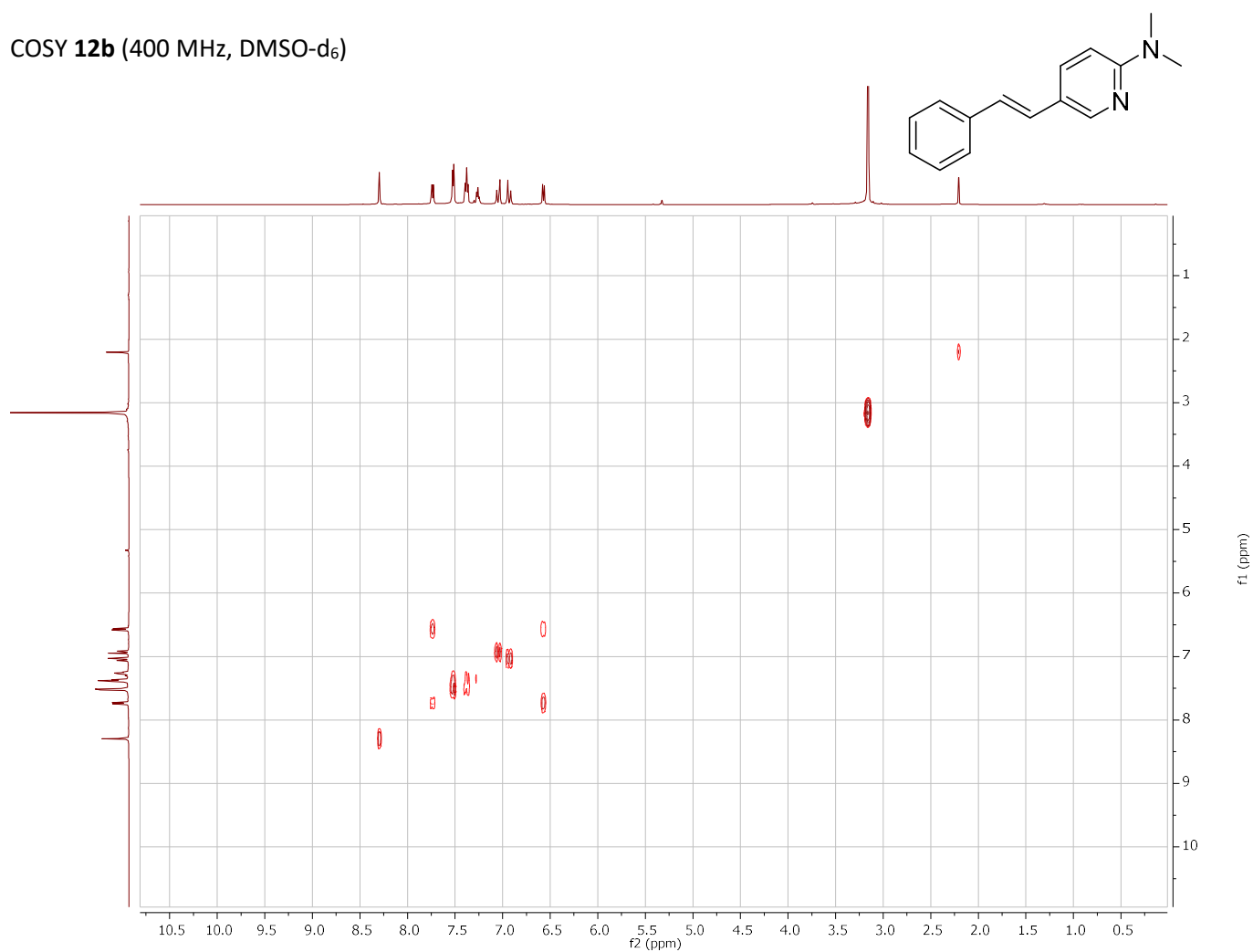
^1H NMR **12b** (500 MHz, CDCl_3)



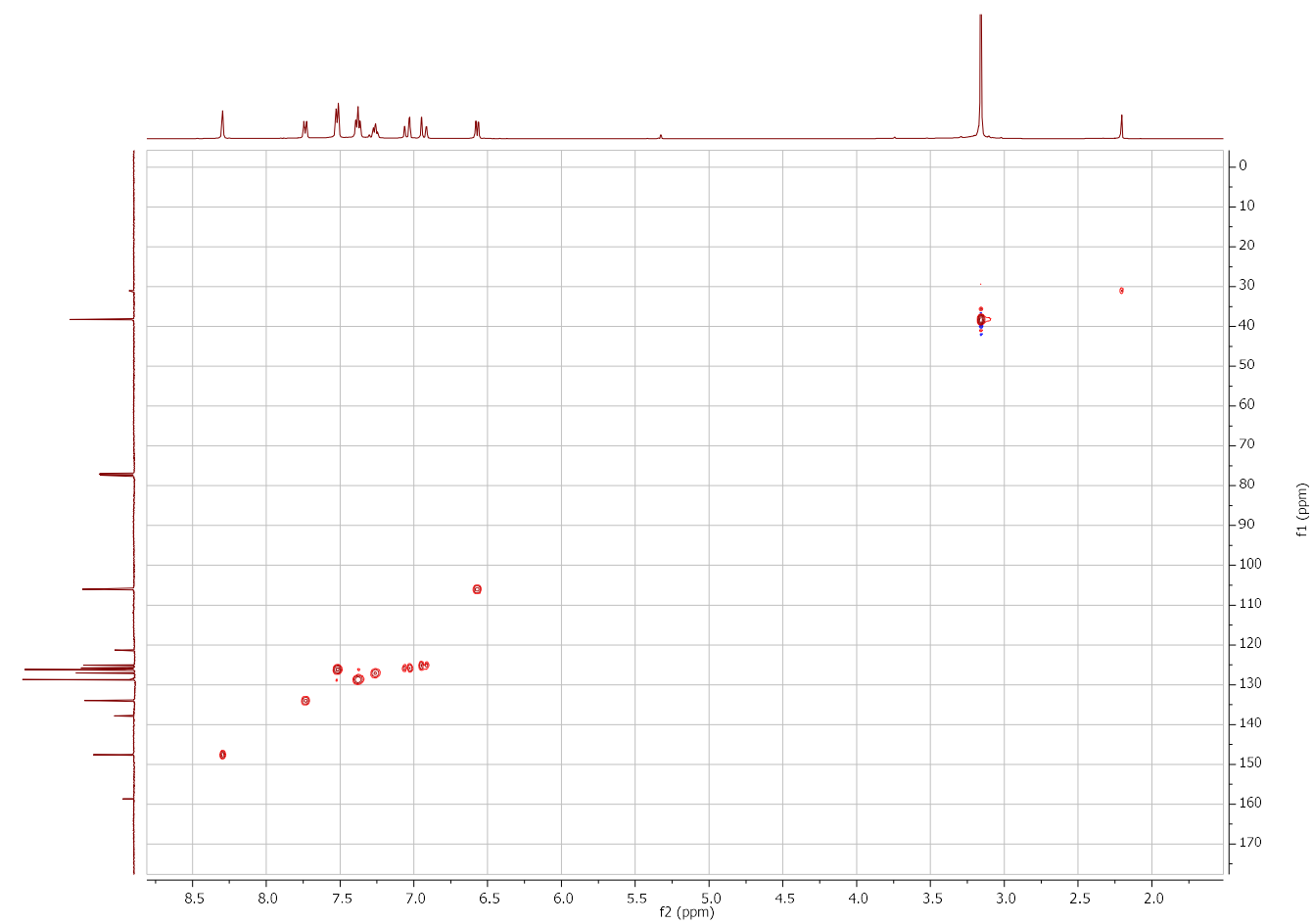
^{13}C NMR **12b** (126 MHz, $\text{DMSO}-d_6$)



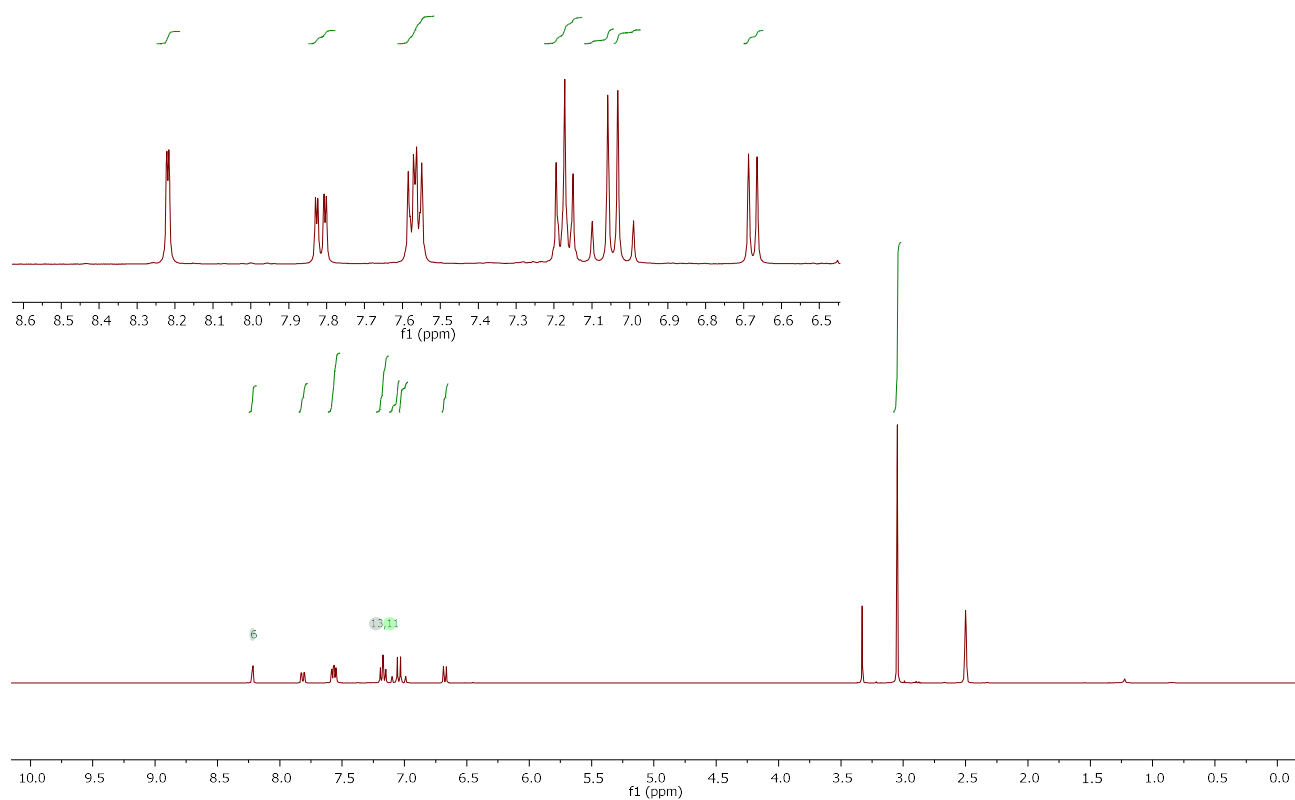
COSY **12b** (400 MHz, DMSO-d₆)



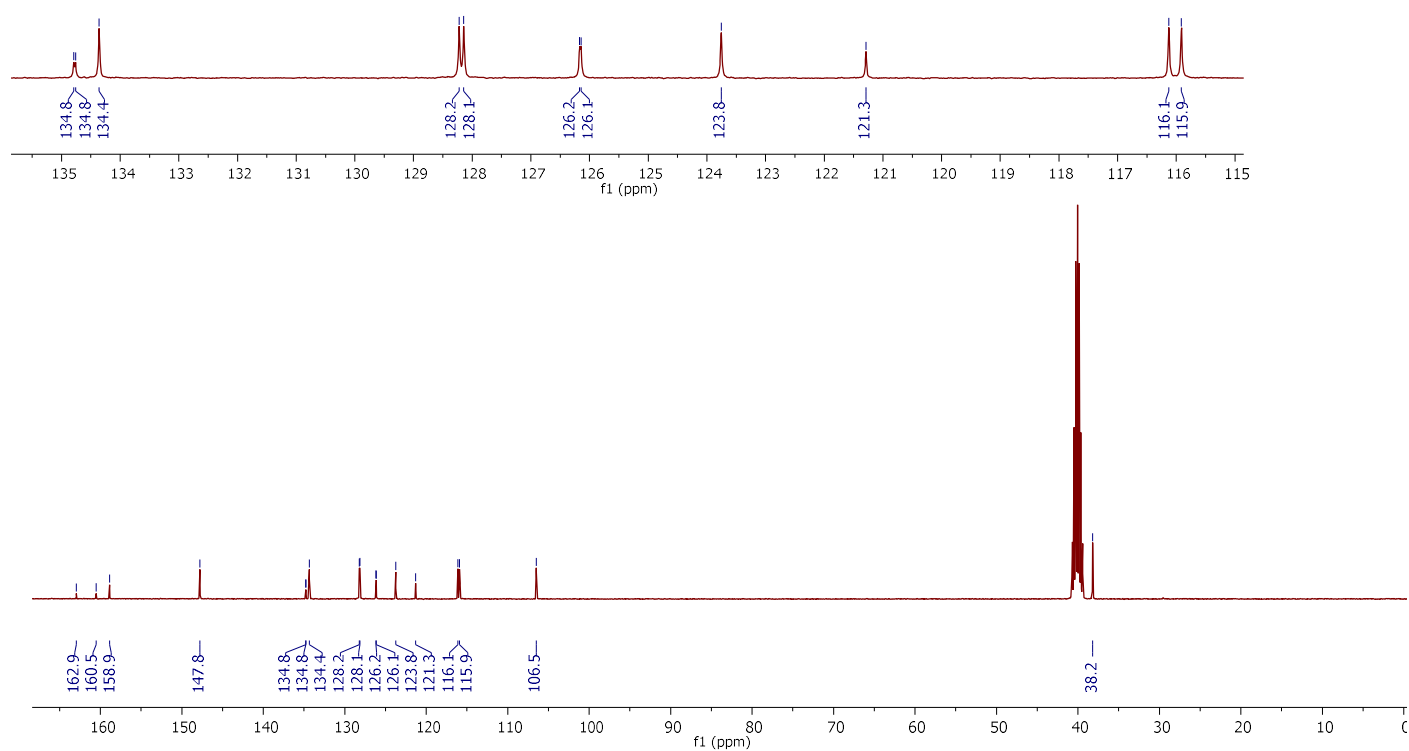
HSQC **12b** (400 MHz, DMSO-d₆)



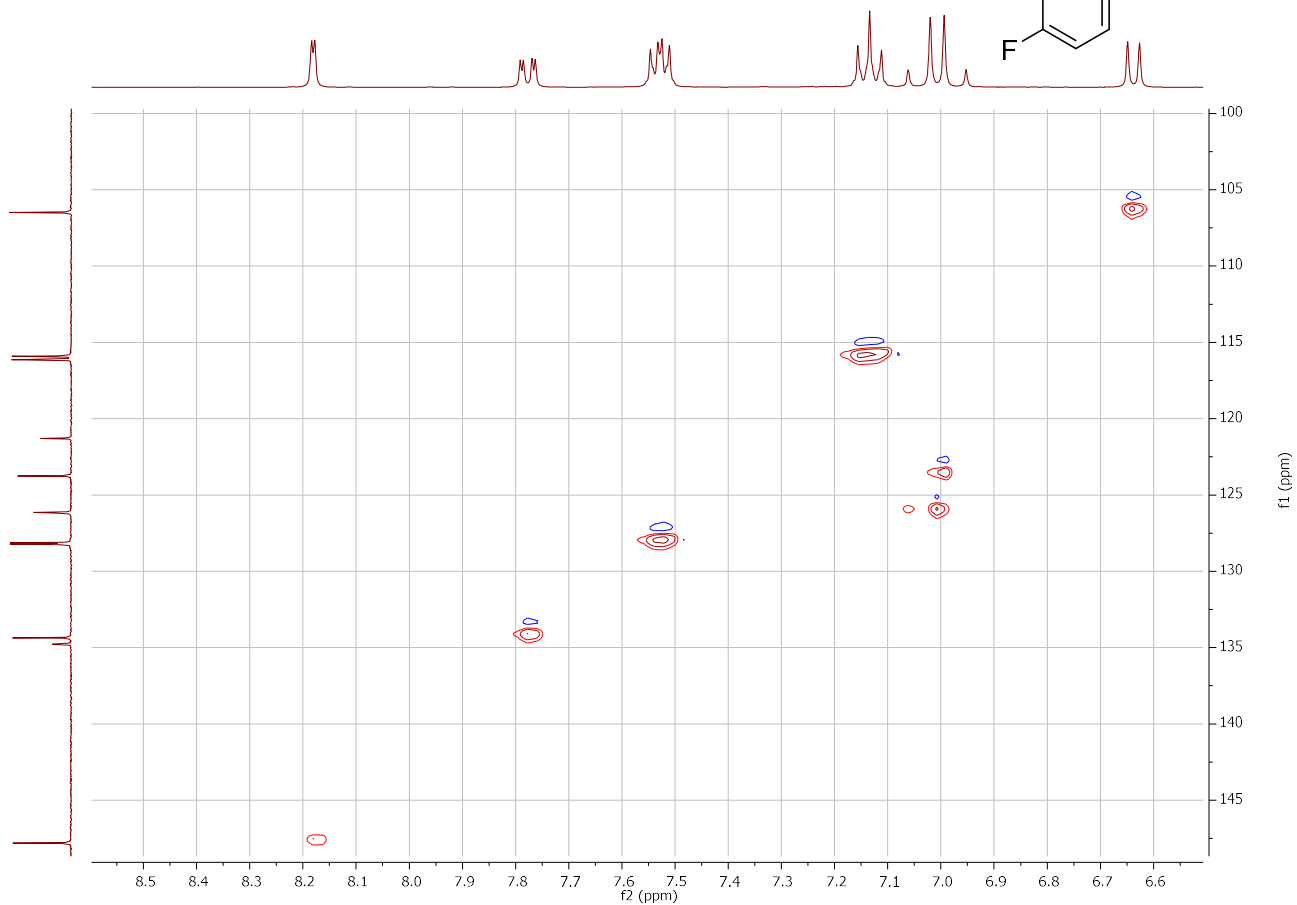
¹H NMR **12c** (400 MHz, DMSO-d₆)



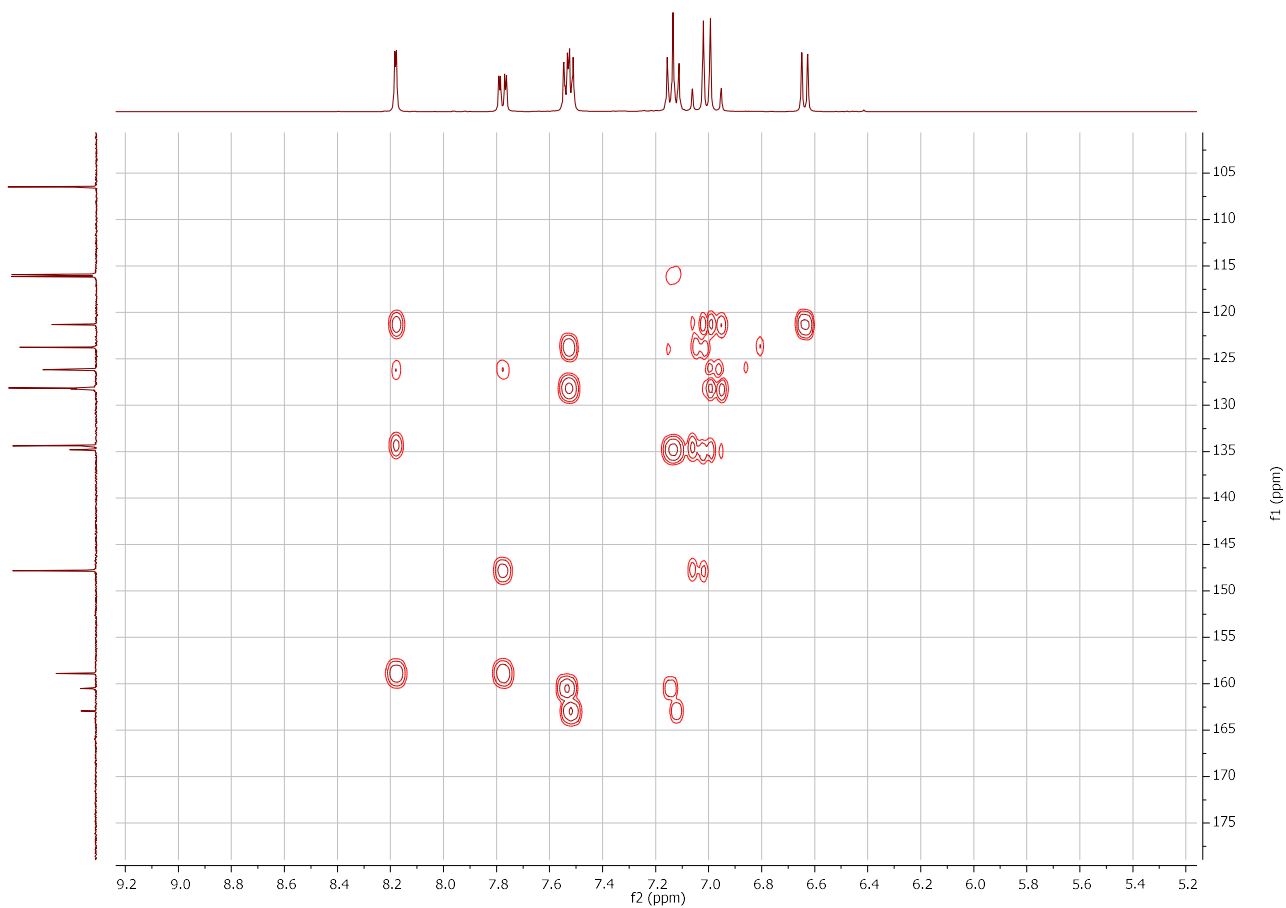
¹³C NMR **12c** (126 MHz, DMSO-d₆)



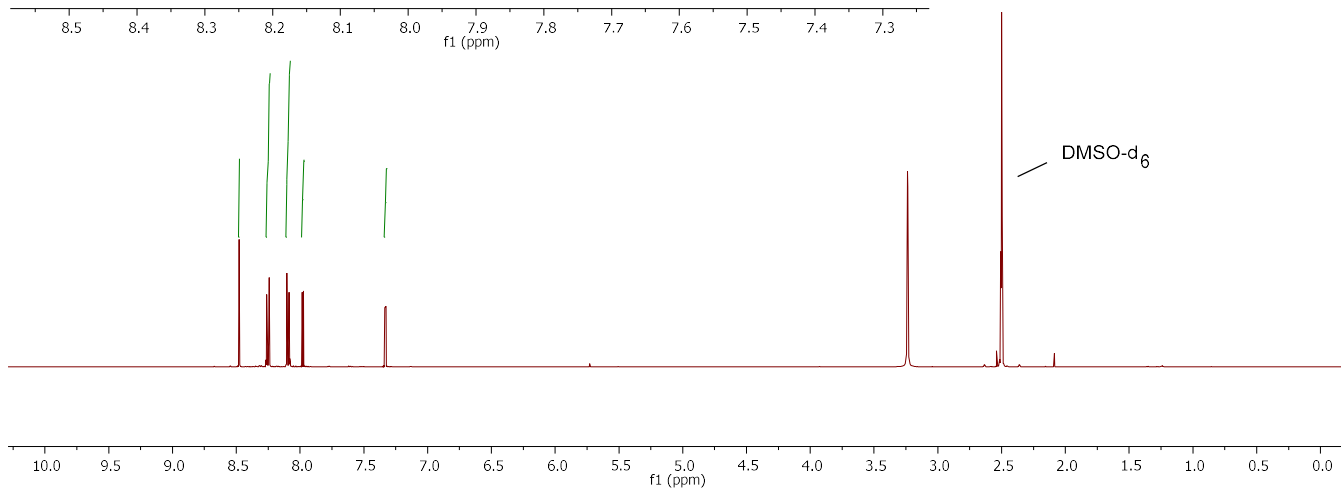
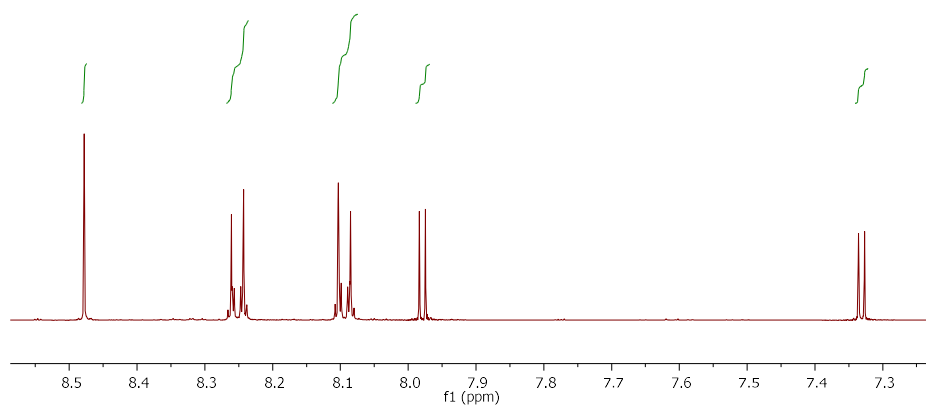
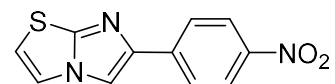
HSQC **12c** (400 MHz, DMSO-d₆)



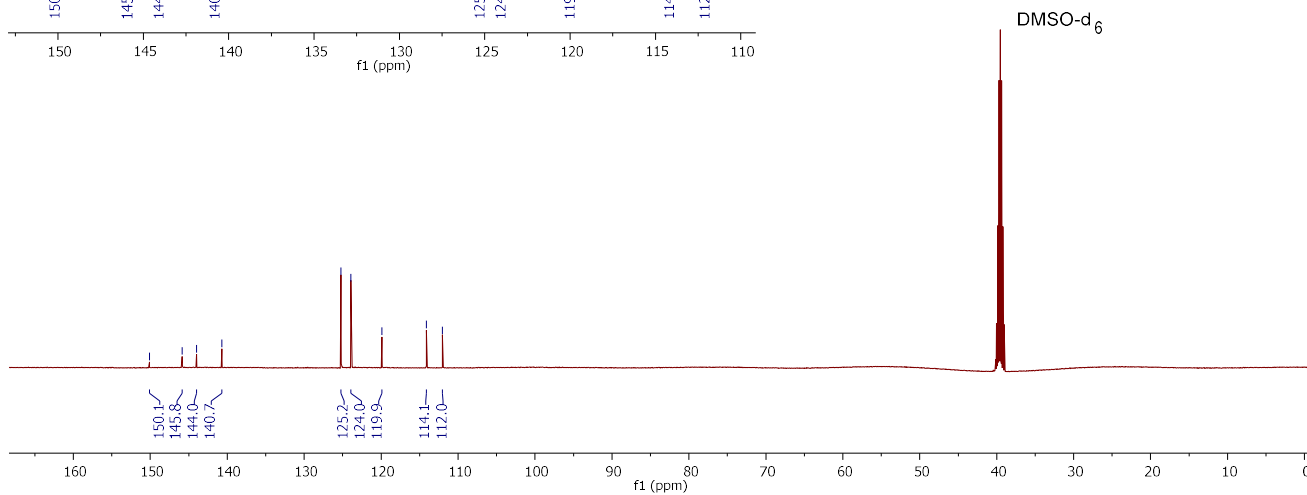
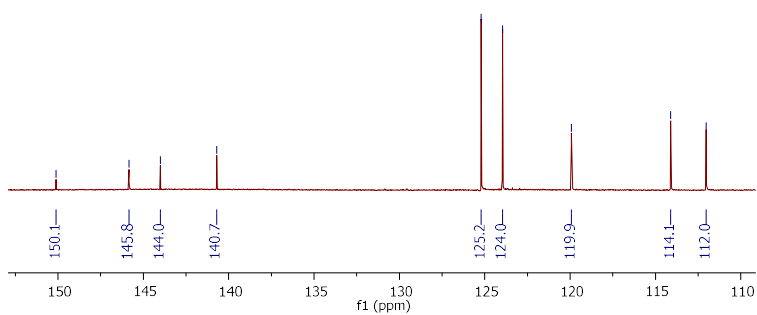
HMBC **12c** (400 MHz, DMSO-d₆)



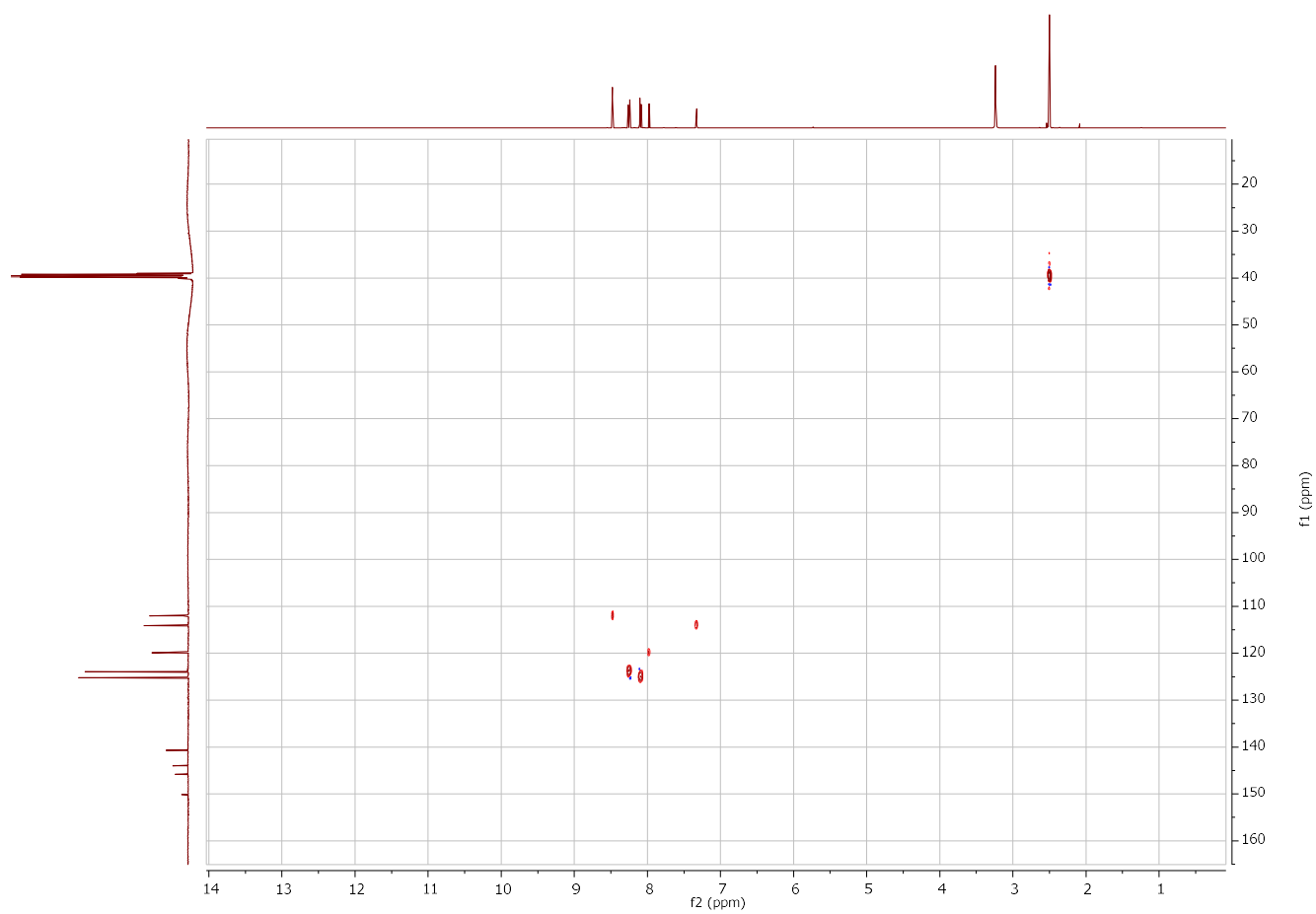
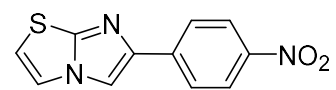
^1H NMR GBG235 **14a** (500 MHz, DMSO- d_6)



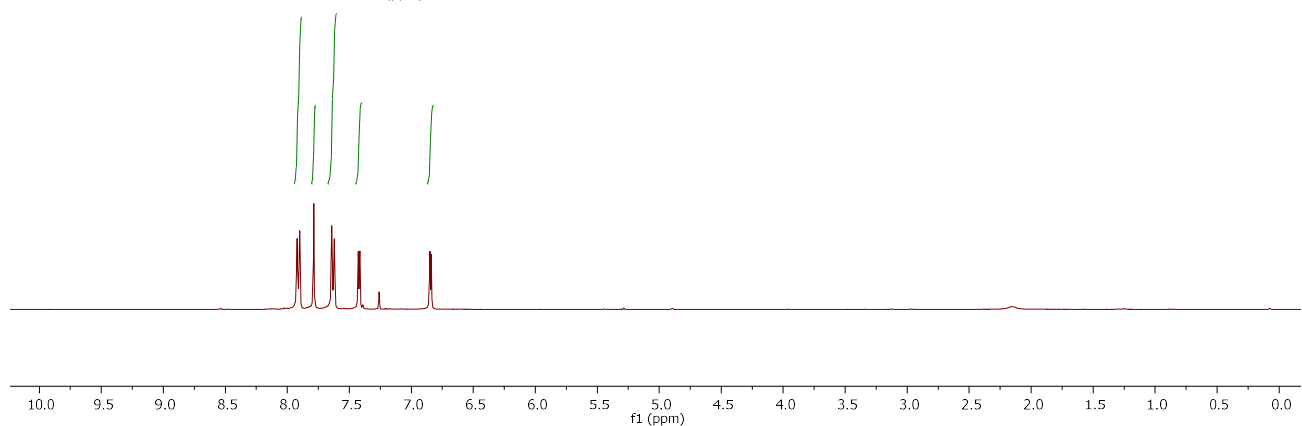
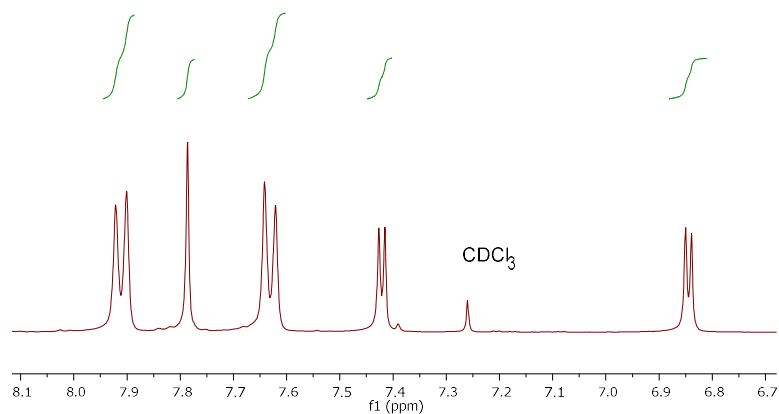
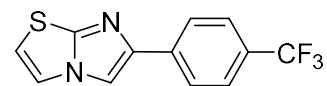
^{13}C NMR GBG235 **14a** (126 MHz, DMSO- d_6)



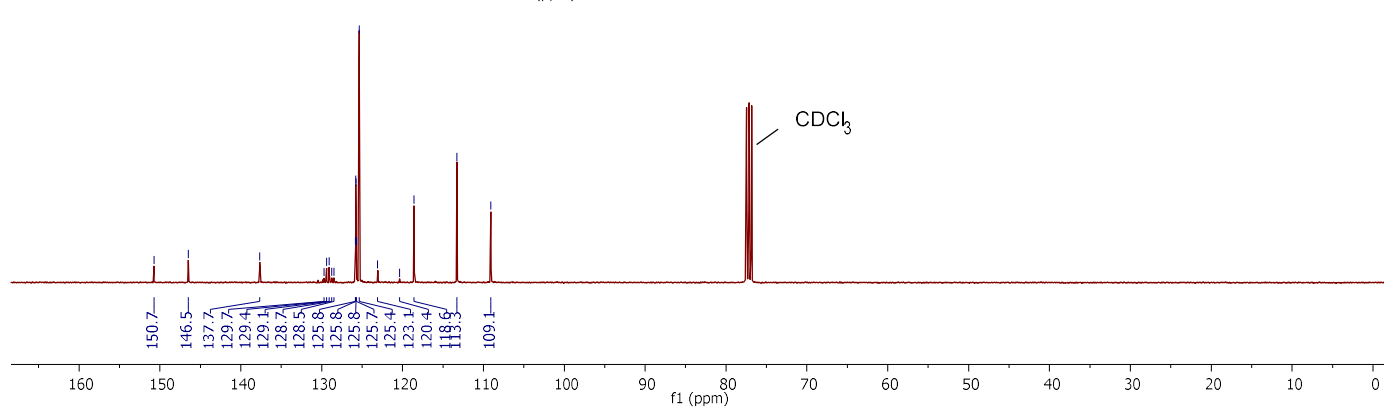
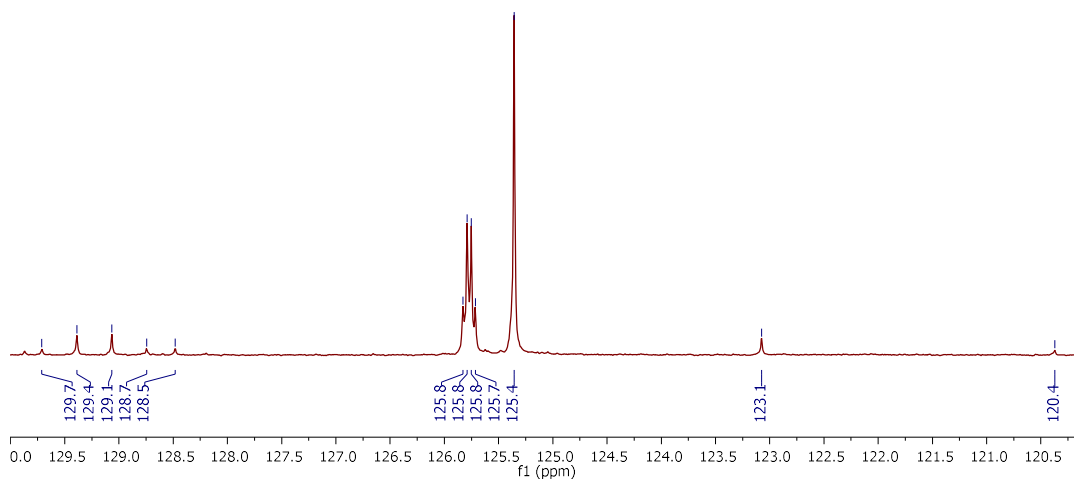
HSQC **14a** (400 MHz, DMSO-d₆)



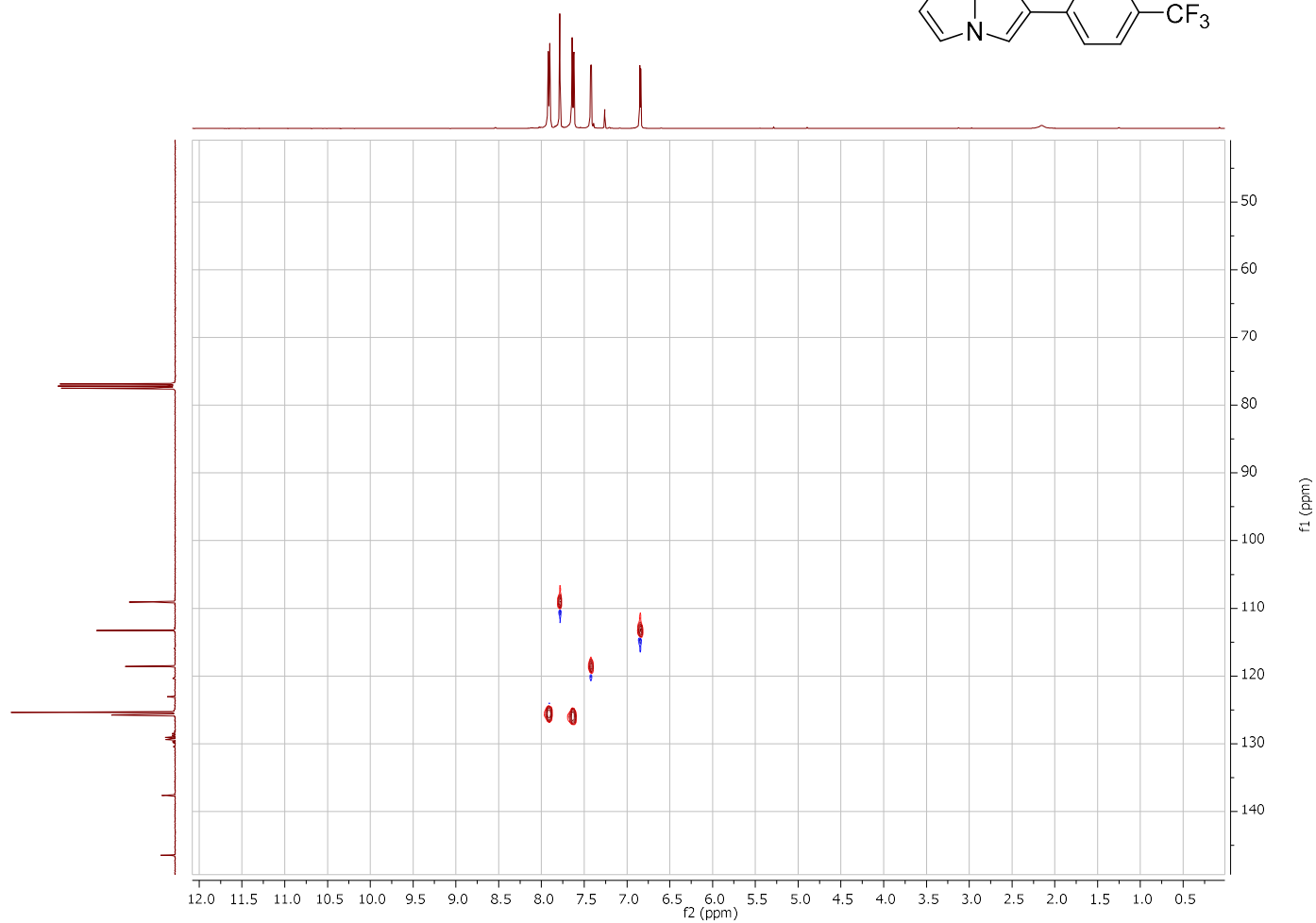
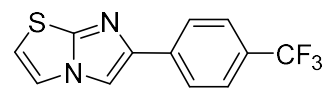
^1H NMR **14b** (400 MHz, CDCl_3)

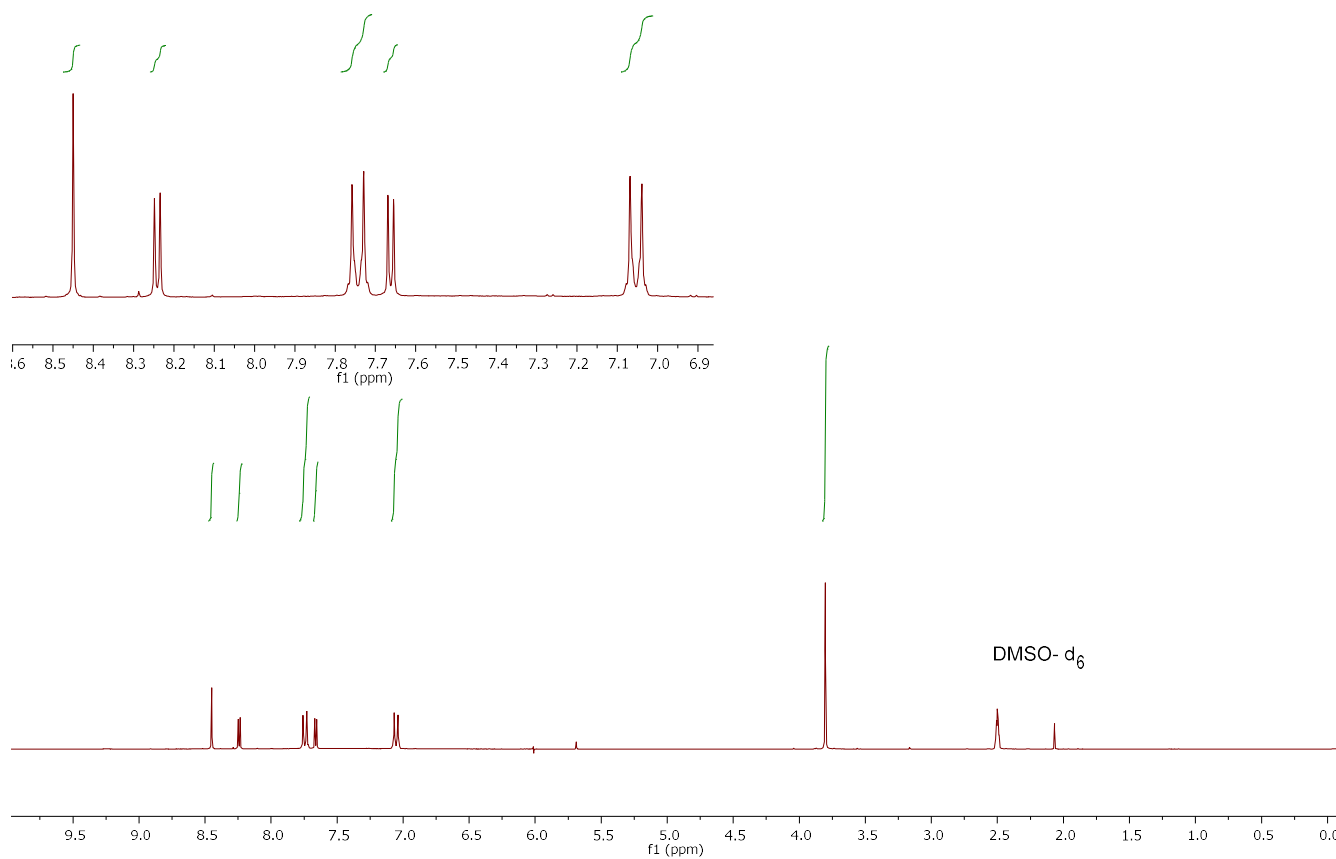
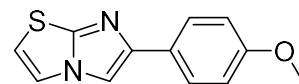
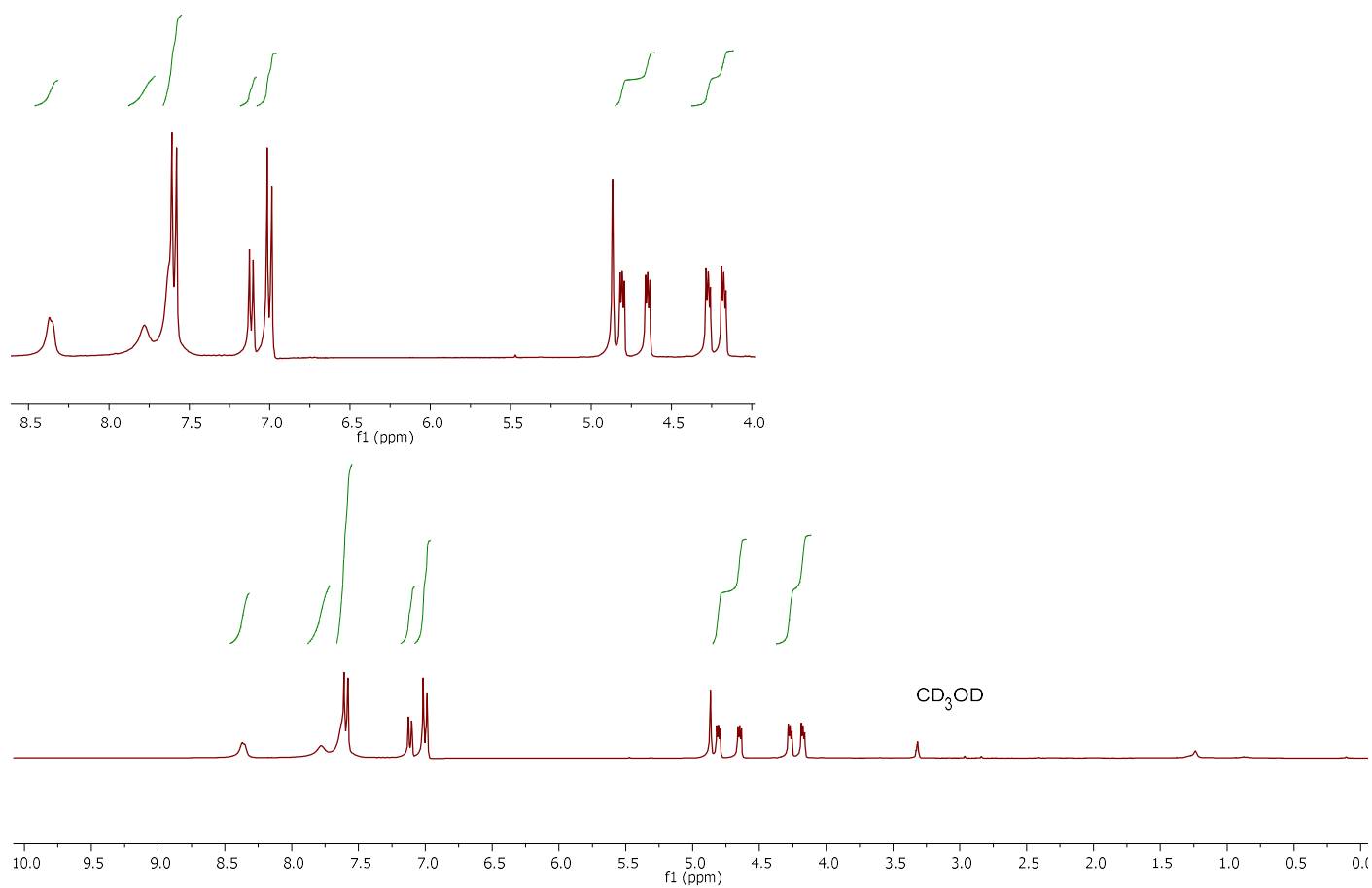


^{13}C NMR **14b** (101 MHz, CDCl_3)

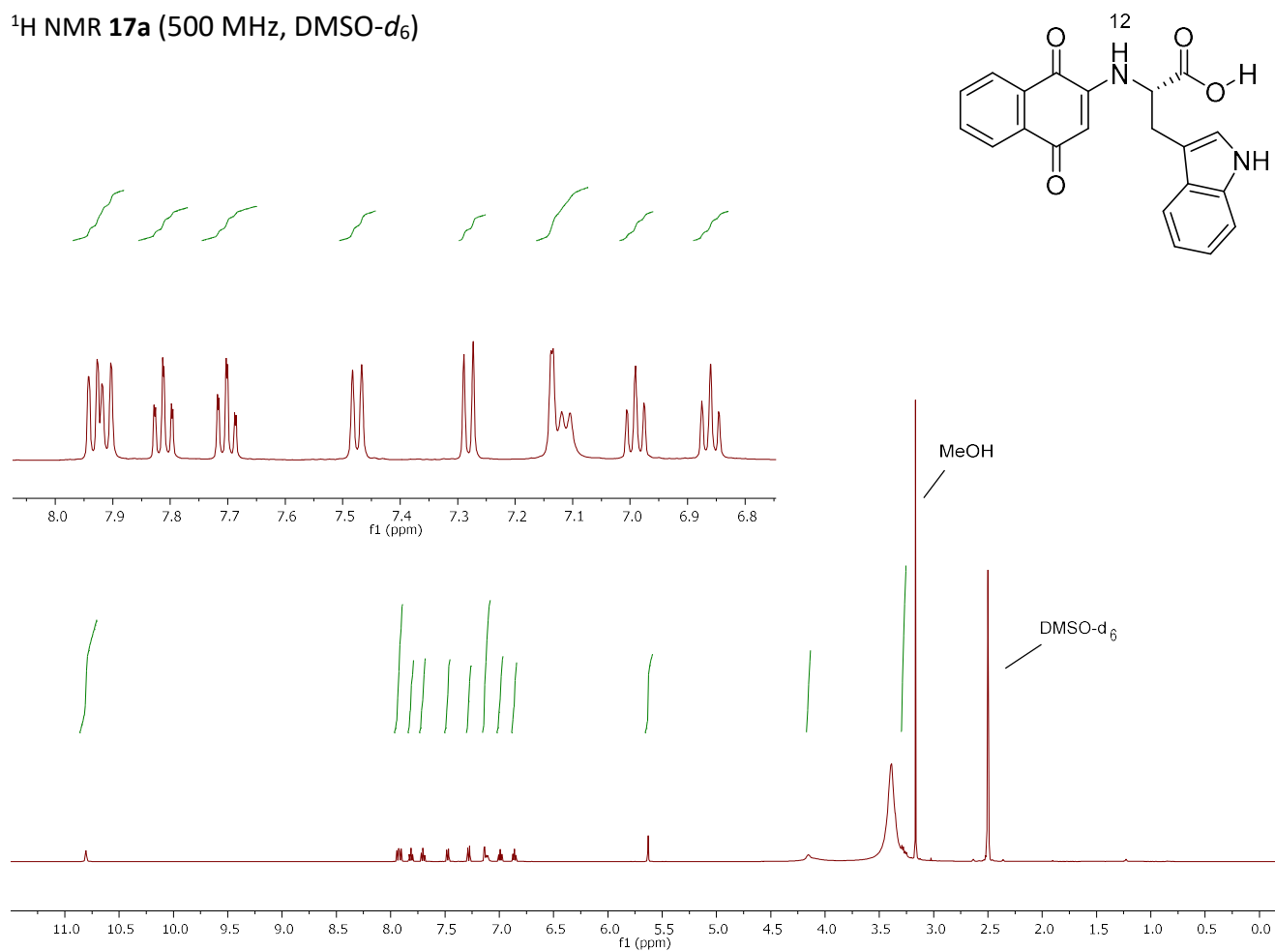


HSQC **14b** (400 MHz, CDCl₃)

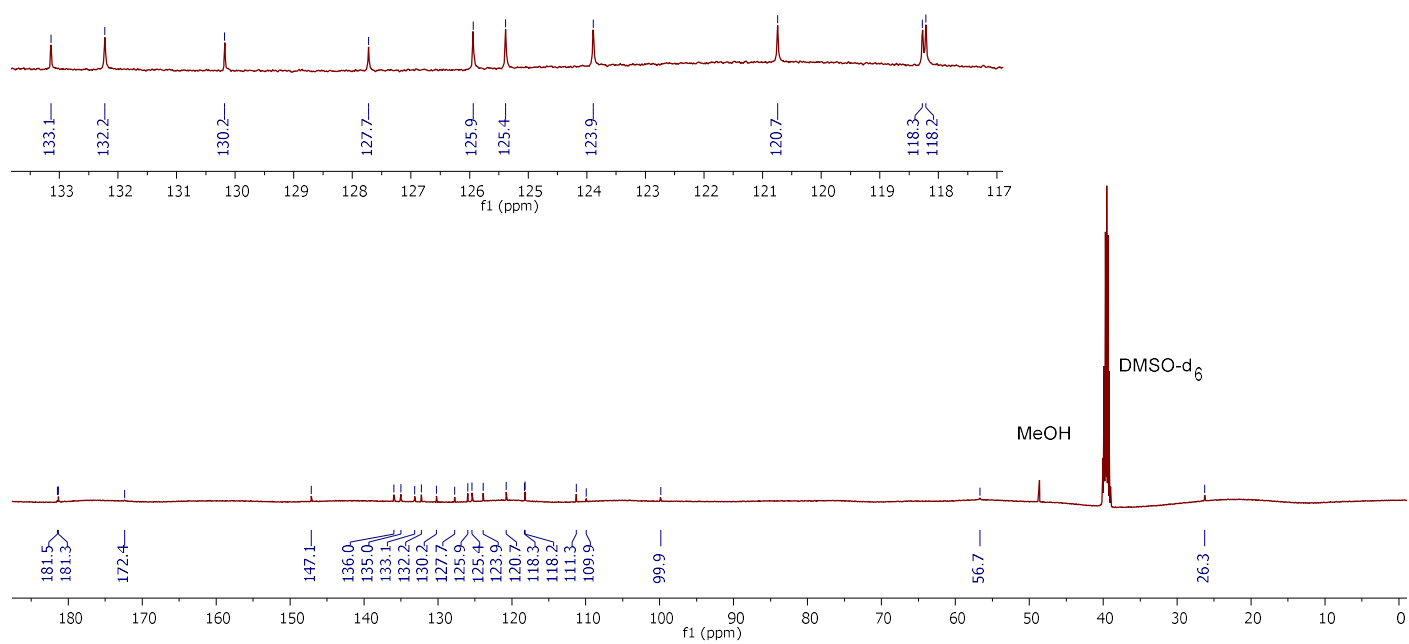


¹H NMR **14c** (400 MHz, CDCl₃)¹H NMR **1n** (400 MHz, CDCl₃)

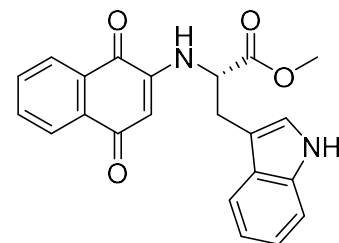
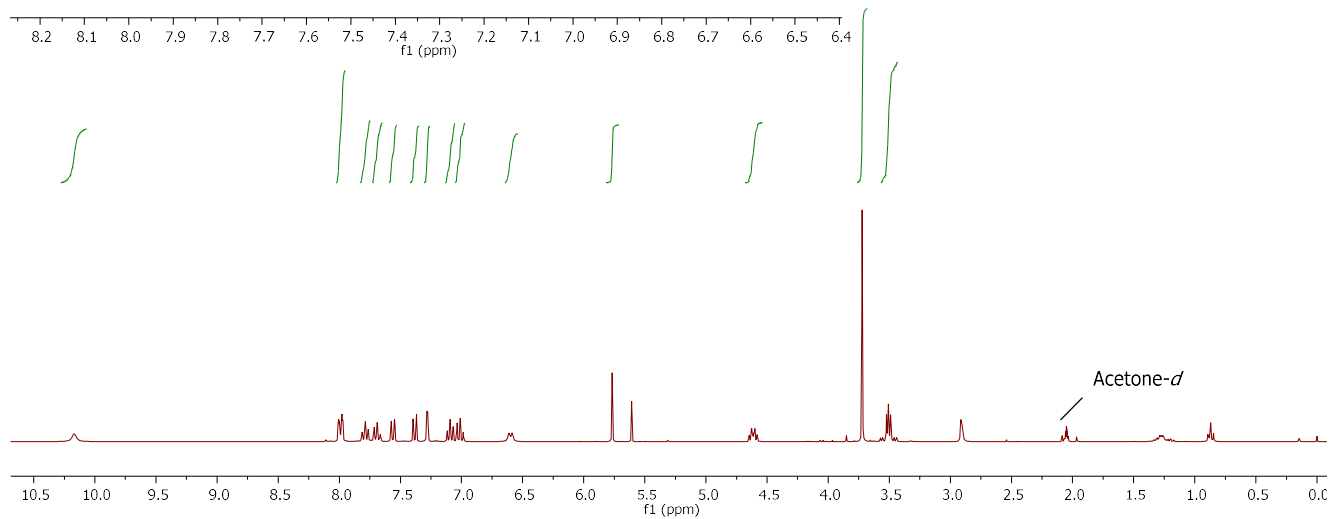
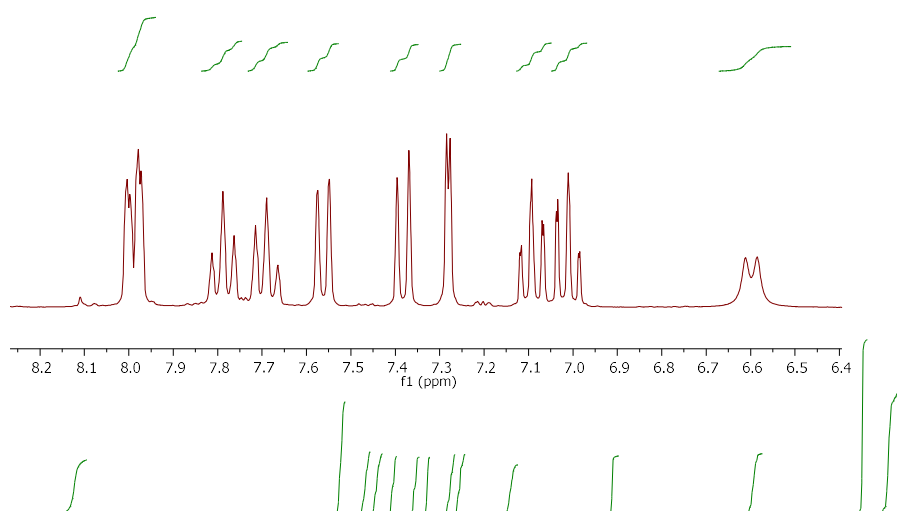
¹H NMR **17a** (500 MHz, DMSO-*d*₆)



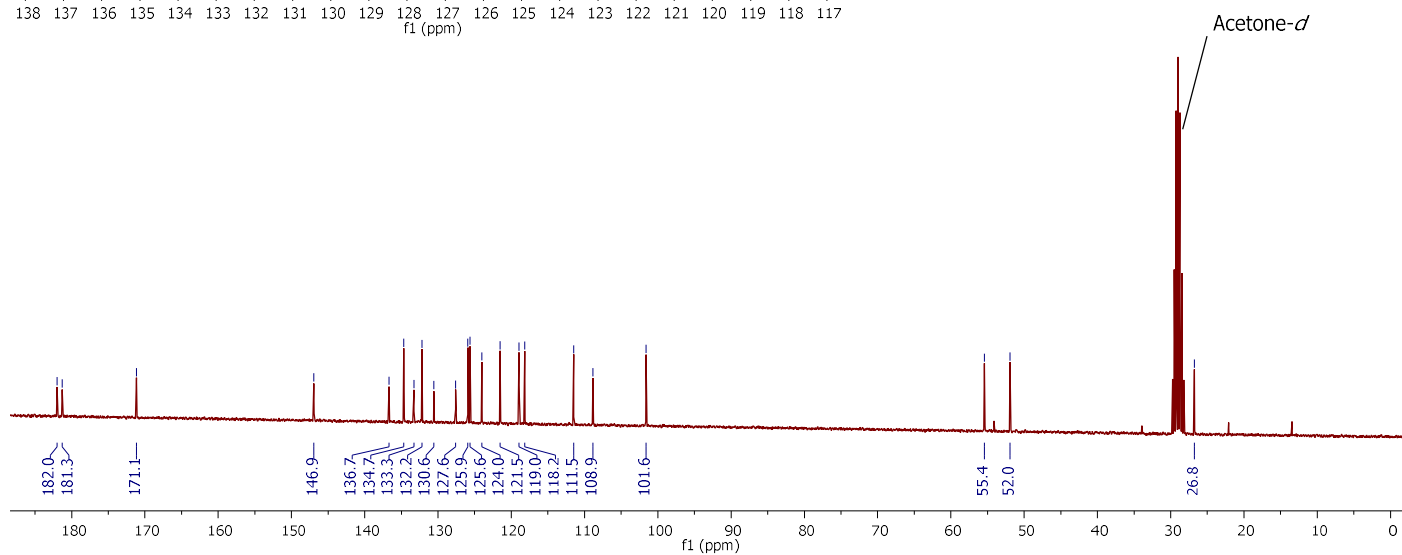
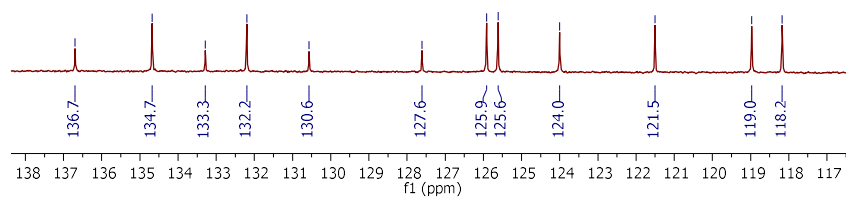
¹³C NMR **17a** (126 MHz, DMSO-*d*₆)

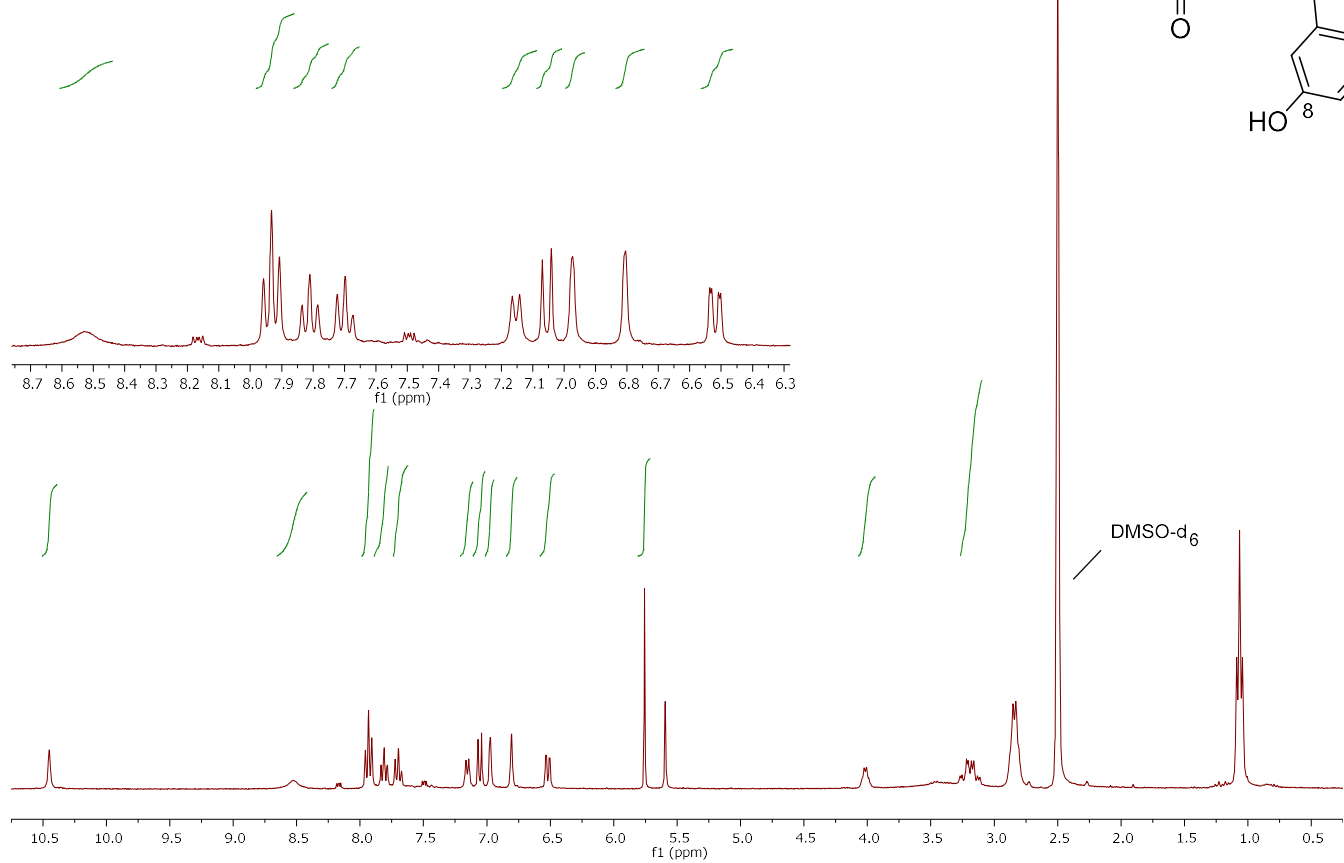
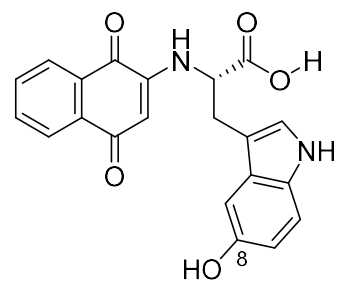
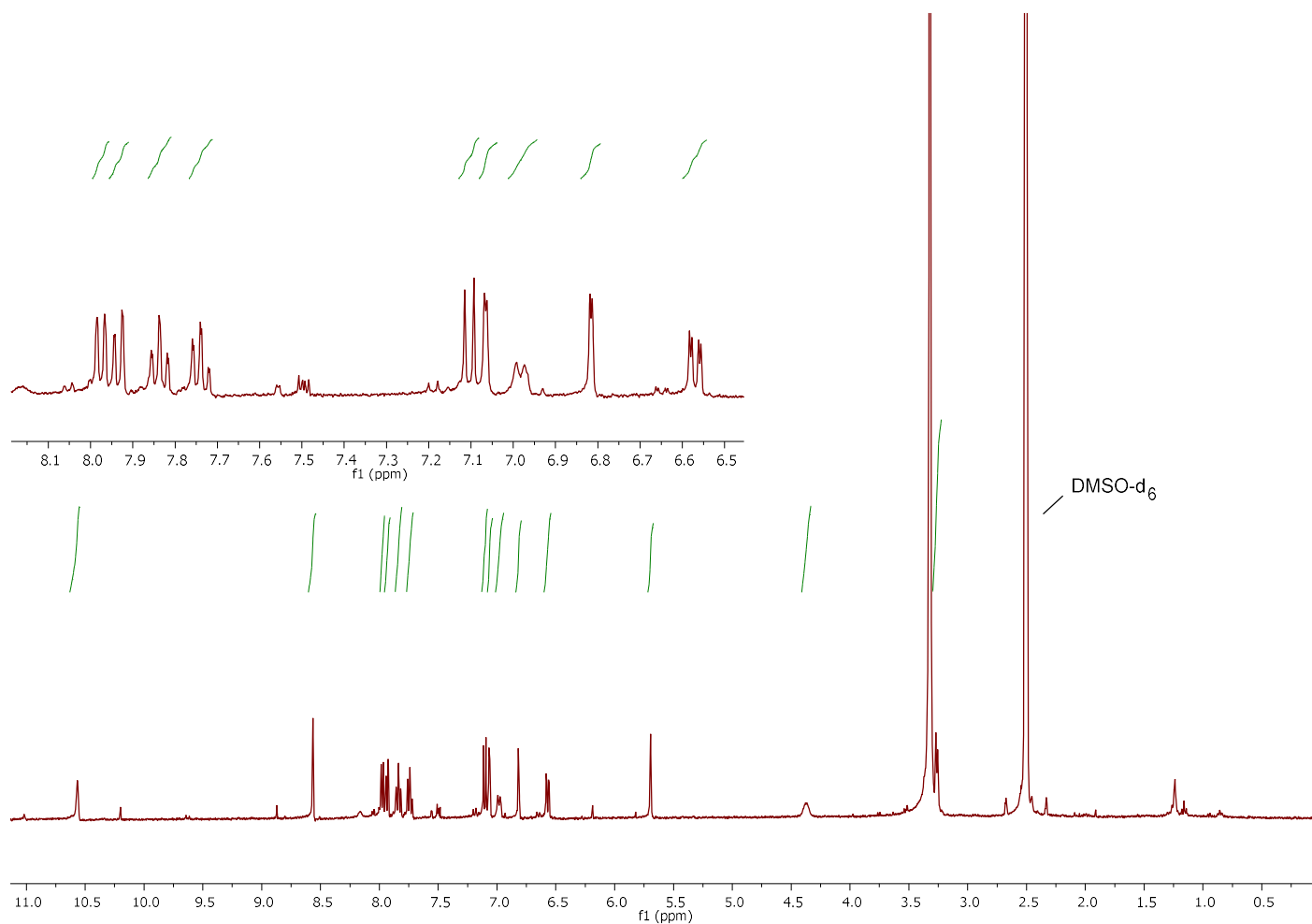


¹H NMR **17b** (500 MHz, DMSO-*d*₆)

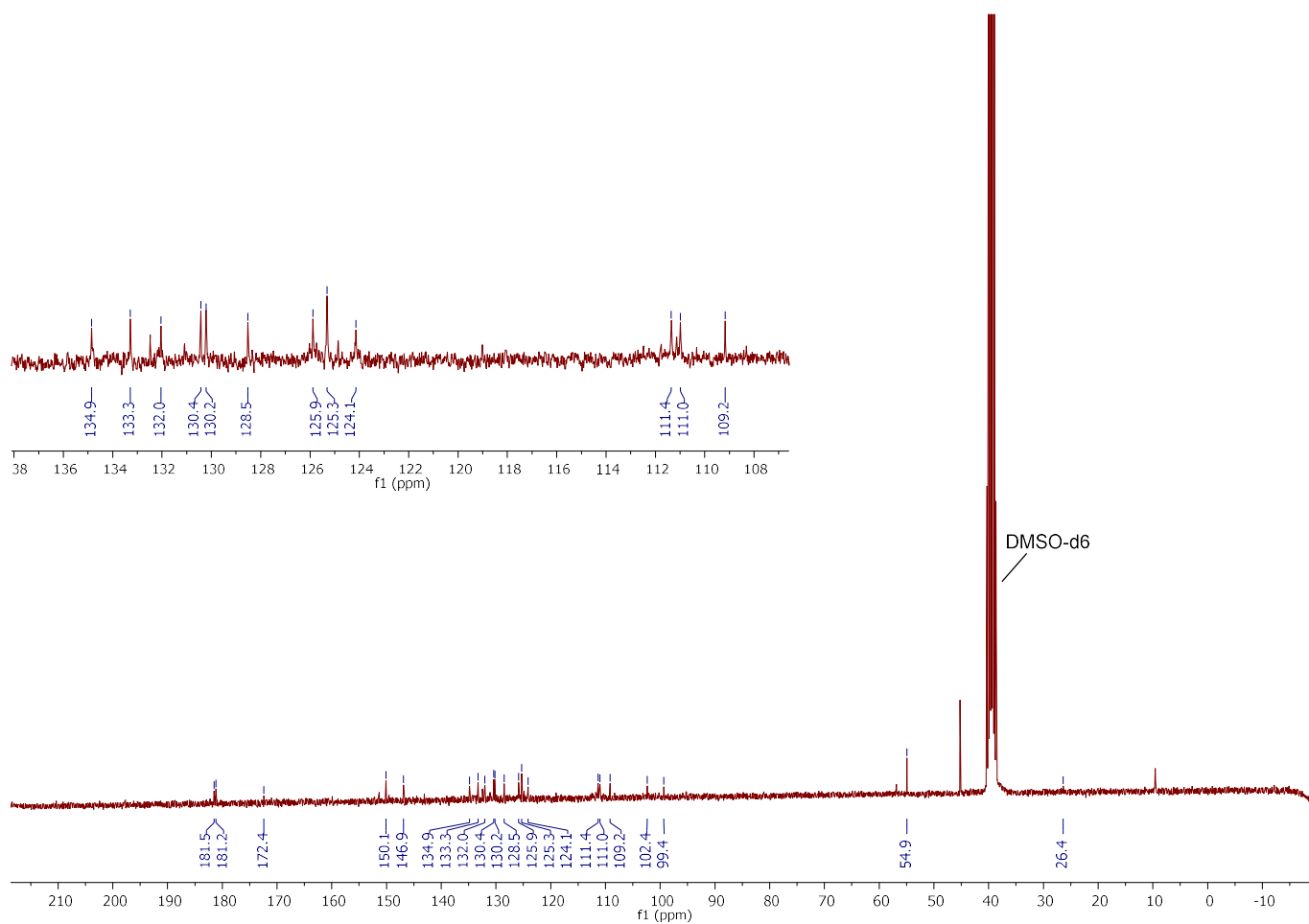


¹³C NMR **17b** (75 MHz, DMSO-*d*₆)

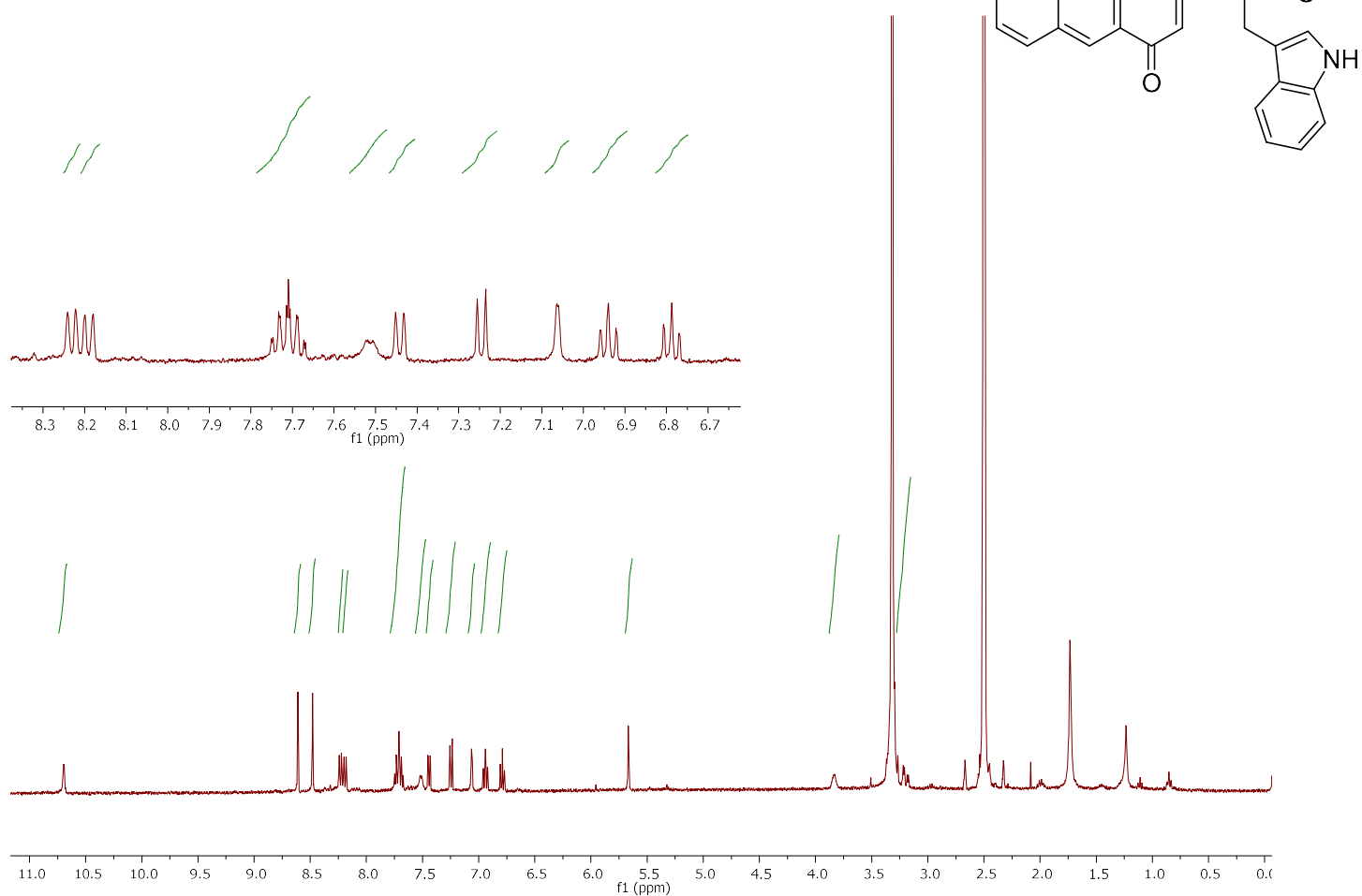


^1H NMR **17c** (300 MHz, DMSO- d_6)¹H NMR **17c** (400 MHz, DMSO-*d*₆) -Post lyophilization

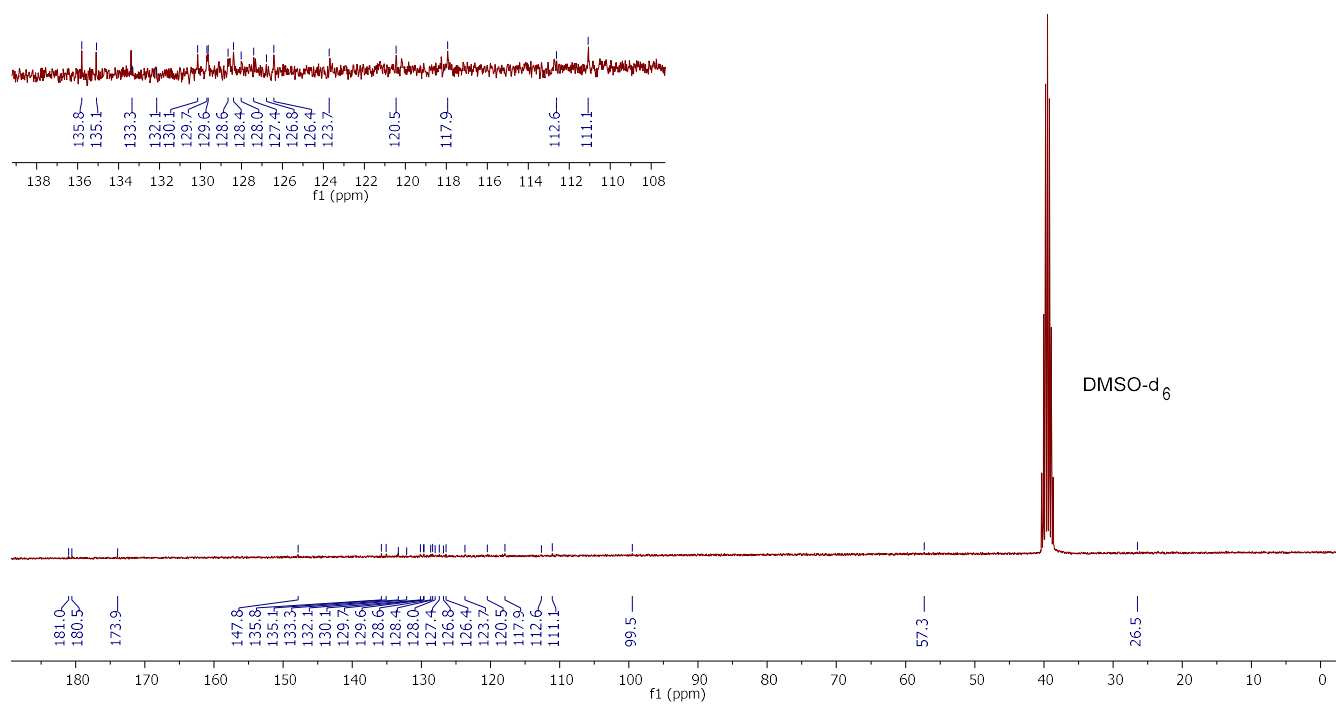
^{13}C NMR **17c** (75 MHz, $\text{DMSO-}d_6$)



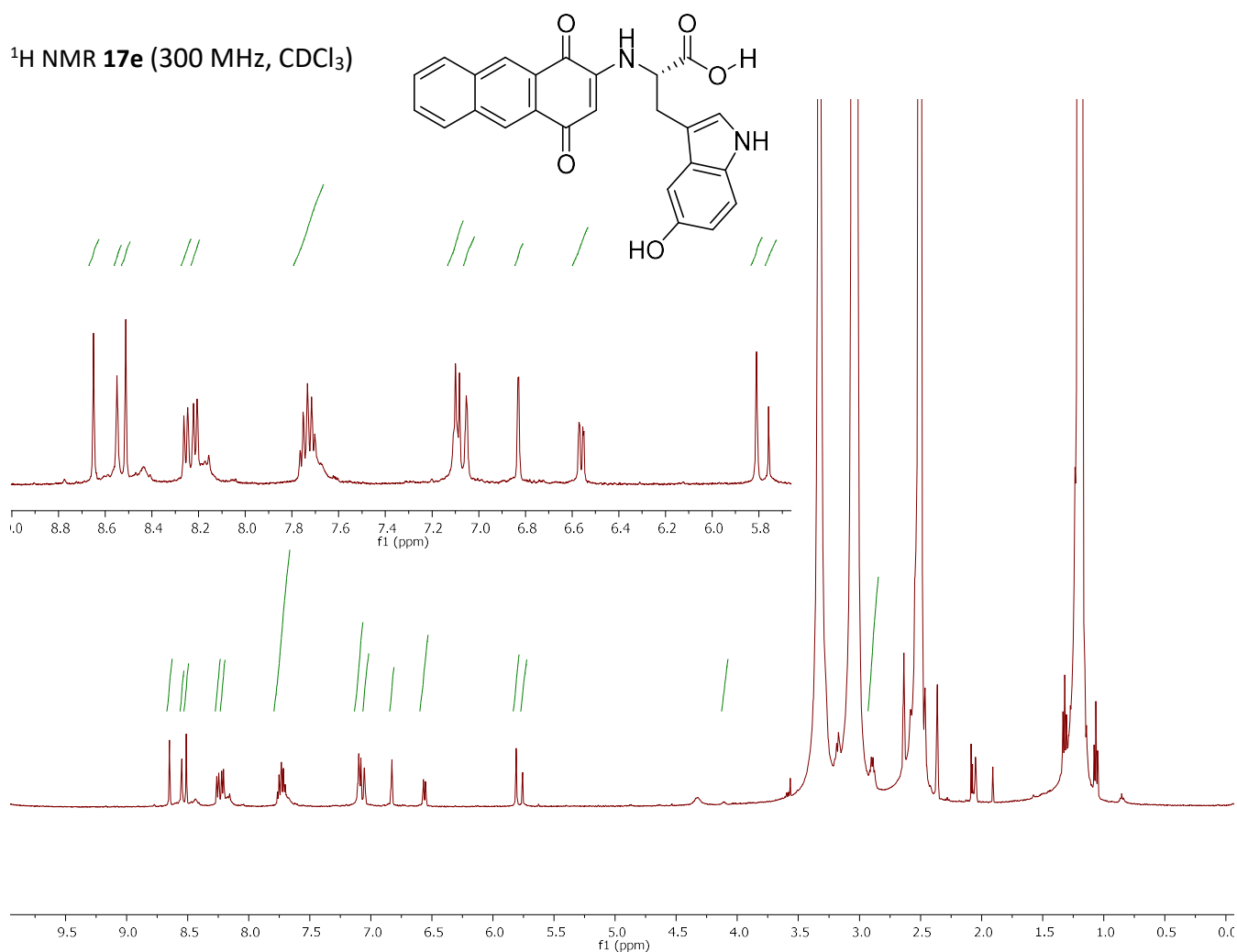
¹H NMR **17d** (400 MHz, DMSO-*d*₆)



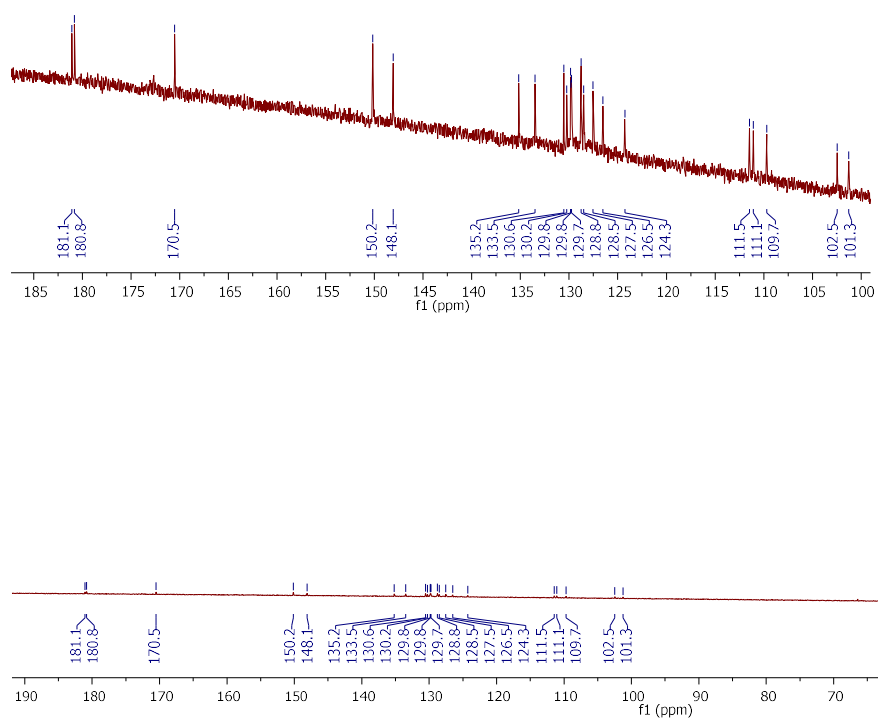
¹³C NMR **17d** (75 MHz, DMSO-*d*₆)



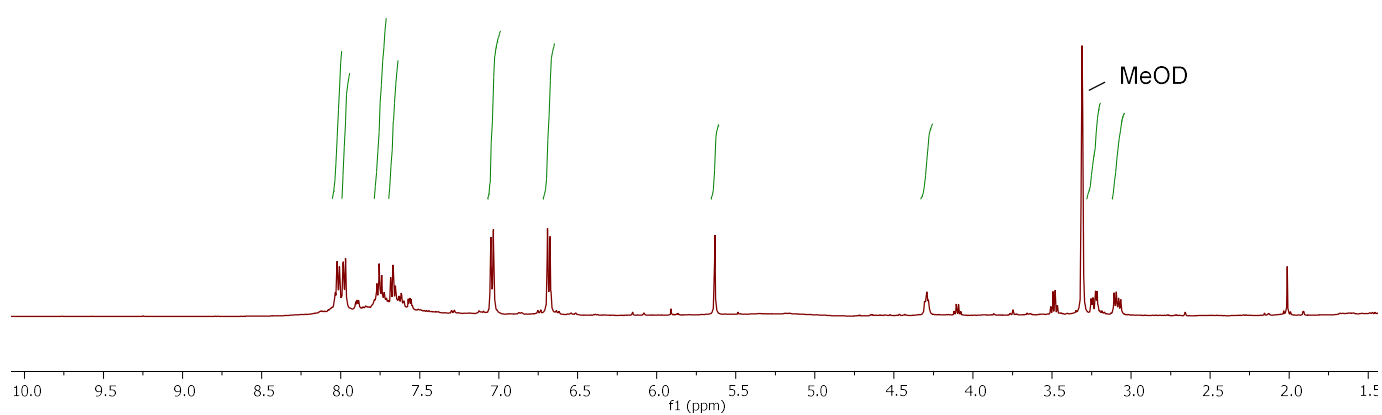
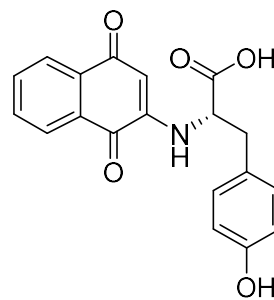
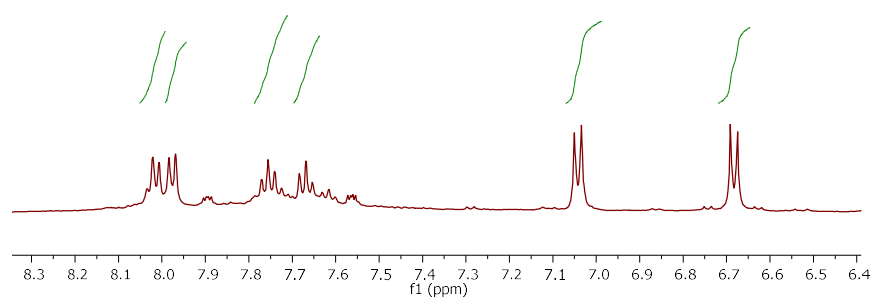
^1H NMR **17e** (300 MHz, CDCl_3)



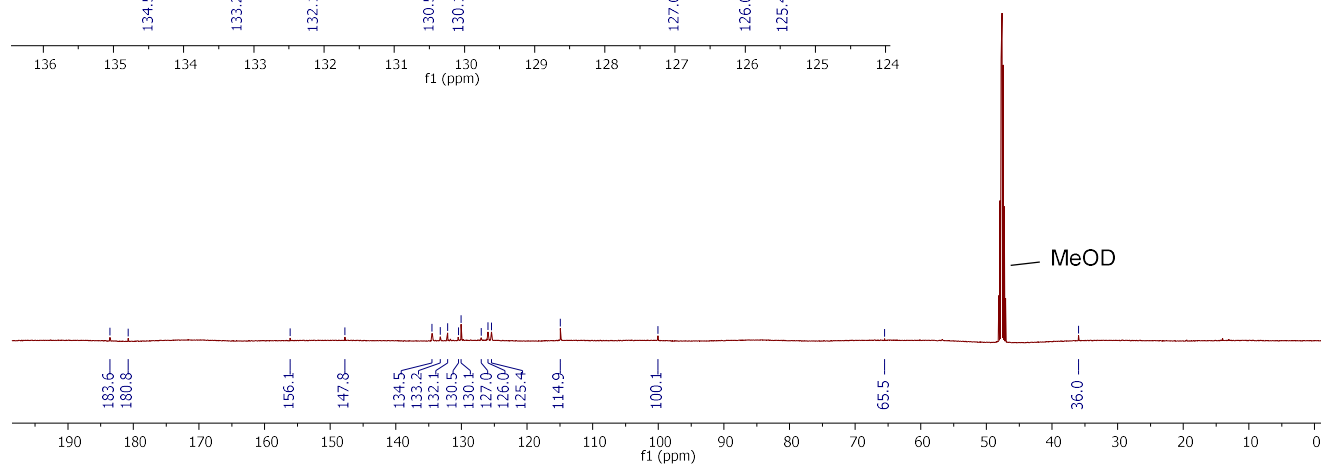
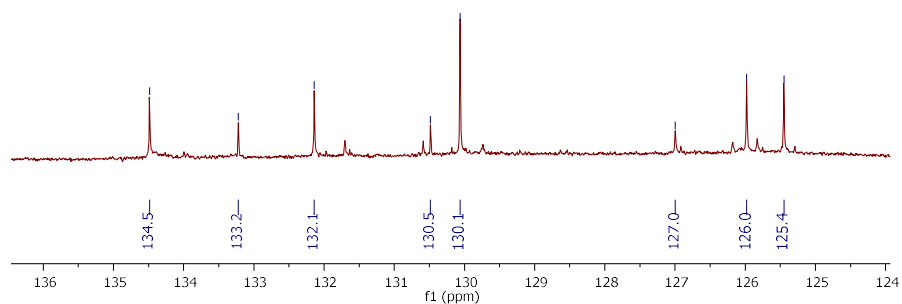
^{13}C NMR **17e** (75 MHz, CDCl_3)

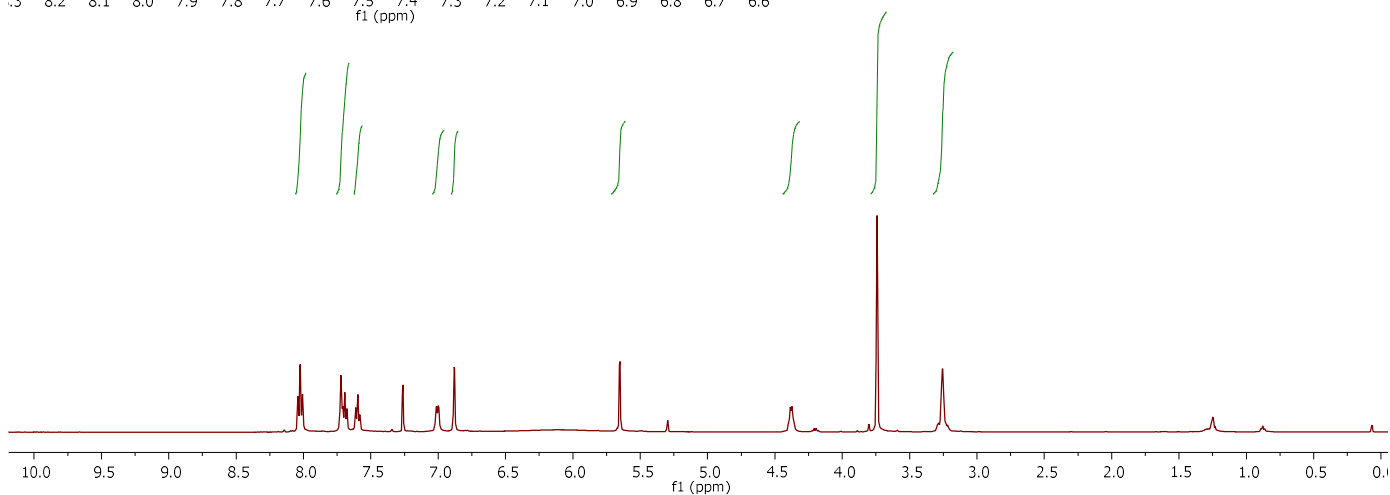
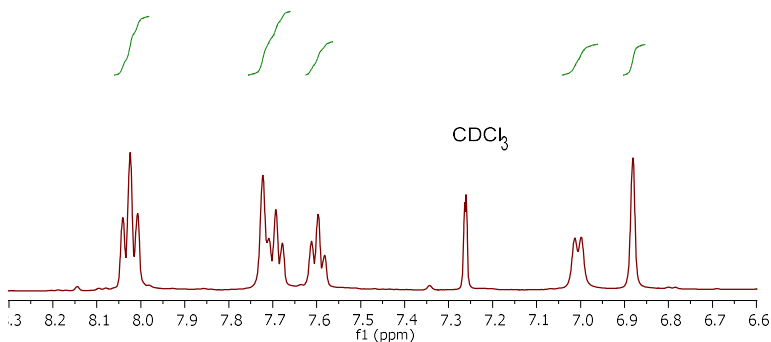
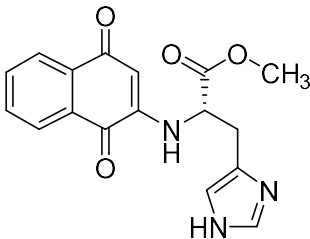
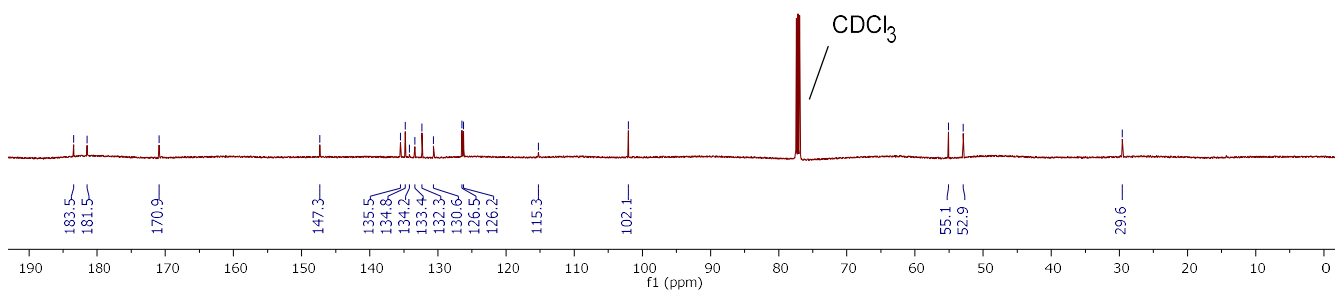
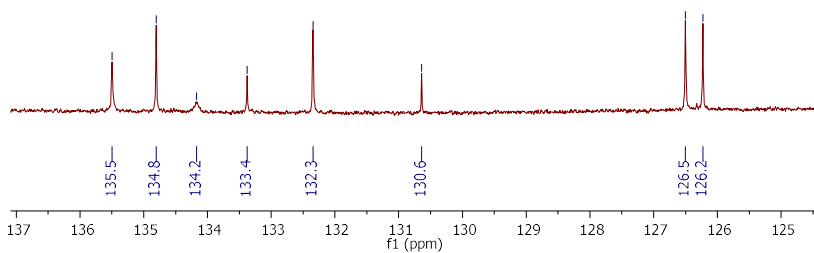


¹H NMR **17f** (500 MHz, MeOD)



¹³C NMR **17f** (75 MHz, DMSO-*d*₆)



^1H NMR **17h** (500 MHz, CDCl_3) ^{13}C NMR **17h** (126 MHz, CDCl_3)

Structural elucidation of compound 17x

Calculated molecular formulae by UNIFI software in ESI+ based on the m/z of the three detected adducts:

512.1467 m/z [M+H]⁺

Results (6 found)											
	Composition	i-FIT Confidence (%)	m/z RMS (PPM)	Intensity RMS (%)	Predicted m/z	m/z error (PPM)	m/z error (mDa)	DBE	i-FIT	Normalized i-FIT	
1	C ₂₈ H ₂₁ N ₃ O ₇	57.910612	2.652870	2.359611	512.145227	2.941467	1.503500	20.000000	523.333447	0.546270	
2	C ₂₉ H ₁₇ N ₇ O ₃	41.732274	0.357341	4.723778	512.146564	0.324935	0.166087	25.000000	523.661073	0.873895	
3	C ₁₆ H ₂₅ N ₅ O ₁₄	0.285113	0.776762	18.703438	512.147077	-0.678873	-0.346999	7.000000	528.647216	5.860039	
4	C ₁₇ H ₂₁ N ₉ O ₁₀	0.027700	3.226807	14.149584	512.148414	-3.295405	-1.684411	12.000000	530.978674	8.191496	
5	C ₁₅ H ₂₉ N ₉ O ₁₈	0.023838	1.811519	24.517184	512.145740	1.937659	0.990414	2.000000	531.128801	8.341624	
6	C ₁₄ H ₁₃ N ₁₉ O ₄	0.017476	1.018752	17.218645	512.147066	-0.658326	-0.336496	18.000000	531.439248	8.652070	

534.1275 m/z [M+ Na]⁺

Results (2 found)											
	Composition	i-FIT Confidence (%)	m/z RMS (PPM)	Intensity RMS (%)	Predicted m/z	m/z error (PPM)	m/z error (mDa)	DBE	i-FIT	Normalized i-FIT	
1	C ₂₉ H ₁₇ N ₇ O ₃	83.545574	1.971018	1.341395	534.128509	-1.953563	-0.998541	25.000000	51.405238	0.179778	
2	C ₂₈ H ₂₁ N ₃ O ₇	16.453604	0.713788	2.398402	534.127171	0.662975	0.338872	20.000000	53.030086	1.804626	

550.1011 m/z [M+K]⁺

Results (6 found)											
	Composition	i-FIT Confidence (%)	m/z RMS (PPM)	Intensity RMS (%)	Predicted m/z	m/z error (PPM)	m/z error (mDa)	DBE	i-FIT	Normalized i-FIT	
1	C ₂₈ H ₂₁ N ₃ O ₇	79.290509	0.833773	4.038959	550.101108	-0.016372	-0.008368	20.000000	5.498967	0.232052	
2	C ₂₉ H ₁₇ N ₇ O ₃	20.443327	2.101265	4.191081	550.102446	-2.632912	-1.345781	25.000000	6.854429	1.587514	
3	C ₁₅ H ₂₉ N ₉ O ₁₈	0.182074	0.899385	27.473570	550.101621	-1.020183	-0.521454	2.000000	11.575427	6.308512	
4	C ₁₆ H ₂₅ N ₅ O ₁₄	0.041466	2.999840	21.178848	550.102959	-3.636722	-1.858867	7.000000	13.054957	7.788042	
5	C ₁₄ H ₁₃ N ₁₉ O ₄	0.028913	3.001713	15.561992	550.102948	-3.616176	-1.848365	18.000000	13.415565	8.148650	
6	C ₁₃ H ₁₇ N ₁₅ O ₈	0.011713	1.786317	20.605289	550.101611	-0.999636	-0.510952	13.000000	14.319151	9.052237	

Although C₂₉H₁₇N₇O₃ was calculated as a potential molecular formula, C₂₈H₂₁N₃O₇ was selected as the most probable calculated molecular formula for **2x** since its elemental composition matched better with the starting materials.

Fragmentation analysis and collision cross section (CCS) determination

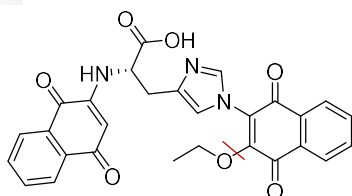
With the aim of confirming the chemical structure of the unknown molecule, a fragmentation analysis was carried out. The total number of fragments are shown in the figure below. The most probable cleavage sites are indicated with red lines.

The fragment m/z 466.1038 correlated with the loss of a molecule of ethanol, which could be associated with the loss of the ethoxy group (Figure 27).

Another observed fragment of high intensity was m/z 354.1082. The predicted molecular formula for this m/z value correlated with the loss of the 1,4-naphthoquinone scaffold.

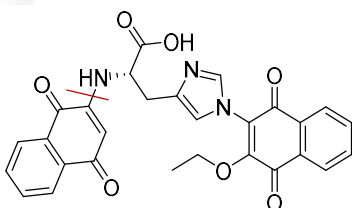
466.1038 m/z [M+H-C₂H₆O]⁺

Results (2 found)								
	Composition	i-FIT Confidence (%)	m/z RMS (PPM)	Intensity RMS (%)	Predicted m/z	m/z error (PPM)	m/z error (mDa)	DBE
1	C ₂₆ H ₁₅ N ₃ O ₆	99.979406	0.996202	0.327677	466.103362	0.899412	0.418314	21.000000
2	C ₂₇ H ₁₁ N ₇ O ₂	0.020590	1.776497	3.144784	466.104699	-1.976147	-0.919099	26.000000



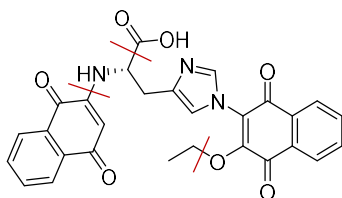
354.1082 m/z [M+H-Naphthoquinone]⁺

Results (3 found)								
	Composition	i-FIT Confidence (%)	m/z RMS (PPM)	Intensity RMS (%)	Predicted m/z	m/z error (PPM)	m/z error (mDa)	DBE
1	C ₁₈ H ₁₅ N ₃ O ₅	99.922440	0.847983	0.988662	354.108447	-0.784660	-0.277064	13.000000
2	C ₂₀ H ₁₇ O ₆	0.040517	4.734240	3.023608	354.109790	-4.587154	-1.619728	12.500000
3	C ₁₆ H ₁₃ N ₆ O ₄	0.037042	3.319491	2.958691	354.107104	3.017834	1.065600	13.500000



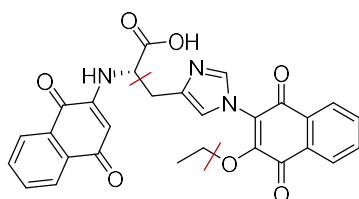
280.0720 m/z

Results (3 found)								
	Composition	i-FIT Confidence (%)	m/z RMS (PPM)	Intensity RMS (%)	Predicted m/z	m/z error (PPM)	m/z error (mDa)	DBE
1	C ₁₇ H ₁₁ O ₄	55.458359	4.675649	48.336115	280.073010	-3.691947	-1.030292	12.500000
2	C ₁₅ H ₉ N ₃ O ₃	30.518591	4.528643	55.831745	280.071668	1.119353	0.312372	13.000000
3	C ₁₃ H ₇ N ₆ O ₂	14.023050	8.262297	65.158522	280.070325	5.930652	1.655036	13.500000



253.0611 m/z

Results (2 found)								
	Composition	i-FIT Confidence (%)	m/z RMS (PPM)	Intensity RMS (%)	Predicted m/z	m/z error (PPM)	m/z error (mDa)	DBE
1	C ₁₄ H ₈ N ₂ O ₃	71.550878	4.780578	56.780560	253.060769	1.314843	0.331411	12.000000
2	C ₁₂ H ₆ N ₅ O ₂	28.449122	8.852643	66.803937	253.059426	6.641736	1.674075	12.500000



The m/z 280.0720 generated by the loss of the naphthoquinone group, the ethyl fragment and the carboxylic acid group.

The m/z 253.0611 can be explained by the loss of (1,4-dioxo-1,4-dihydronaphthalen-2-yl)glycine neutral fragment and the ethyl group.

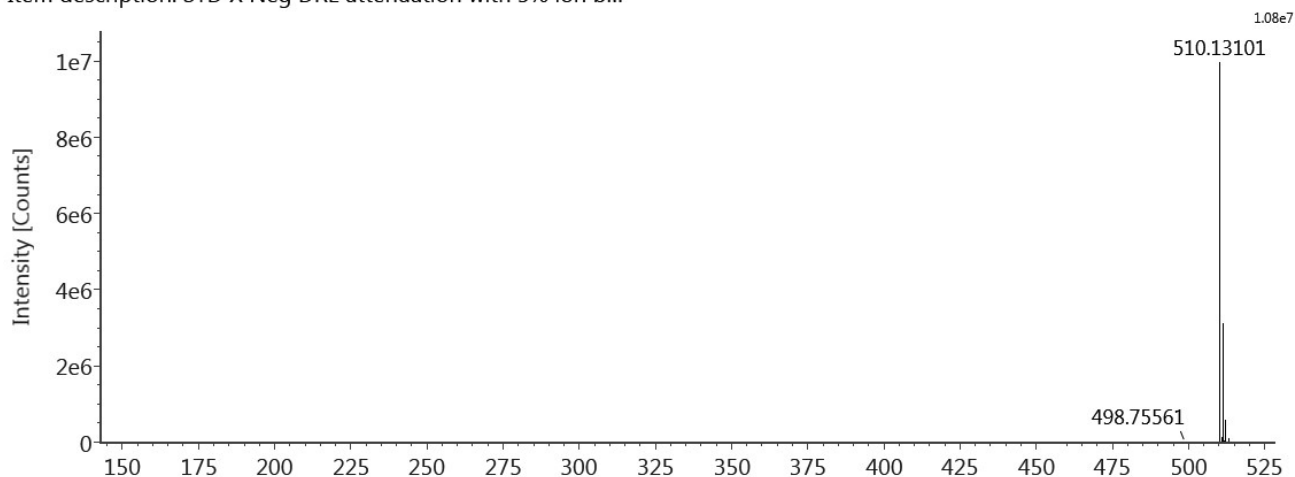
In both cases, the rearrangement of the product ions in the gas phase can occur, leading to hydride shifts followed by potential cyclizations of the molecule to more stable fragments.

From the ESI- analysis, in addition to the [M-H]⁻ adduct (m/z 510.1310, 0.3 mDa error) the same fragments were found, which represent the deprotonated species of the above-mentioned ions.

Item name: 200312_003_StdX_NEG

Channel name: Low energy : Time 6.4683 +/- 0.0187 minute...

Item description: STD X Neg DRE attenuation with 5% ion b...



Item name: 200312_003_StdX_NEG

Channel name: High energy : Time 6.4683 +/- 0.0187 minut...

Item description: STD X Neg DRE attenuation with 5% ion b...

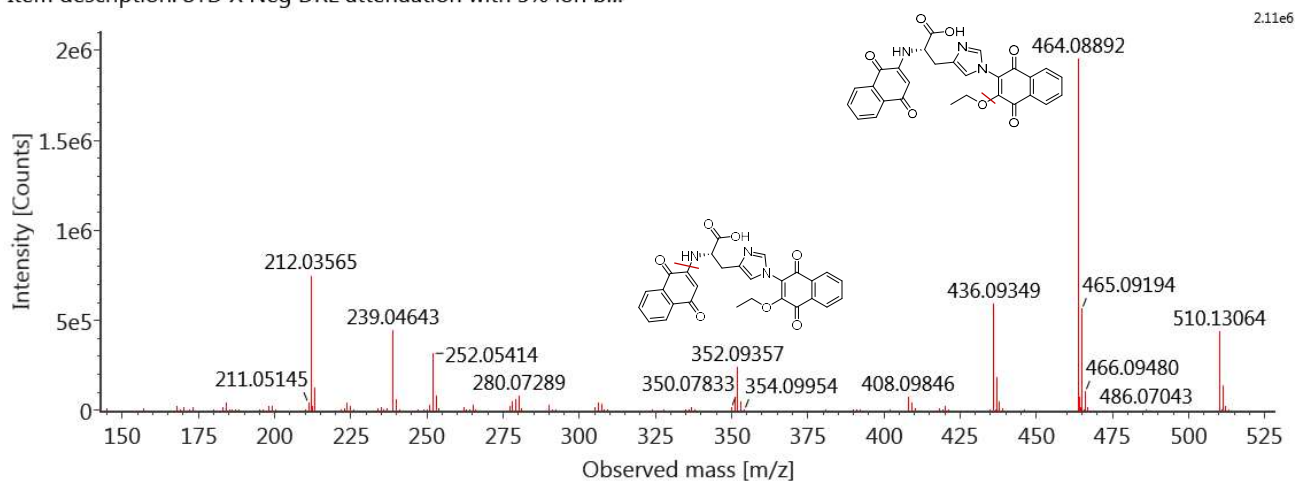


Figure- High resolution mass spectrum of compound **17x** in negative mode (ESI⁻). Low energy (**A**) and high energy spectrum (**B**). Fragments m/z 464 and 352 corresponds to m/z 466 and 354 in ESI⁺.

In addition to the accurate mass of the precursor and fragment ions, it was possible to measure the collision cross section (CCS, or Ω) of the adducts in positive and negative ion mode for the candidate molecule. This parameter can be related to the mobility of ions according to the Mason-Schamp equation,¹³ and describes the momentum transfer between ions and drift gas particles. The mobility of an ion depends on experimental conditions such as drift gas composition, temperature, and reduced field strength (E/N , where E represents the electric field and N is the gas number density). In this work the technology adopted was the traveling-wave ion mobility spectrometry (TWIMS) embedded in the Vion system, which has extensively been described elsewhere.¹⁴ By the use of a calibration equation, it is possible to derive the CCS of an ion from its arrival time (t_A). In general terms, the CCS is related to the chemical structure and three-dimensional conformation of an ion where the projection of the rotating three-dimensional volume into a two-dimensional space represents the average cross section. Since this parameter is ultimately determined by the mass, shape and size of an ionized molecule, ion mobility separation and CCS are being utilized by researches for three main purposes:

1. As an added dimension of separation for increasing the peak capacity and partitioning the chemical noise from analyte signals of interest (in our case, the drift time alignment allowed cleaner spectra to be obtained by the removal of noise peaks and potential co-eluting components).
2. As an additional measurement for analyte identification and characterization.
3. As a structural measurement technique, where the ion mobility information is used to infer some details regarding the structure (either primary or higher-order) of the analyte.

The latter two strategies, analyte identification and structural measurement, are achieved by converting the ion mobility measurement (typically arrival time, also called “drift time”), to an ion-neutral collision cross section value (CCS), which represents a fundamental property of the analyte comparable across different laboratories and different instrumental platforms.¹⁵

Herein we measured the $^{TW}CCS_{N_2}$ of the compound **17x** in triplicate for the adducts $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M-H]^-$. We then calculated the theoretical $^{TW}CCS_{N_2}$ value for each of these adducts using a prototype model that is based on a Gradient Boosting machine learning algorithm¹. We obtained a total of 198 chemical descriptors via RDKit Library (<https://www.rdkit.org/>), of which only 70 are used for model training and prediction. Preliminary data from previous cross-validation of the model, we verified that the prediction capability leads to CCS percentage errors $< \pm 5\%$ in all cases (nested 10-fold cross-validation)¹⁶.

¹¹ R. Bouwmeester, L. Martens, S. Degroeve, K. Richardson, J.P.C. Vissers, Predicting ion mobility collision cross sections by combining conventional and data driven modelling, ASMS Proc. (2019) MP 366.

Because the prediction model is in a beta-validation stage, no additional references are available at present for the currently used algorithm.

The relative standard deviation of the measured CCS values was used as precision index, while the percentage CCS deviation of the measured values (mean, $n = 3$) to the theoretical ones was calculated in order to gain additional insights on the chemical structure of the candidate molecule.

Table - Summary table with measured and predicted CCS values using the TWIMS technology for the compound 2x. Relative Standard Deviation % (RSD%) of measured values, percentage deviation between measured and predicted CCS values (Δ CCS%).

	Adduct			
	$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	$[M-H]^-$
Arrival time (ms)	6.95	7.18	7.27	7.32
Measured $^{TW}CCS_{N_2}$ ($\text{\AA}^2$)	218.4	224.5	227.1	225.6
Predicted $^{TW}CCS_{N_2}$ ($\text{\AA}^2$)	222.4	226.0	225.3	222.0
RSD%	0.34	0.10	0.09	0.21
Δ CCS%	-1.8	-0.7	0.8	1.6

The RSDs are below 0.35% in all cases, which is an indication of very good precision of the experimental values.

The maximum Δ CCS% is -1.8%, whilst average $|\Delta$ CCS%| is 1.2%. All deviations are below the generally accepted threshold value for the CCS measurements ($\pm 2\%$)¹⁴, which adds more confidence in the assignment of the chemical structure for the compound **17x**.

It is worth noting that deviations higher than $\pm 1.5\%$ are usually encountered for protonated species with multiple equivalent protonation sites. The same concept stands for the $[M-H]^-$ species. This is caused by the presence of potential charge-isomers, which are not separated in the ion mobility dimension.