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Desarrollo de sistemas poliméricos de liberación prolongada de cannabinoides como estrategia para potenciar su actividad antitumoral

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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UNIVERSIDAD COMPLUTENSE DE MADRID
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DESARROLLO DE SISTEMAS POLIMÉRICOS DE LIBERACIÓN PROLONGADA DE CANNABINOIDES COMO ESTRATEGIA PARA POTENCIAR SU ACTIVIDAD ANTITUMORAL

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DEVELOPMENT OF CONTROLLED RELEASE POLYMERIC SYSTEMS CONTAINING CANNABINOIDS AS A STRATEGY TO INCREASE THEIR ANTITUMORAL EFFICACY

MEMORIA

Presentada para optar al grado de Doctor en Farmacia con Mención Internacional por la Universidad Complutense de Madrid por:

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ÍNDICE DE CONTENIDOS/INDEX

Índice de contenidos

RESUMEN/SUMMARY

Desarrollo de sistemas poliméricos de liberación prolongada de cannabinodies como estrategia para potenciar su actividad antitumoral

Introducción

En las últimas décadas, los cannabinoides han surgido como potenciales activos terapéuticos en el tratamiento del cáncer, al ser capaces de inhibir el crecimiento, migración e invasión de una gran variedad de tumores. Uno de los cannabinoides más prometedores es el cannabidiol (CBD), que carece de efectos psicoactivos.

De entre todas las neoplasias, el cáncer de mama y ovario representan un importante problema de salud en la población femenina. El cáncer de mama, el más frecuente en este colectivo, constituye una de las principales causas de mortalidad por cáncer en mujeres. El cáncer de ovario, aunque menos frecuente, es altamente letal al ser diagnosticado en estados avanzados. La actividad antitumoral del CBD en monoterapia ha sido evaluada por algunos autores en cáncer de mama, pero no existen datos en cáncer de ovario.

No obstante, a pesar del potencial que presentan los cannabinoides, su elevada liposolubilidad e inestabilidad dificultan el desarrollo de formulaciones eficaces de administración parenteral. La micro y nanoencapsulación constituyen potenciales estrategias tecnológicas para solventar estos problemas y mejorar su eficacia.

Por todo ello, el objetivo principal de la presente tesis doctoral ha sido diseñar, desarrollar y evaluar sistemas de liberación modificada de CBD eficaces para el tratamiento de cáncer de mama y de cáncer de ovario en monoterapia y/o en combinación con agentes antineoplásicos convencionales. Este objetivo en general se desglosa en los siguientes objetivos parciales:

1. Evaluar el potencial antitumoral del CBD en solución en monoterapia y en combinación con agentes quimioterápicos convencionales (paclitaxel, doxorubicina y cisplatino) en diferentes cultivos celulares de cáncer de mama y ovario y en modelos in ovo de cáncer de ovario.

2. Evaluar la estabilidad del CBD en solución frente a diversos factores: temperatura, presencia de oxígeno y luz.

3. Diseñar, desarrollar y caracterizar micropartículas poliméricas de PLGA cargadas con CBD para su administración parenteral.

4. Evaluar el potencial antitumoral de las micropartículas de CBD desarrolladas en monoterapia y en combinación con agentes quimioterápicos convencionales en cultivos celulares de cáncer de mama y ovario y en modelos in ovo de cáncer de ovario.

5. Diseñar, desarrollar y caracterizar nanopartículas poliméricas de PLGA cargadas con CBD para su administración intraperitoneal.

6. Evaluar el potencial antitumoral de las nanopartículas de CBD desarrolladas en monoterapia en cultivos celulares y en modelos in ovo de cáncer de ovario

Resultados

Los principales resultados obtenidos se desglosan a continuación:

1. El CBD en solución administrado en monoterapia inhibe in vitro la proliferación de células tumorales de ovario (SKOV-3, IGROV-1 y OAW-42) e in ovo el crecimiento de tumores de ovario desarrollados sobre la membrana corioalantoidea de embriones de pollo.

2. El CBD en solución potencia el efecto antitumoral del paclitaxel en cáncer de ovario. Por un lado, la administración previa de este cannabinoide sensibilizó las células SKOV-3 a la acción de paclitaxel. Por otro, la co-administración de ambos agentes también fue efectiva, observándose un efecto sinérgico entre ellos.

3. El CBD en solución potencia el efecto antitumoral del paclitaxel y la doxorrubicina en cáncer de mama. Por un lado, la administración previa de este compuesto sensibilizó las células MDA-MB-231 (células tumorales de mama triple negativas) y MCF-7 (células tumorales de mama positivas a los receptores de estrógenos) a la acción de doxorrubina y paclitaxel. Por otro lado, la co-administración de CBD en solución y paclitaxel o doxorrubicina demostró un efecto sinérgico en células MCF-7 y un efecto aditivo o sinérgico dependiente de la concentración de estos antineoplásicos en células MDA-MB-231.

4. La estabilidad del cannabidiol en solución se encuentra comprometida por la temperatura, la luz y la presencia de oxígeno. El disolvente utilizado influye significativamente en la estabilidad de este compuesto, siendo las soluciones acuosas altamente inestables.

5. El desarrollo de micropartículas poliméricas de CBD constituye una buena estrategia tecnológica para vehiculizar este compuesto. Las micropartículas de PLGA desarrolladas presentan un tamaño inferior a 25µm, adecuado para su administración parenteral, una elevada eficacia de encapsulación (superior al 90%) y una liberación controlada del activo durante más de un mes. Conservadas en refrigerador y protegidas de la luz, las micropartículas elaboradas con un ratio CBD: polímero 10:100 son física y químicamente estables durante al menos 1 año.

6. Las micropartículas de CBD elaboradas con un ratio CBD: polímero 10:100 presentan una actividad antiproliferativa prolongada durante al menos 10 días, tras una única administración, en células de cáncer de mama (MCF-7 y MDA-MB-231) y de cáncer de ovario (SKOV-3). Estas formulaciones de CBD son también útiles en terapia de combinación al potenciar el efecto antitumoral in vitro del paclitaxel y la doxorrubicina en todas las líneas celulares de cáncer de mama y de ovario mencionadas. Esto permitiría reducir la concentración de estos antineoplásicos necesaria para conseguir un mismo efecto antitumoral. Finalmente, estas micropartículas potencian el efecto antitumoral del paclitaxel en los tumores de ovario generados en la membrana corioalantoidea de embriones de pollo.

7. El desarrollo de nanopartículas poliméricas constituye una buena estrategia para vehiculizar el CBD a tumores de ovario. Las nanopartículas de PLGA desarrolladas presentan un tamaño inferior a 250nm, una elevada eficacia de encapsulación y una liberación controlada del CBD durante 96 horas. Estas formulaciones son eficazmente inernalizadas por células SKOV-3 a partir de las 2 horas de incubación.

8. Las nanopartículas de CBD desarrolladas inhiben la proliferación de las células de cáncer de ovario SKOV-3 y el crecimiento de los tumores de ovario generados en la membrana de embriones de pollo.

Conclusión

Las micropartículas y las nanopartículas poliméricas de CBD desarrolladas suponen una herramienta útil para aumentar la eficacia antitumoral del cannabidiol frente a células de cáncer de mama y de ovario, en monoterapia o en terapia de combinación con paclitaxel o doxorrubicina.

Development of controlled release polymeric systems containing cannabinoids as a strategy to increase their antitumoral efficacy

Introduction

In the last decades, cannabinoids have emerged as a promising therapeutic tool in cancer treatment due to their ability to inhibit the proliferation, migration and invasion of several tumour types. Cannabidiol (CBD), that lacks of psychoactive effects, is one of the most promising cannabinoids.

Breast and prostate carcinomas are major health problems in women worldwide. Breast cancer is the most frequent tumour and one of the leading causes of cancer death in female population. Ovarian cancer, although less frequent, is a highly lethal disease. The antitumor activity of CBD in monotherapy has been demonstrated in breast cancer. However, it has not been reported in ovarian tumours.

In spite of the therapeutic potential of cannabinoids, they show a high liphophilicity and several stability problems that hinder the development of effective parenteral formulations. Micro and nanoencapsulation are promising technological strategies to overcome these challenges and improve CBD antitumor efficacy.

Against this background, the main aim of this thesis is to design, develop and characterise controlled release systems of cannabidiol to improve its antitumor efficacy in monotherapy or in combination with conventional antineoplastics in breast and ovarian cancer. This global aim can be broken down into three specific aims:

1. To evaluate the antitumor potential of CBD in solution in monotherapy or in combination with conventional antineoplastic agents in breast and ovarian cancer cell lines and *in ovo* models of ovarian cancer.

2. To evaluate the stability of CBD against several factors: temperature, light and oxygen.

3. To design, develop and characterise parenteral PLGA polymeric microparticles loaded with CBD.

4. To evaluate the antitumor activity of CBD microparticles in monotherapy or in combination with paclitaxel and doxorubicin in breast and ovarian cancer cell line and *in ovo* models of ovarian cancer.

5. To develop, design and characterised PLGA polymeric nanoparticles loaded with CBD for intraperitoneal administration.

6. To evaluate the antitumor activity of CBD nanoparticles in monotherapy using *in vitro* and *in ovo* models of ovarian cancer.

Results

The main results can be summarized in the following highlights:

1. CBD in solution as monotherapy inhibits *in vitro* the proliferation of ovarian cancer cells **(**SKOV-3, IGROV-1 y OAW-42) and *in ovo* the growth of SKOV-3 derived tumours formed on the chorioallantoic membrane of the chick fertilized eggs.

2. CBD in solution potentiates the antitumor effect of paclitaxel in ovarian cancer cells. On the one hand, the pre-adminsitration of this cannabinoid sensitizes SKOV-3 cells to paclitaxel. On the other hand, the co-adminsitration of both actives was also effective, showing a synergistic effect.

3. CBD in solution potentiates the antitumor effect of paclitaxel and doxorubicin in breast cancer cells. On the one hand, the pre-administration of this cannabinoid sensitizes MDA-MB-231 (triple negative breast cancer cells) and MCF-7 (oestrogen receptor positive breast cancer cells) to doxorubicin and paclitaxel. On the other hand, the co-administration of CBD in solution plus paclitaxel or doxorubicin shows synergism in MCF-7 cells and an additive or synergistic effect in MDA-MB-231 cells, depending on the concentration of these antineoplastics.

4. The stability of CBD in solution is influenced by several factors such as temperature, light and oxygen. The solvent affects significantly the stability of this compound, being aqueous solutions highly unstable.

5. The microencapsulation of CBD into PLGA microparticles is a good strategy to vehiculizate this active. PLGA-microparticles developed show a mean particle size lower than 25 µm, that is suitable for parenteral administration, a high encapsulation efficiency (above 90%) and a controlled CBD release for more than one month. Stored into the refrigerator and protected from light microparticles prepared with a CBD:polymer ratio of 10: 100 are physical and chemically stable for at least one year.

6. Microparticles prepared with a CBD:polymer ratio of 10:100 and administered as monotherapy show an extended antiproliferative activity for at least 10 days after a single administration in breast (MCF-7 y MDA-MB-231) and ovarian (SKOV-3) cancer cells. This formulation is also useful in combination with paclitaxel and doxorubicin, potentiating the *in vitro* activity of these antineoplastics in ovarian and breast cancer cells. This would allow the reduction of their doses to get the same antiproliferative effect. CBD microparticles also enhance the antitumor effect of paclitaxel *in ovo* against ovarian tumours.

7. The nanoencapsulation of cannabidiol into PLGA nanoparticles is a good strategy to vehiculizate this agent to the therapeutic target. The nanoparticles developed show a particle size below 250 nm, a high encapsulation efficiency and a controlled drug release for 96 hours. These nanoparticles are efficiently internalized by SKOV-3 cells after 2 hours of incubation.

8. CBD nanoparticles developed inhibit *in vitro* the proliferation of SKOV-3 ovarian cancer cells and *in ovo* the growth of SKOV-3 derived tumours formed on the chorioallantoic membrane of the chick fertilized eggs.

Conclusion

Polymeric micro and nanoparticles loaded with CBD are good tools to increase the antitumor efficacy of cannabidiol against breast and ovarian cancer cells either as monotherapy or as a part of combination with paclitaxel or doxorubicin.

INTRODUCCIÓN/INTRODUCTION

El término "cannabinoides" inicialmente se utilizaba para designar a un grupo de compuestos terpenofenólicos presentes en las plantas del género Cannabis, especialmente *Cannabis sativa* y *Cannabis indica*; y cuyos extractos se han utilizado en terapéutica desde la antigüedad para el tratamiento de diversas patologías como neuralgias, convulsiones y dolor [1-3]. Sin embargo, debido a sus propiedades psicoactivas y a la dificultad de preparar formulaciones estandarizadas, con el consiguiente riesgo de sobredosificación, los cannabinoides se retiraron del arsenal terapéutico en la primera mitad del siglo XX [1, 4].

Los cannabinoides más relevantes son el Δ^9-tetrahidrocannabinol (Δ^9-THC), el cannabinoide más abundante y el principal responsable de los efectos psicoactivos del cannabis; y el cannabidiol (CBD), el segundo en abundancia y carente de efectos psicótropos. En un principio, su elevada liposolubilidad llevó a pensar que ejercían su actividad modificando los fosfolípidos de las membranas plasmáticas. Sin embargo, el aislamiento y caracterización de receptores específicos de cannabinoides demostraron que estos compuestos ejercían su efecto a través de receptores de membrana. Este descubrimiento llevó a la búsqueda de ligandos endógenos que los activaran, los llamados cannabinoides endógenos o endocannabinoides; que junto con las enzimas responsables de su síntesis y degradación, y los receptores específicos de cannabinoides constituyen el sistema cannabinoide endógeno (SCE). Además, han surgido numerosos cannabinoides de síntesis. Por ello, en la actualidad el término "cannabinoides" designa no sólo a los cannabinoides naturales, sino también a los endocannabinoides y los análogos sintéticos de ambos grupos.

Se ha demostrado que el SCE interviene en numerosas funciones fisiológicas, tales como el metabolismo energético, control del apetito, naúseas y vómitos, memoria, sistema inmune y analgesia entre otras [5-7]. Además, alteraciones en el SCE se han relacionado con ciertas patologías, como enfermedades neurodegenerativas (Parkinson, Alzheimer, esclerosis múltiple o enfermedad de Huntington), anorexia o cáncer. De esta manera, el SCE se ha convertido en una prometedora diana terapéutica en todos estos trastornos, y por consiguiente, los cannabinoides en potenciales agentes medicinales [8-10]. De hecho, en las últimas décadas han surgido como una prometedora herramienta terapéutica, existiendo incluso diversas formulaciones comercializadas para el

tratamiento de la espasticidad asociada a esclerosis múltiple, las naúseas y vómitos asociados a la quimioterapia y la anorexia y el dolor relacionados con cáncer**. El potencial uso terapéutico de los cannabinoides está recogido en la publicación 1.**

Entre todas las patologías, una de las áreas terapéuticas donde los cannabinoides presentan un importante potencial es la terapia antitumoral. En cáncer, los cannabinoides no sólo son útiles en terapia adyuvante, para combatir las náuseas, vómitos, dolor y pérdida de apetito relacionados con el cáncer y los tratamientos quimioterápicos, sino que en los últimos años son numerosos los estudios sobre su eficacia antitumoral per se debido a su capacidad para inhibir la proliferación, migración e invasión de las células tumorales, así como para ejercer un efecto antiangiogénico**. En la publicación 2 se describen las alteraciones del SCE en cáncer y el potencial uso de los cannabinoides como novedosos agentes terapéuticos en este patología.**

Uno de los cannabinoides más prometedores a este nivel es el CBD, el cual carece de propiedades psicoactivas y presenta una toxicidad muy baja. El CBD ha demostrado inhibir *in vitro* e *in vivo* el crecimiento de diversos tipos de tumores, tales como gliomas, cáncer de mama, próstata y pulmón, y neoplasias gastrointestinales [11, 12]. Así mismo, ha demostrado ser útil como terapia de combinación, incrementando la eficacia de los tratamientos antitumorales. Por ejemplo, se ha demostrado que este compuesto aumenta la citotoxicidad de vinblastina y vincristina en modelos *in vitro* de leucemia, de la temozolomida en modelos *in vivo* de gliomas y la acción de la radioterapia en cáncer de próstata, colón y glioma [13-15]. De hecho diversos ensayos clínicos se están llevando a cabo actualmente para evaluar la eficacia y seguridad del CBD en combinación con temozolomida en gliomas y con radioterapia en mieloma múltiple y tumores gastrointestinales (NCT03246113, NCT0360764, NCT03529448 and NCT03246113).

El cáncer mama y ovario son dos de las principales neoplasias femeninas. El cáncer de mama es la neoplasia más común en mujeres, con 2.08 millones de casos y 627.000 muertes globales en el 2018. Aunque en las últimas décadas el uso de técnicas de diagnóstico precoz y la aparición de novedosas terapias han mejorado significativamente la tasa de supervivencia en este tipo de tumores, esta patología continúa siendo una de las principales causas de mortalidad por cáncer en mujeres. En cuanto al cáncer de ovario, es el tumor ginecológico más común, con 295.414 casos y

184.799 muertes globales en el año 2018 [16]. Debido a sus características asintomáticas y la falta de tests de diagnóstico precoz, en el 85% de los casos es diagnosticado en estadios avanzados de la enfermedad, lo que dificulta su tratamiento y aumenta considerablemente el riesgo de muerte. De hecho, presenta una tasa de supervivencia de 5 años en torno al 45% [17, 18]. De esta manera, tanto el cáncer de mama como de ovario constituyen importantes problemas de salud.

La actividad antitumoral del CBD en cáncer de mama está ampliamente establecida *in vitro*, en modelos celulares de mama e *in vivo* en modelos tumorales xenográficos desarrollados en ratones [19-21]; siendo de hecho uno de los tumores donde este cannabinoide presenta una mayor acción. **En la publicación 3 se describe el potencial uso de los cannabinoides en el tratamiento de cáncer de mama**. Sin embargo, aún no se han publicado datos sobre la actividad antitumoral de los cannabinoides en células de cáncer de ovario. No obstante, en tumores de ovario invasivos se ha detectado un aumento de los receptores específicos de cannabinoides. Esta sobreexpresión se ha relacionado con una mayor agresividad [22]. Esto sugiere la implicación del SCE en el desarrollo y avance de cáncer de ovario y el posible potencial anticancerígeno de los cannabinoides en este tipo de tumores.

El uso de nanosistemas como trasportadores de agentes antitumorales constituye una de las principales estrategias disponibles hoy en día para mejorar y solventar los problemas de los tratamientos quimioterápicos convencionales. Por un lado, permite vehiculizar compuestos lipófilos, eliminando la necesidad de usar disolventes orgánicos. Por otro lado, debido al efecto de permeabilidad y retención que caracteriza a los tumores, estos nanosistemas tienden a acumularse a nivel tumoral, disminuyendo los efectos asociados a los agentes antitumorales y aumentando su eficacia [26-28]**. En la publicación 4 se describe el uso de nanomedicinas en el tratamiento de cáncer de mama.**

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Ana Isabel Fraguas-Sánchez &Ana Isabel Torres-Suárez

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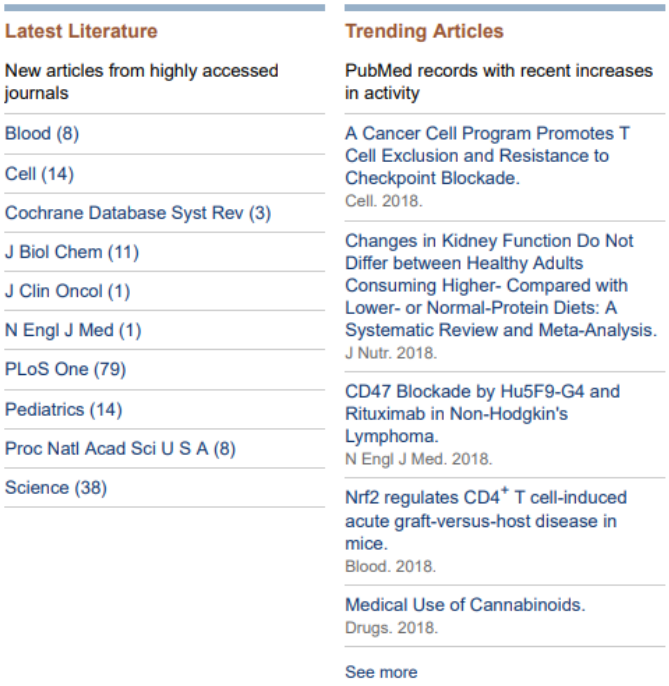

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REVIEW ARTICLE

Medical Use of Cannabinoids

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Abstract
Cannabinoid receptors, endocannabinoids and the enzymes responsible for their biosynthesis and degradation constitute the endocannabinoid system. In recent decades, the endocannabinoid system has attracted considerable interest as a potential therapeutic target in numerous pathological conditions. Its involvement in several physiological processes is well known, such as in energy balance, appetite stimulation, blood pressure, pain modulation, embryogenesis, nausea and vomiting control, memory, learning and immune response, among others, as well as in pathological conditions where it exerts a protective role in the development of certain disorders. As a result, it has been reported that changes in endocannabinoid levels may be related to neurological diseases such as Parkinson's disease, Huntington's disease, Alzheimer's disease and multiple sclerosis, as well as anorexia and irritable bowel syndrome. Alterations in the endocannabinoid system have also been associated with cancer, affecting the growth, migration and invasion of some tumours. Cannabinoids have been tested in several cancer types, including brain, breast and prostate cancers. Cannabinoids have shown promise as analgesics for the treatment of both inflammatory and neuropathic pain. There is also evidence for a role of the endocannabinoid system in the control of emotional states, and cannabinoids could prove useful in decreasing and palliating post-traumatic stress disorder symptoms and anxiolytic disorders. The role of the endocannabinoid system in addictions has also been examined, and cannabinoids have been postulated as alternative and co-adjuvant treatments in some abuse syndromes, mainly in ethanol and opioid abuses. The expression of the endocannabinoid system in the eye suggests that it could be a potential therapeutic target for eye diseases. Considering the importance of the endocannabinoid system and the therapeutic potential of cannabinoids in this vast number of medical conditions, several clinical studies with cannabinoid-based medications are ongoing. In addition, some cannabinoid-based medications have already been approved in various countries, including nabilone and dronabinol capsules for the treatment of nausea and vomiting associated with chemotherapy, dronabinol capsules for anorexia, an oral solution of dronabinol for both vomiting associated with chemotherapy and anorexia, a Δ^9-tetrahydrocannabinol/cannabidiol oromucosal spray for pain related to cancer and for spasticity and pain associated with multiple sclerosis, and an oral solution of cannabidiol for Dravet and Lennox–Gastaut syndromes. Here, we review the available efficacy, safety and tolerability data for cannabinoids in a range of medical conditions.

1 Introduction

Initially, the term 'cannabinoids' was used to designate a group of specific compounds present in the *Cannabis sativa* plant, which is known for its psychoactive effects and which has been used in medicine since ancient times [1, 2]. For example, in traditional Chinese medicine, cannabis was used for the treatment of asthma, malaria and gout, and in India for neuralgias, convulsions and migraines [3, 4]. In the nineteenth century, the use of cannabis became very popular in Europe and USA, where ethanolic extracts of cannabis (known as cannabis tincture) were also utilised to treat various disorders such as convulsions in infants, tetanus, cholera and rabies, among others. However, these disappeared from therapeutic use in the first half of the twentieth century owing to an inability to prepare standardised cannabis preparations, which resulted in the risk of producing over- or under-dosed formulations [4–7].

The most relevant cannabinoids are Δ^9-tetrahydrocannabinol (Δ^9-THC), the most abundant cannabinoid and the one mainly responsible for the psychoactive properties of cannabis, and cannabidiol (CBD), the second

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Publicación 2: Insights into the effects of the endocannabinoid system in cancer: a review

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REVIEW ARTICLE

Insights into the effects of the endocannabinoid system in cancer: a review

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In the last few decades, the endocannabinoid system has attracted a great deal of interest in terms of its applications to clinical medicine. In particular, its applications in cancer probably represent one of the therapeutic areas with most promise. On the one hand, expression of the endocannabinoid system is altered in numerous types of tumours, compared to healthy tissue, and this aberrant expression has been related to cancer prognosis and disease outcome, suggesting a role of this system in tumour growth and progression that depends on cancer type. On the other hand, cannabinoids exert an anticancer activity by inhibiting the proliferation, migration and/or invasion of cancer cells and also tumour angiogenesis. However, some cannabinoids, at lower concentrations, may increase tumour proliferation, inducing cancer growth. Enough data has been provided to consider the endocannabinoid system as a new therapeutic target in cancer, although further studies to fully establish the effect of cannabinoids on tumour progression are still needed.

Abbreviations

2-AG, 2-arachidonoylglycerol; ABCP, breast cancer resistance protein; AM-356, methanandamide; CBD, cannabidiol; ECS, endocannabinoid system; HER-2, human epidermal growth factor receptor; MDR1, multidrug resistance protein 1; THC, Δ^9-tetrahydrocannabinol

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Introduction

Nowadays, the term "endocannabinoid system" (ECS) comprises cannabinoid (CB) receptors, endogenous cannabinoids, also called endocannabinoids, and the enzymes involved in their biosynthesis, transport and degradation. The most important endocannabinoids are N-arachidonoylethanolamine (AEA), also called anandamide, and 2-arachidonoylglycerol (2-AG), which are derived from arachidonic acid and synthesized on demand. The main synthesis and degradation pathways are shown in Figure 1 (Fraguas-Sanchez *et al.*, 2016; Schurman and Lichtman, 2017). Other minor endocannabinoids have also been identified, such as oleamide, virodhamine and 2-arachidonyl glyceryl ether, also known as noladin-ether.

The CB receptors belong to the superfamily of GPCRs and two distinct receptors have been identified and characterized to date, CB_1 and CB_2, exhibiting around 44% homology. The CB_1 receptor (first cloned from a rat brain in 1990) has an extended distribution, and although it is mostly expressed in the CNS, it is also found in peripheral nerve terminals and extraneuronal tissues, including the vascular endothelium, adipose tissue, lungs, liver, spleen, kidneys, uterus, prostate, testis and stomach. The CB_2 receptor (isolated for the first time in 1993 from human promyelocytic HL-60 cells) has a more localized distribution, being found predominantly in the immune system (tissues and cells). Nonetheless, it is also detected in the CNS, primarily after certain circumstances such as inflammation (Console-Bram *et al.*, 2012; Kendall and Yudowski, 2016). Finally, some endocannabinoid effects are mediated by non-CB receptors, particularly the orphan receptors GPR55 and GPR18, which have also been postulated to be members of the ECS (Okuno and Yokomizo, 2011; Pyszniak *et al.*, 2016; Morales and Reggio, 2017).

Since the discovery of the ECS, it has attracted a great deal of interest in therapeutics due to its involvement in several physiopathological processes including energy balance, appetite stimulation, nociception, embryogenesis, immune response and control of nausea and vomiting (Cunha *et al.*, 2011; Katchan *et al.*, 2016; Laprairie *et al.*, 2017). Alterations of ECS expression have been found in many different disease conditions including cancer and neurological disorders such as Parkinson's disease, Huntington's disease and multiple sclerosis (Hasenoehrl *et al.*, 2016; Ligresti *et al.*, 2016; Bridgeman and Abazia, 2017). This review focuses on the role of the ECS in cancer disease progression and as a novel therapeutic target for anticancer treatments.

Endocannabinoid system expression in cancer

Cannabinoid receptors

The expression of both CB_1 and CB_2 receptors is altered in numerous types of tumours (summarized in Figure 2) and has been related to cancer prognosis. The altered expression

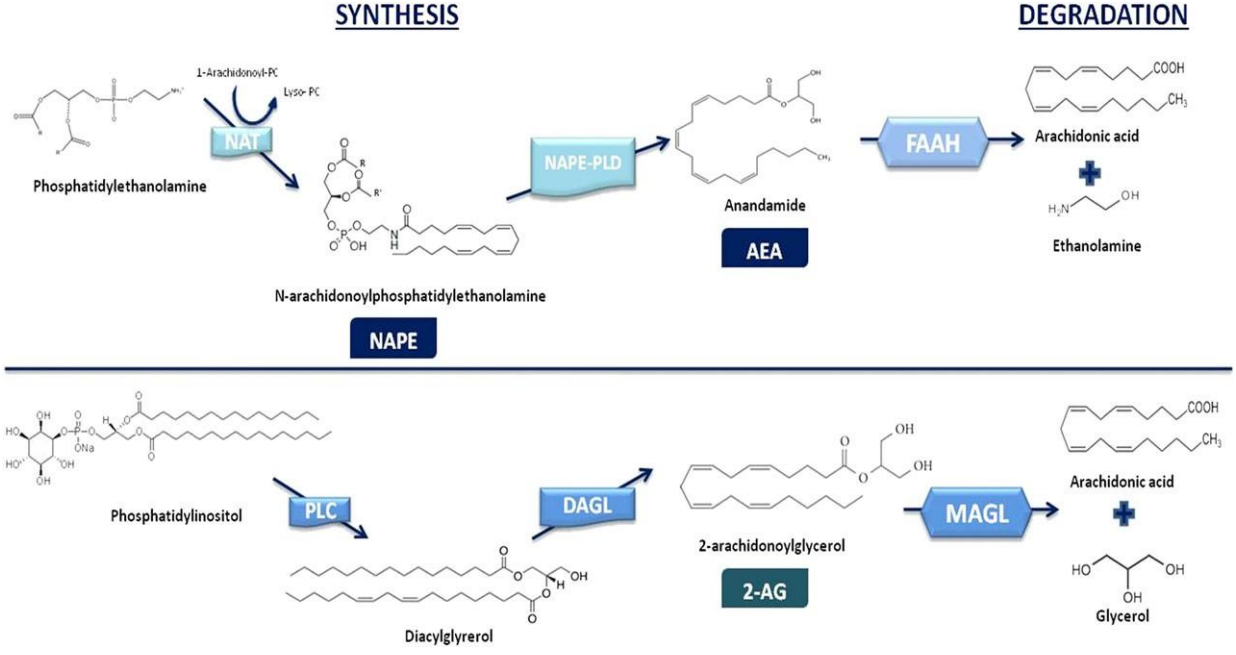

Figure 1

Diagram showing the main biosynthetic and degradation pathways of both AEA and 2-AG. Anandamide is synthesized *via* a phospholipase-D (NAPE-PLD), which converts N-arachidonoylphosphatidylethanolamine (NAPE), formed by the transfer of an arachidonoyl group to phosphatidylethanolamine by the action of N-acetyltransferase (NAT), to AEA. 2-AG is formed from diacylglycerol, *via* diacylglycerol lipase (DGL). Diacyl- glycerol is synthesized from phosphatidylinositol by the action of a PLC. In terms of degradation pathways, FAAH and MAGL are the most important enzymes responsible for inactivation of AEA and 2-AG respectively. However, other pathways also participate in their degradation in- cluding lipoxygenases, cytochrome P450, COX-2 and the domains 6 and 12 of serine lipases a/ß hydrolases.

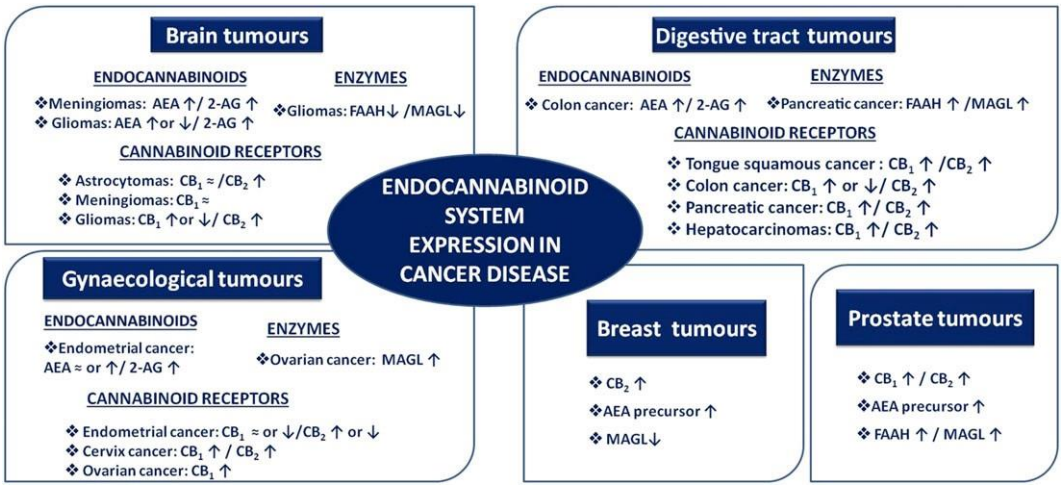

Figure 2

Altered expression of endocannabinoid system in brain, breast, digestive tract, gynaecological and prostate carcinomas.

is correlated with positive and negative survival indicators, depending on the origin of the cancer.

In brain carcinomas, both CB_1 and CB_2 receptor expression is different from that in healthy tissue. Although, in astrocytomas and meningiomas, no differences in the expressionof CB_1 receptors have been detected, some studies have reported that in glioblastoma multiforme, their expression is lower (De Jesus *et al.*, 2010). However, recent studies have demonstrated opposite results, showing CB_1 receptors to be highly expressed in high-grade gliomas compared to low-grade tumours and healthy brain samples (Wu *et al.*, 2012). Interestingly, these results have also been found in samples of low-grade paediatric glioma, where overexpression has been associated with tumour regression, due to the apoptosis and cell cycle arrest induced by activation of CB_1 receptors by endocannabinoids (Sredni *et al.*, 2016). Concerning CB_2 receptors, Schley *et al*. (2009) revealed an up-regulation of these receptors in the endothelial cells of blood vessels of glioblastoma tissues. Finally, Wu *et al*. reported an overexpression of both CB_1 and CB_2 receptors in gliomas compared to the healthy brain. Whereas up- regulation of CB_1 receptors was associated with low-grade tumours, overexpression of CB_2 receptors was related to high-grade gliomas (Wu *et al.*, 2012).

In breast tumours, which are mainly grouped into three categories depending on the molecular profile, hormone-receptor positive, human epidermal growth factor receptor 2 (HER-2) and triple negative, an increased expression of CB_2 receptors has been detected. More than 90% of HER-2-positive tumours overexpressed this receptor (Caffarel *et al.*, 2010) and this finding was related to a poor prognosis, probably due to the activation of HER-2 pro- oncogenic pathways (Perez-Gomez *et al.*, 2015). However, Elbaz *et al*. (2016) reported that the expression of these receptors in oestrogen-receptor-positive and oestrogen- receptor-negative mammary tumours was related to a better prognosis. In fact, they suggested CB_2 receptors as a potential therapeutic target for treating breast cancer metastases. They demonstrated *in vitro* and *in vivo* (using mice orthotopic models) that the activation of CB_2 receptors decreased the migration and invasion of oestrogen-positive and -negative breast cancer cells, suppressing epidermal growth factor and insulin-like growth factor tumourigenic pathways. Similar results were also found by Murase *et al*. (2014) who reported in mice models of breast cancer that the activation of CB_2 receptors by O-1665, a resorcinol compound analogue of cannabidiol (CBD), reduced gene expression of the Id-1 protein, associated with breast cancer metastases, and also increased the survival rate in advanced stages of carcinoma.

In prostate carcinomas, expressions of both CB_1 and CB_2 receptors were increased compared to the normal prostatic tissue (Orellana-Serradell *et al.*, 2015), and the overexpres- sion of CB_1 receptors has been associated with a higher Gleason score and metastasis incidence, being a negative marker of disease outcome. (Chung *et al.*, 2009; Cipriano *et al.*, 2013).

With respect to cancers of the digestive tract, the overexpression of CB receptors has also been related to cancer prognosis. In this sense, in tongue squamous tumour cells, both CB_1 and CB_2 receptors were overexpressed and this up-regulation has been postulated to be an indicator of cancer outcome, with high levels of CB_1 receptors being a better marker of disease survival than the overexpression of CB_2, or of both CB receptors (Theocharis *et al.*, 2016). Similarly, in colon tumours, both CB receptors were detected. While some studies reveal that CB_1 receptors were down-regulated in colon cancer compared to normal mucosa (Cianchi *et al.*, 2008), others indicate that some tumours had high levels of this CB receptor, and it was an indicator of a poor disease outcome in patients with stage II microsatellite-stable (Gustafsson *et al.*, 2011) or stage IV (Jung *et al.*, 2013) tumours. Targeting the CB_1 receptors could be a good strategy to increase the efficacy of anticancer treatments. Thus their inactivation with rimonabant, an inverse agonist at CB_1 receptors. decreased the growth of colon cancer tumours *in vivo* by inhibiting the canonical Wnt/β catenin- pathway through the inhibition of p300-histone

acetyltransferase activity (Proto *et al.*, 2017). In fact, a recent paper has reported, using 3D cultures of colon cancer cells that this compound acted specifically in tumour cells. Whereas in HTC_{116} cells, it demonstrated a strong synergism with 5-fluorouracil in overcoming tumour resistances, in GTG7 cells, it slightly increased 5-fluorouracil efficacy and showed an antagonism with oxaliplatin, with further stud- ies being necessary (Fiore *et al.*, 2017). As for CB_2 receptors, an up-regulated expression has also been associated with lower survival (Martinez-Martinez *et al.*, 2015). In pancreatic cancer, expression of both CB_1 and CB_2 receptors was increased compared to the normal pancreas (Carracedo *et al.*, 2006), and CB_1 up-regulation has been related to a worse cancer prognosis (Michalski *et al.*, 2008). In contrast, in hepatocarcinoma, where CB_1 and CB_2 receptors were also over-expressed comparing to normal liver (307- and 5.44-fold respectively) (Suk *et al.*, 2016), high levels of both receptors have been associated with better disease-free survival rates (Xu *et al.*, 2006).

In several gynaecological tumours, the expression of CB receptors was also altered. In this context, studies performed in biopsies of patients with endometrial cancer showed that CB_2 receptors, which are barely expressed in the healthy endometrial tissue, were up-regulated, but no significant differences in CB_1 receptor expression were detected (Guida *et al.*, 2010). However, other researchers found low levels of both CB_1 and CB_2 receptors in endometrial carcinoma (Ayakannu *et al.*, 2014). On the other hand, in cervix cancer, high levels of both CB were detected (Contassot *et al.*, 2004a). In invasive ovarian tumours, only higher levels of CB_1 were found and this up-regulation was related to the invasiveness of ovarian cancer (Messalli *et al.*, 2014).

Finally, in melanoma cells (Blazquez *et al.*, 2006) and lung carcinomas, CB_1 and CB_2 receptors are present in 24% and 55% of patients with non-small cell lung cancer re- spectively (Preet *et al.*, 2011). In leukaemia and lymphomas, the opposite results have been reported. While some murine leukaemia cell lines expressed both CB_1 and CB_2 receptors, human leukaemia cells only expressed CB_2 receptors (high levels) (McKallip *et al.*, 2002). A high CB_1 receptor expres- sion has also been found in Hodgkin lymphoma cells (Benz *et al.*, 2013).

Table 1 summarizes the alterations in expression of CB_1 and CB_2 receptors in several types of tumours.

Endocannabinoid levels

The levels of endocannabinoids, especially AEA and 2-AG, are also abnormal in some tumours, compared with normal tissues. Regarding brain tumours, conflicting information has been documented. While some authors have reported that AEA levels were lower in gliomas compared to non-tumour tissues (Maccarrone *et al.*, 2001; Wu *et al.*, 2012), others have detected higher levels of this endocannabinoid in gliomas and also in meningiomas (Petersen *et al.*, 2005). With respect to 2-AG levels, they were up-regulated in both kinds of brain tumours (Petersen *et al.*, 2005; Wu *et al.*, 2012). In prostate tumours, increased levels of AEA have also been reported (Schmid *et al.*, 2002). However, in breast carcinoma, AEA levels were not increased. Nevertheless, high levels of the AEA precursor, *N*-acylphosphatidylethanolamine, have been detected. In colon cancer, several authors have also reported that levels of both AEA and 2-AG were increased, threefold and twofold respectively (Ligresti *et al.*, 2003). Interestingly, especially AEA levels were increased in lymphatic metastasis (Chen *et al.*, 2015). In patients with endometrial carcinoma, elevated levels of 2-AG have also been shown compared to healthy tissues, but with respect to AEA levels, the opposite results were detected. While some authors showed no significant differences in AEA levels (Guida *et al.*, 2010), in other studies, high levels have been reported (Schmid *et al.*, 2002). Finally, elevated levels of both AEA and 2-AG have been found in pituitary adenomas, correlated with the presence of CB_1 receptors. While in CB_1 receptor-positive samples, higher endocannabinoid levels have been detected, in sam- ples with a low expression of CB_1, endocannabinoid levels were lower (Pagotto *et al.*, 2001).

Endocannabinoid degrading enzymes

Taking into account that, in some tumours, endocannabinoid levels were increased compared to normal tissues, an inhibition of the main enzymes responsible for their degradation could be expected. This happens in gliomas, where the expression and the activity of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) enzymes were reduced, compared with nor- mal brain tissue (Wu *et al.*, 2012). However, in some studies, an increase of these enzymes has been reported. For example, in breast ductal carcinomas, MAGL expression was increased (Gjerstorff *et al.*, 2006), and in prostate tumours, high levels of AEA and its major degradative enzyme were detected compared to normal prostate tissue (Endsley *et al.*, 2008). FAAH overexpression has been associated with cancer invasion and disease outcome (Thors *et al.*, 2010). In androgen-independent prostate tumours, elevated levels of MAGL have also been found (Nithipatikom *et al.*, 2005). The expression and the activity of the MAGL enzyme were also increased in invasive ovarian and melanoma tumours, and interestingly, the up-regulation of MAGL in ovarian, melanoma and non-aggressive prostate cancer was associated with higher tumour cell migration and invasion (Van Dross *et al.*, 2013; Qin and Ruan, 2014). Nevertheless, the opposite results were found in pancreatic ductal adenocarcinomas, where both FAAH and MAGL enzymes were overexpressed and related to a good prognosis (Michalski *et al.*, 2008).

Table 2 summarizes the levels of endocannabinoids and expression of endocannabinoid degradative enzymes in several cancer types.

Non-cannabinoid receptors: GPR55

Levels of the orphan GPR55 were associated with high proliferation rates of tumour cells. In this context, *in vitro* and *in vivo* studies undertaken in several tumour models, including pancreas, breast, brain (Andradas *et al.*, 2011; Andradas *et al.*, 2016), prostate, ovary (Pineiro *et al.*, 2011) and skin carcinomas (Perez-Gomez *et al.*, 2013) reported that GPR55 receptors were implicated in the proliferation and progression of cancer. This may be attributed to GPR55 activation by the agonist L-α-lysophosphatidylinositol (LPI) (Ford *et al.*, 2010; Hofmann *et al.*, 2015) and, in patients with ovarian cancer, elevated levels of LPI were detected in plasma (Xiao *et al.*, 2001; Sutphen *et al.*, 2004).

Table 1

Expression of CB_1 and CB_2 receptors in several carcinomas, compared with that in normal tissue.

	Cancer type	CB_1	CB_2	Relation to disease outcome	Reference
Brain tumours	Astrocytomas	≈	↑	–	(De Jesus *et al.,* 2010)
	Meningiomas	≈	–	–	
	Gliomas	↑	↑	• CB_1 receptor overexpression is associated with tumour regression in glioblastoma and paediatric-low-gliomas • CB_2 receptors; higher levels are related to tumour grade	(Schley *et al.,* 2009, Wu *et al.,* 2012, Sredni *et al.,* 2016)
–	Breast	–	↑	• CB_2 receptors overexpression in more than 90% of HER-2 positive tumours; is a negative prognosis marker. • CB_2 receptor overexpression is a good prognosis marker in oestrogen negative and positive tumours.	(Caffarel *et al.,* 2010, Perez-Gomez *et al.,* 2015)
	Prostate	↑	↑	• CB_1 receptor overexpression is a good prognosis marker	(Chung *et al.,* 2009, Cipriano *et al.,* 2013, Orellana-Serradell *et al.,* 2015)
Digestive tract tumours	Tongue squamous	↑	↑	• CB_1 receptor overexpression is a positive marker of disease outcome	(Theocharis *et al.,* 2016)
	Colon	↑ or ↓	↑	• CB_1 and CB_2 receptor overexpression is related to a poor disease outcome.	(Cianchi *et al.,* 2008, Gustafsson *et al.,* 2011, Jung *et al.,* 2013, Martinez-Martinez *et al.,* 2015)
	Pancreas	↑	↑	• CB_1 receptor overexpression is a negative marker of disease outcome	(Carracedo *et al.,* 2006, Michalski *et al.,* 2008)
	Hepatocarcinoma	↑	↑	• CB_1 and CB_2 receptor overexpression is a good indicator of survival	(Xu *et al.,* 2006, Suk *et al.,* 2016)
Gynaecological tumours	Endometrial	≈ or ↓	↑ or ↓	–	(Guida *et al.,* 2010, Ayakannu *et al.*)
	Cervix	↑	↑	–	(Contassot *et al.,* 2004a)
	Ovary	↑	–	• CB_1 receptor up-regulation is associated with a higher tumour aggressiveness	(Messalli *et al.,* 2014)
–	Hodgkin lymphoma	↑	–	–	(Benz *et al.,* 2013)

In the Table, ≈ denotes a similar expression, ↑ higher levels and ↓ lower levels of expression compared with normal tissues

The overexpression of these receptors has also been related to cancer disease aggressiveness and a poor prognosis. For example, in glioblastoma, increased levels of GPR55 were correlated with higher tumour grades and a lower survival rate, and provided a marker of a negative cancer prognosis. This relationship has also been shown in breast and pancreatic tumours (Andradas *et al.,* 2011). Hasenoehrl *et al*. (2018) reported a pro-tumour activity of these recep- tors in a recent study undertaken in mouse models of colon carcinoma. Although no effect on cell proliferation was detected with an agonist or antagonist of GPR55 receptors, they interfered with the composition of the leukocyte population during carcinogenesis, triggering a tumour-promoting micro-environment with the increase of tumourigenic factors (COX-2, STAT3 and NF-κB). Knockout mice exhibited reduced levels. In samples obtained from colon cancer patients, a correlation between high levels of GPR55 and a decrease in relapse-free survival has been also reported, supporting the implication of GPR55 in carcinogenesis (Hasenoehrl *et al.,* 2018).

Interestingly, some studies have reported that the heterodimerization between GPR55 and CB_2 receptors (Balenga *et al.,* 2014) may modulate, in breast and brain tumours, the antitumour activity of some cannabinoids.

Table 2

Expression of the major endocannabinoids and their major degradative enzymes in several carcinomas, compared with normal tissue.

	Cancer type	AEA	2-AG	FAAH	MAGL	Observations	References
Brain cancer	Meningioma	↑	↑	–	–	–	(Maccarrone *et al.*, 2001, Petersen *et al.*, 2005, Wu *et al.*, 2012)
	Glioma	↑ or ↓	↑	↓	↓	–	
–	Pituitary adenomas	↑	↑	–	–	Correlation with CB_1 receptor expression	(Pagotto *et al.*, 2001)
	Breast	Precursor ↑	–	–	↑	Higher levels of AEA precursor have been detected	(Gjerstorff *et al.*, 2006)
	Prostate	↑	–	↑	↑	FAAH and MAGL overexpression related to cancer invasion and disease outcome	(Schmid *et al.*, 2002, Nithipatikom *et al.*, 2005, Endsley *et al.*, 2008, Thors *et al.*, 2010)
Digestive tract tumours	Colon	↑	↑	–	–	High levels of AEA in lymphatic metastases	(Ligresti *et al.*, 2003, Chen *et al.*, 2015)
	Pancreas	–	–	↑	↑	FAAH and MAGL overexpression is a good cancer prognosis	(Michalski *et al.*, 2008)
Gynaecological tumours	Endometrial	≈ or ↑	↑	–	–	–	(Schmid *et al.*, 2002, Guida *et al.*, 2010)
	Ovarian	–	–	–	↑	MAGL up-regulation associate with cancer invasiveness.	(Van Dross *et al.*, 2013, Qin and Ruan, 2014)
–	Melanoma	–	–	–	↑		

In the Table, ≈ denotes a similar expression, ↑ higher levels and ↓ lower levels of expression compared with normal tissues

Endocannabinoid antitumour activity

A large number of studies have been undertaken to evaluate the anticancer activity of plant and synthetic cannabinoids (Guzman, 2003; Fraguas-Sanchez *et al.*, 2016; Velasco *et al.*, 2016; Bogdanovic *et al.*, 2017). Regarding endocannabinoids, their exogenous administration has also been reported to reduce the proliferation, migration and invasion of tumours. Finally, inhibition of the MAGL and FAAH enzymes has also shown anticancer activity.

Several authors have demonstrated that endocannabinoids exert an anticancer activity in brain tumours. Thus, AEA inhibited the proliferation of C6 glioma cells in a mechanism that involves both TRPV-1 channels and CB receptors (Fowler *et al.*, 2003). Contassot *et al*. (2004b) reported that this compound also inhibited the proliferation of U87, U251, C6 and H4 glioma cells. However, these authors found that the blockade of both CB_1 and CB_2 receptors did not protect from this activity; on the contrary, blocking these receptors seemed to exacerbate proliferation of these cells. The use of TRPV-1 channel antagonists significantly decreased the antiproliferative effect, suggesting that this was responsible for AEA action. The involvement of these receptors was also demonstrated by Bari *et al*. (2005), who found that the pretreatment of C6 glioma cells with the lipid raft disruptor, methyl-β-cyclodextrin, decreased the apoptosis induced by AEA. While co-incubation with the TRPV-1 channel antagonist significantly reduced the apoptotic effect, CB_1 antagonists almost doubled it, also preventing methyl-β-cyclodextrin activity. All these data support the participation of TRPV-1 channels in AEA apoptotic activity. Finally, the involvement of the COX-2 enzyme in cell death induced by this endocannabinoid has also been suggested in H4 cells (Hinz *et al.*, 2004). Ma *et al*. (2016) also found *in vitro* that AEA decreased not only the proliferation but also the migration and invasion of U251 glioma cancer cells, showing, in agreement with earlier results, that the inhibition of cell growth was due to induction of apoptosis and even a cell cycle arrest at the G_0/G_1 phase. They also demonstrated the antiproliferative effect of this cannabinoid in mice, reporting a significant tumour growth inhibition compared to the control.

In addition to AEA, other endocannabinoids have been investigated. 2-AG also decreased the proliferation of C6 glioma cells, showing a similar IC_{50} value to AEA (1.8 and 1.6 μM respectively) (Fowler *et al.*, 2003). Jacobsson *et al*. (2001) also reported that 1-AG and 2-AG inhibited the proliferation of C6 glioma cells. Interestingly, both endocannabinoids showed practically the same inhibitory profile and sensitivity as TRPV-1 and CB receptor antagonists; blocking completely their activity. All these data suggest that the action of 2-AG may be secondary to its conversion to 1-AG (Jacobsson *et al.*, 2001).

With respect to breast cancer, both the major endogenous cannabinoids, AEA and 2-AG, and also minor compounds such as oleamide, inhibited the proliferation of breast cancer cells (EFM-19, MCF-7 T-47D and BT4744–6) *in vitro* by cell cycle arrest and/or the induction of apoptosis. The involvement

of CB_1 receptors has also been reported (Bisogno *et al*., 1998; Melck *et al*., 2000). The nerve growth factor (NGF) induced proliferation was also inhibited by both AEA and 2-AG suppressing NGF/Trk receptor levels, and the co- administration of several endocannabinoids, such as the combination of AEA with oleamide, potentiated this antiproliferative effect (De Petrocellis *et al*., 1998).

Concerning prostate carcinomas, various studies have reported the anti-proliferative activity of endocannabinoids. In a CB_1 receptor-dependent manner, AEA has been shown to inhibit the proliferation of PC-3, DU-145 and LNCaP cells, including the proliferation induced by epidermal growth factor, by decreasing the expression of its receptors and blocking the cell cycle at the G1 phase (Mimeault *et al*., 2003; Nithipatikom *et al*., 2011). The growth of primary cul- tures of prostate tumours was also inhibited by AEA, triggering apoptosis (Orellana-Serradell *et al*., 2015). It has been reported that AEA and also 2-AG decreased the prolactin-induced growth of DU-145 prostate cancer cells. All these effects seem to involve CB_1 receptors. On the other hand, noladin ether, a minor endogenous cannabinoid, also reduced the proliferation of prostate tumour cells, but this action was not mediated by CB receptors (Nithipatikom *et al*., 2011).

The effect of endocannabinoids on prostate tumour invasion has also been investigated. The increase in endogenous 2-AG levels *via* diacylglycerol lipase inhibition enhanced the invasion of PC-3 and DU-145 cells (androgen-indepen- dent) (4.2- and 2.0-fold increase respectively). However, in LNCaP (androgen-dependent), the opposite results were found, suggesting that this endocannabinoid may be a potential inhibitor of androgen-dependent prostate tumours. This anti-invasive effect was related to inhibition of adenyl cyclase and a reduction of PKA activity in a mechanism that seems to involve CB_1 receptors (Nithipatikom *et al*., 2004). Interestingly, Endsley *et al*. (2007) showed endogenous 2- AG to be an anti-invasive compound in PC-3 cells, while its exogenous administration had the opposite effect, stimulating the invasion capacity of these cells. The administration of exogenous arachidonic acid also showed this effect, suggesting that the rapid hydrolysis of 2-AG may be responsible for the increase in the invasion rate. In fact, Nomura *et al*. (2011) reported that MAGL inhibitors decreased the invasion capacity of prostate carcinomas and that this effect was partially reversed by blocking CB_1 receptors. The disrup- tion of MAGL activity also interfered with the expression of the epidermal growth factor receptor, decreasing the proliferation induced by epidermal growth factor (Cipriano *et al*., 2014). Finally, noladin ether has also been reported to inhibit the invasion of androgen-independent prostate tumours, inhibiting PKA activity *via* the CB_1 receptors (Nithipatikom *et al*., 2004).

Regarding tumours of the digestive tract, AEA and 2-AG have been demonstrated to reduce the proliferation of several lines of colon cancer cells (DLD-1, HT-29, SW620 and CaCo-2). The involvement of CB receptors in this antiproliferative effect is disputed and may depend on cell type. While some researchers reported that these effects were mediated by CB_1 receptors and CB_2 receptors in the case of DLD-1 cells (Ligresti *et al*., 2003; Linsalata *et al*., 2010), others showed that CB were not involved (Gustafsson *et al*., 2009b; Patsos *et al*., 2010). Interestingly, Gustafsson *et al*. (2009b) demonstrated the involvement of oxidative stress due to the use of α-tocopherol, and a NO synthase inhibitor attenuated the AEA antiproliferative activity. Linsalata *et al*. (2010) postulated that the antiproliferative activity of AEA may be due to the reduction of polyamine levels that play a critical role in cell proliferation. Finally, the inhibition of FAAH also reduced the viability, migration and invasion of Colo-205 cells *in vitro* (Wasilewski *et al*., 2017).

In gastric carcinomas, some researchers have reported that AEA diminished the proliferation of cancer cells, inducing cell cycle arrest at the G_0/G_1 phase (Park *et al*., 2011; Ortega *et al*., 2016). It also enhanced the pro-apoptotic effect of paclitaxel, being a promising combination drug in chemotherapy (Miyato *et al*., 2009). In hepatocarcinomas, the opposite results were found. While anandamide showed an *in vitro* antiproliferative activity in cholangiocarcinoma cells, 2-AG stimulated cell growth. Both effects were not mediated by CB receptors and involved lipid rafts. AEA seemed to exert its action by stabilizing lipid rafts and recruiting Fas and FasL. 2-AG disrupted the lipid raft structure (DeMorrow *et al*., 2007). *In vivo* studies undertaken in mice supported the antiproliferative activity of AEA and re- ported the activation of non-canonical Wnt signalling pathway as one of the mechanisms responsible for its ac- tion, increasing the expression of Wnt5a. The increase of this protein induced the triggering of calcium-independent pathways that involved the orphan receptor Ror2 (DeMorrow *et al*., 2008).

With regard to gynaecological cancers, AEA has been reported to inhibit the proliferation of several cervical cell lines (CC299, CasKi and HeLa) by the induction of apoptosis (activating the cleavage of caspase-7). Interestingly, CB receptors were not involved in this induction of apoptosis, although they were expressed by these cells. By contrast, the blockade of both CB_1 and CB_2 receptors with selective agonists did not prevent the induction of apoptosis but potentiated it, suggesting that these receptors may have a protective role in death of cervical cancer cells and the apoptotic effect was attributed, at least in part, to TRPV-1 receptors (Contassot *et al*., 2004a). Nevertheless, CB receptors were involved in the reduction of the migration and invasion of cervical cancer cells triggered by some cannabinoid com- pounds. For example, Rudolph *et al*. (2008) reported that CB_1 receptors mediated the anti-migratory effect of 2-AG in SW 756 cancer cells.

The synthetic analogue of anandamide, methanandamide (AM-356), has been reported to inhibit the growth of established thyroid cancer *in vivo* and also to decrease the expression of VEGF, producing anti-angiogenic effects. All these actions were attenuated by CB_1 receptor antagonists, so CB_1 receptors are involved in the anti-tumour activity of AM-356. The same authors also demonstrated that AM-356 inhibited the *in vitro* proliferation of metastasis- derived thyroid cancer cells, especially lung metastasis cells (Portella *et al*., 2003). Interestingly, *in vivo* studies in thyroid tumour xenografts induced in mice reported that arachidonyl-5-HT, an FAAH inhibitor, and VDM-11, an inhibitor of endocannabinoid re-uptake, decreased tumour growth, involving both non-cannabinoid and CB_1 receptors (Bifulco *et al*., 2004).

In lymphomas, some murine cell lines that express both CB_1 and CB_2 receptors were sensitive to the action of AEA and also to other CB receptor agonists which induce apoptosis (McKallip *et al.*, 2002). AM-356 also decreased the proliferation of mantle cell lymphoma, normally a very aggressive carcinoma, *via* the activation of the novo ceramide synthesis pathway in a CB_1 receptor-dependent manner (Gustafsson *et al.*, 2009a).

Finally, AEA has been shown to reduce the proliferation of human leukaemia cells, such as Jurkat, Mol-4 and Sup-1 cells, inducing apoptosis with the involvement of CB_2 receptors (McKallip *et al.*, 2002).

The anti-tumour activities of endocannabinoids are summarized in Table 3.

Involvement of the endocannabinoid system in tumour progression

Effect on tumour neovascularization

The ECS has also been postulated as a modulator of tumour angiogenesis, being implicated in anti-angiogenic action by decreasing the survival and migration of endothelial cells and/or reducing the expression of pro- angiogenic factors. In fact, several cannabinoids, including AEA and CBD, have been shown to have anti-angiogenic properties (Rajesh *et al.*, 2010; Thapa *et al.*, 2011; Solinas *et al.*, 2012). This effect is involved in the inhibition of the growth of numerous kinds of tumours such as lung, breast, skin and brain carcinomas. For example, *in vitro* and *in ovo* (in the chick chorioallantoic neovascularization model), AM-356 exerted an anti-angiogenic effect, decreasing the proliferation and inducing apoptosis of endothelial cells, with the diminution of metalloproteinase-2 activity. The mechanisms responsible for these effects involved CB_1 receptors and were also involved in the anti-tumour activity of cannabinoids in thyroid cancer (Pisanti *et al.*, 2007). Similar results were previously reported by Portella *et al*. (2003), showing that the inhibition of angiogenesis in thyroid tumours by AM-356 involved a reduction of the expression of VEGF, a pro-angiogenic factor, also showing the participation of CB_1 receptors. This effect was attributed to inhibition of p21ras activity (necessary for VEGF action). AEA was also reported to reduce pro- angiogenic pathways in breast cancer cells. In an *in vitro* model of angiogenesis, AEA decreased the MDA-MB-231- induced proliferation of endothelial cells and reduced the levels of a significant number of pro-angiogenic factors, especially those of IFNγ, leptin, TGFβ1, TIMP1, TIMP2, thrombopoietin and VEGF (Picardi *et al.*, 2014).

CB receptors are also implicated in the anti-angiogenic activity of other synthetic cannabinoids such as WIN-55212−2 and JWH-133, in brain tumours (gliomas and astrocytomas). Blazquez *et al*. (2003) reported that these compounds impaired tumour neovascularization by inhibiting cell survival with the induction of apoptosis and the migration of vascular endothelial cells. They also reduced the expression of several angiogenesis stimulation factors, specifically VEGF and angiotensin II.

The inhibition of the MAGL enzyme with the consequent increase of 2-AG levels was also reported to decrease tumour growth, exerting an anti-angiogenic activity. *In vivo* studies reported that MAGL inhibitors down-regulated pro-angiogenic factors, particularly VEGF and FGF-2, and also reduced the number of vessels (Pagano *et al.*, 2017).

In spite of this anti-angiogenic effect of cannabinoids, minor endocannabinoid compounds have been postulated as pro-angiogenic factors, stimulating angiogenesis in a GPR55 receptor-dependent manner (Zhang *et al.*, 2010). Indeed, this effect has also been reported for AEA. In the nanomolar range, this cannabinoid induced the angiogenesis stimulated by FGF-2, in a pathway that involved CB_1 receptors, which were found to be overexpressed during the angiogenesis process. Their involvement in the angiogenesis process has been corroborated *in vivo*. On the one hand, studies in CB_1 receptor knockdown mice reported an inactivation of the proliferation, migration and capillary-like tube formation induced by pro-angiogenic factors (particularly FGF-2). On the other hand, CB_1 blockage also reduced the FGF-2-induced neovascular proliferation in the rabbit cornea assay (Pisanti *et al.*, 2011). The implication of these receptors in carcinogenesis has also been reported by other authors. Malfitano *et al*. (2012) demonstrated using an ascitic tumour model (Meth- A cells specifically injected into mice) that CB_1 receptor blockade reduced tumour growth. Ciaglia *et al*. (2015) found similar results in an *in vivo* model of glioma, also involving the participation of STAT3 pathway. Additionally, in glioma samples from patients, they related low levels of active STAT3 to a lower expression of CB_1 receptors. Nevertheless, Wang *et al*. (2008) found in mice models of colon cancer that the loss or inhibition of CB_1 receptors induced tumour growth. So the involvement of these receptors in cancer probably de- pends on the origin of the tumour.

Stimulation of tumour growth by cannabinoids

In spite of the establishment of anticancer activity of cannabinoids and the involvement of the ECS, the implication of the ECS in cancer progression has also been reported in some tumours. This biphasic effect seems to depend on the cannabinoid concentration and involves CB receptors, specifically CB_2.

Thus, AEA at concentrations of 1 μM stimulated the proliferation of gastric cancer cells (Miyato *et al.*, 2009), and its analogue, AM-356, at lower concentrations also exerted a mitogenic activity in prostate carcinomas. While, in the micromolar range, AM-356 inhibited the proliferation of LNCap prostate cancer cells, at nanomolar concentrations (100−200 nM), it stimulated cell growth. Similarly, Δ^9-tetrahydrocannabinol (THC) and JWH-133 exerted pro-proliferative effects in prostate cancer (Sanchez *et al.*, 2003).

The proliferation induced by THC has also been reported in lung, breast and even in brain tumours. This phytocannabinoid, in concentrations of 100−300 nM, stimulated the proliferation of lung tumours and gliomas *in vitro* (Hart *et al.*, 2004). *In vivo*, in mice models, Δ^9-THC also improved the growth and metastases formation of breast tumours (McKallip *et al.*, 2005). Interestingly, this pro-cancer activity has been related to the immune response against the tumour and involves CB_2 receptors. By the activation of

Table 3

Anti-tumour actions of endocannabinoids

Cancer type	Cannabinoid	Cancer cells	Anti-tumour effect	Mechanism	References
Glioma	AEA	U87, U251, C6, H4	Inhibition of cell proliferation	• Apoptosis induction • Involvement of TRPV-1 channels	(Hinz *et al.,* 2004, Contassot *et al.,* 2004b, Bari *et al.,* 2005)
		U251	Inhibition of cell proliferation, migration and invasion	• Induction of apoptosis and cell cycle arrest at G_0/G_1 phase	(Ma *et al.,* 2016)
	2-AG 1-AG	C6	Inhibition of cell proliferation	• Involvement of CB receptors and TRPV-1 channels. • The action of 2-AG seems to be secondary to its conversion to 1-AG	(Jacobsson *et al.,* 2001, Fowler *et al.,* 2003)
Breast cancer	AEA, 2-AG	EFM-19, MCF-7, T47-D, BT-47446	Inhibition of cell proliferation	• Induction of apoptosis and cell cycle arrest at G_1/S phase *via* CB_1 receptors • Suppression of NGF induced proliferation probably due to a reduction of NGF/TrK receptor levels • Oleamide potentiates the antiproliferative activity of AEA.	(Bisogno *et al.,* 1998, De Petrocellis *et al.,* 1998, Melck *et al.,* 2000)
Prostate cancer	AEA	PC-3, DU-145 and LNCaP	Inhibition of cell proliferation.	• Apoptosis induction	(Mimeault *et al.,* 2003, Nithipatikom *et al.,* 2011, Orellana-Serradell *et al.,* 2015)
	AEA, 2-AG	DU-145	Reduction of prolactin induced proliferation	–	(Nithipatikom *et al.,* 2011)
	Noladin-Ether	DU-145	Inhibition of cell proliferation	• In a CB receptor independent manner	
	2-AG	Androgen-independent tumour cells	Inhibition of cell invasion	• Involvement of CB_1 receptors	(Endsley *et al.,* 2007)
Colon cancer	AEA, 2-AG	–	Inhibition of cell proliferation	• Opposite results have been reported in the participation of CB receptors.	(Ligresti *et al.,* 2003, Gustafsson *et al.,* 2009b, Linsalata *et al.,* 2010, Patsos *et al.,* 2010)
Gastric cancer	AEA	AGS	Inhibition of cell proliferation	• Cell cycle arrest at $G_0/G_1$and apoptosis induction.	(Ortega *et al.,* 2016)
Hepatocarcinoma	AEA	–	Inhibition of cell proliferation. Reduction of tumour growth *in vivo*	• CB receptor independent mechanism	(DeMorrow *et al.,* 2007, DeMorrow *et al.,* 2008)
Cervical cancer	AEA	CC299, CasKi, HELA	Inhibition of cell proliferation	• Cell cycle arrest at G_0/G_1 phase and apoptosis induction.	(Contassot *et al.,* 2004a)

continues

Table 3
(Continued)

Cancer type	Cannabinoid	Cancer cells	Anti-tumour effect	Mechanism	References
				• Involvement of non-CB receptors in the generation of apoptosis	
	2-AG	SW-756	Inhibition of cell migration and invasion	• Involvement of CB_1 receptors	(Rudolph *et al.*, 2008)
Thyroid cancer	AM-356	–	*In vivo* inhibition of tumour growth	• Inhibition of tumour angiogenesis due to the reduction of VEGF expression *via* CB_1 receptors	(Portella *et al.*, 2003)
Lymphomas	AEA	Murine cells	Inhibition of cell proliferation	–	(McKallip *et al.*, 2002)
Leukaemia	AEA	Jurkat, Mol-4 and Sup-1	Inhibition of cell proliferation	• Induction of apoptosis *via* CB_2 receptors	

these receptors, THC increased the production of IL-4 and IL-10, stimulating a Th-2-type immune response and inhibiting the Th-1 response (Zhu *et al.*, 2000; McKallip *et al.*, 2005).

ECS and drug resistance

Multidrug resistance is probably one of the major problems in chemotherapy of cancer. Tumours become resistant to a great number of different drugs without structural or functional similarities, including anthracyclines, taxanes and *Vinca* alkaloids, and this hampers cancer treatments (An *et al.*, 2017). Much of such resistance to cancer chemotherapy involves the multidrug resistance protein 1 (MDR1), an efflux pump that belongs to the family of ATP-binding cassette transporters (ABC) (Lopes-Rodrigues *et al.*, 2016) and other proteins of the ABC superfamily such as the breast cancer resistance protein (ABCP or ABCG2) (Chen *et al.*, 2010). Several studies have re- ported the involvement of the ECS in the mediation of resistance to anticancer drugs.

In T-lymphoblastic leukaemia cells (CEM/VLB100), the two major plant cannabinoids, THC and CBD, modulate the expression of MDR1. While, at shorter incubation times (4 h), they increased its expression and consequently the activity of MDR1, at longer times (72 h), they decreased MDR1 action. The ECS is involved in these actions, although differences in the mechanisms of both cannabinoids have been reported. Although the THC effect was only mediated by CB_2 receptors, the activity of CBD was mediated by both CB_2 and non-CB receptors, specifically TRPV-1 channels (Arnold *et al.*, 2012). Holland *et al*. also reported that both cannabinoids and also cannabinol reduced the expression of MDR1 protein in these leukaemia cells, without inhibiting its efflux activity. In breast cancer (MCF-7 cells), CBD also inhibited the expression of MDR1, but it increased expression of ABCP (Feinshtein *et al.*, 2013).

By contrast, other authors have shown that several plant cannabinoids, including CBD, THC and cannabinol, inhibited ABCP (Holland *et al.*, 2007; Tournier *et al.*, 2010) and also MDR1, with CBD being the most potent inhibitor (Holland *et al.*, 2008).

Concluding remarks

In tumours, the abnormal expression of the different components of the ECS, especially CB_1 and CB_2 receptors, compared with that healthy tissues reveals its involvement in cancer. However, this aberrant expression is not consistent and varies with cancer type. Although in some tumours these receptors are up-regulated, in others, their expression is lower. Therefore, it is important to consider the participation of CB_2 receptors, whose levels seem to be up-regulated after certain pathological conditions. The correlation of the aberrant expression in the ECS with cancer outcome reinforces its involvement in tumourigenesis. The ECS either participates in disease progression or exerts a protective role and becomes a potential therapeutic target. In general, for example, in prostate, colon and pancreatic carcinomas, an altered ECS is a negative marker for cancer, being related to a more invasive

tumour and a lower survival rate, except in hepatocarcinoma where it is an indicator of good prognosis.

CB receptors also mediate anticancer activity, at least in part, of other cannabinoid compounds, including phytocannabinoids such as THC, CBD and cannabinol, and even synthetic cannabinoids, including AM-356. The efficacy of CBD alone and in combination with THC is being extensively evaluated for the treatment of different solid tumours (Ramer and Hinz, 2016).

Besides CB receptors, endocannabinoid levels are also altered in some carcinomas. In this context, it is important to emphasize that the two major endogenous cannabinoids (AEA and 2-AG) administered exogenously inhibit the growth of several kinds of tumours, demonstrating an antiproliferative, anti-invasive and anti-angiogenic activity. The increase in endocannabinoid levels as a result of inhibiting their degradation pathways, especially the FAAH and MAGL enzymes, has also been useful in reducing cancer proliferation.

In spite of the promising activity of cannabinoids as anticancer treatments, it has to be taken into account that some cannabinoids have also been shown to increase tumour growth, exerting pro-angiogenic and pro-proliferative effects. This biphasic activity has been related to cannabinoid concentration. It seems that while cannabinoids stimulated cancer growth at low concentrations (in the nanomolar range), they inhibited it at higher concentrations (micromolar range). While both CB_1 and CB_2 receptors are involved in cannabinoid anticancer activity, the stimulation of cancer proliferation appears to be mainly attributable to CB_2 receptors.

Finally, cannabinoids have also been associated with resistance to anticancer drugs, interfering in the expression of several ABC drug transporter proteins, especially MDR1, implying that these proteins could provide a good target for cannabinoids to overcome cancer resistance. In fact, some cannabinoids at non-toxic concentrations increase the sensitivity of cancer cells to chemotherapy. For example, THC and CBD potentiate the cytotoxicity of vinblastine (Holland *et al.*, 2006) and temozolomide in leukaemia and glioma respectively. Consequently, cannabinoids could be a good strategy as coadjuvant treatments. In fact, a clinical study is currently being performed to evaluate the efficacy and safety of a cannabinoid-based spray containing the two major natural cannabinoids present in *Cannabis sativa*, THC and CBD, in combination with temozolomide for the treatment of glioma (NCT01812603) (Holland *et al.*, 2006; Zogopoulos *et al.*, 2015).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c,d,e,f).

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Conflict of interest

The authors declare no conflicts of interest.

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Publicación 3: Phyto-, endo- and synthetic cannabinoids: promising chemotherapeutic agents in the treatment of breast and prostate carcinomas

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REVIEW

Phyto-, endo- and synthetic cannabinoids: promising chemotherapeutic agents in the treatment of breast and prostate carcinomas

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ABSTRACT

Introduction: The term 'cannabinoids' designates a family of compounds with activity upon cannabinoid receptors. Cannabinoids are classified in three groups: phytocannabinoids, endocannabinoids, and the synthetic analogues of both groups. They have become a promising tool in the treatment of cancer disease, not only as palliative agents, but also as antitumor drugs, due to their ability to inhibit the proliferation, adhesion, migration, invasion, and angiogenesis of tumour cells. Two of the cancers where they have shown high anticancer activity are breast and prostate tumours. Despite this potential clinical interest, several studies have also reported that cannabinoids can stimulate the proliferation of cancer cells at very low concentrations.

Areas covered: The aim of this review is to evaluate the promising chemotherapeutic utility of phytocannabinoids, endocannabinoids, and synthetic cannabinoids in breast and prostate cancer.

Expert opinion: Cannabinoids, in particular the non-psychoactive CBD, may be promising tools in combination therapy for breast and prostate cancer, due to their direct antitumor effects, their ability to improve the efficacy of conventional antitumor drugs and their usefulness as palliative treatment. Nevertheless, deeper studies to fully establish the mechanisms responsible for their antitumour and pro-tumour properties and their formulation in efficient delivery systems remain to be established.

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Breast cancer; cannabidiol; cannabinoid receptors; endocannabinoids; endocannabinoid system; phytocannabinoids; prostate cancer; Δ^9-tetrahydrocannabinol

1. Introduction

The term 'cannabinoids' refers to a heterogeneous family of compounds that exhibit activity upon cannabinoid receptors and are divided in three groups: natural compounds present in the *Cannabis sativa* plant, which are named phytocannabinoids; endogenous cannabinoids, also known as endocannabinoids; and the synthetic analogs of both groups, called synthetic cannabinoids.

Cannabinol (CBN) constitutes the first plant cannabinoid to be isolated in 1899 by Wood and co-workers [1], although its structure was not elucidated until 1940 [2,3]. Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) were identified later. CBD, the main non-psychotropic phytocannabinoid, was identified in the 1960s by Mechoulam and Shvo [4], but it was elucidated at the first time in 1940 by Adams and colleagues [2]; and Δ^9-THC, which is primarily responsible for the psychoactive effects of cannabis, was first isolated and elucidated by Gaoni and Mechoulam in 1964 [5]. In the last decades, a huge number of cannabinoids have been elucidated, and their potential therapeutic utility in several diseases, including cancer, has been intensively investigated [6].

On the one hand, cannabinoids have proved to be useful in the treatment of chemotherapy side effects such as nausea, vomiting, pain, weight loss, and lack of appetite, and several drugs based on cannabinoids are approved in some countries as palliative agents in anticancer treatments. Dronabinol (synthetic Δ^9-THC) and nabilone (a synthetic cannabinoid similar to Δ^9-THC) are approved in Canada, United States, and several countries of Europe to treat nausea and vomiting associated with chemotherapeutic treatments [7]. Nevertheless, their psychoactive side effects eclipsed their antiemetic potential, and they are not the first-line treatments [8]. An oromucosal spray containing Δ^9-THC and CBD is also approved in Europe and Canada for the treatment of spasticity associated with multiple sclerosis (MS) and also in Canada as an adjunctive analgesic for the treatment of pain in patients with advanced cancer and MS [9] (Table 1). Other medication that contains a high content in CBD is under investigation to the treatment of refractory epilepsies, specifically Dravet and Lennox Gastaut syndromes [10].

On the other hand, in the last years, a significant research work has been undertaken to evaluate the therapeutic utility of cannabinoids in the treatment of tumor progression [11]. The ability of cannabinoids to reduce tumor growth was reported for the first time by Munson et al. in 1975. They showed by *in vitro* and *in vivo* experiments that several phytocannabinoids, including Δ^9-THC, Δ^8-tetrahydrocannabinol and CBN, decreased Lewis lung adenocarcinoma proliferation [12]. Nevertheless, it was not until the 2000s, predominantly due to the work of Guzman's group in gliomas, when resurfaced the interest of cannabinoids as anticancer agents. These first experiments of Guzman's group are compiled in the Guzman et al. 2003 review [13]. Thenceforth, many studies have evaluated the anticancer activity of cannabinoids, and

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Publicación 4:

CURRENT STATUS OF NANOMEDICINE IN THE CHEMOTHERAPY OF BREAST CANCER

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CURRENT STATUS OF NANOMEDICINE IN THE CHEMOTHERAPY OF BREAST CANCER

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ABSTRACT

Despite the efforts that have been made in the field of breast cancer therapy, it is a leading cause of cancer death in women and a major health problem. The current treatments combine several strategies (surgery, radiotherapy, hormone therapy, and chemotherapy) depending on cancer subtype and tumour stage. The use of chemotherapy is required in certain circumstances, like before or after surgery or in advanced stages of the disease. Chemotherapeutic regimens that include anthracyclines (e.g. doxorubicin), taxanes (e.g. paclitaxel), 5-fluorouracil and/or cyclophosphamide show, in general, a high toxicity that limit their clinical use. The use of targeted chemotherapy allows to get a selective location of the drug at tumour mass, decreasing the toxicity of these treatments. An increase of the antitumor efficacy can also be achieved. The use of nanocarriers containing anticancer drugs can be a good strategy to get targeted chemotherapy. In fact, several nanoformulations containing paclitaxel and doxorubicin have been approved or are under clinical trial for breast cancer therapy. The main advantage of these nanomedicines is their lower toxicity compared to conventional formulations, which can be attributed to the elimination of the solvents of the formulation (e.g. Cremophor-EL in paclitaxel conventional formulations) and the more selective location of the drug at tumour site (e.g. cardiotoxicity related to free doxorubicin). However, some adverse events (e.g. hand foot syndrome or infusion reactions) have been related to the administration of some nanomedicines, which have to be considered.

Keywords

Breast cancer, Chemotherapy, Doxorubicin, Micelles, Liposomes, Paclitaxel

1. Introduction

Breast cancer is the most common neoplasm in women with 2.09 million cases and 627,000 of deaths worldwide in 2018[1]. Although in the last decades the implementation of early detection techniques and novel therapies have significantly improved breast cancer survival; this disease is still a leading cause of death in women between 35 and 54 years of age and a major health problem.

Breast cancer is a heterogeneous disease that is divided in several subtypes according to: i) the aggressiveness grade: in situ and infiltrating (invasive) carcinomas; ii) the histopathology, e.g. tubular, medullary and ductal; iii) the molecular profile considering the expression of oestrogen receptor (ER), progesterone receptor (PR), and the overexpression of human epidermal growth factor receptor 2 (HER-2) and iv) the disease progress: in stage 1 (primary tumour within the breast), stage 2-3 (spreading to tissues and lymph nodes nearby) and stage 4 (spreading to distant organs like lung, bone and brain) [2]. Tumours with no expression of ER, PR and HER-2, called triple negative breast cancers (TNBC), are more aggressive and tend to metastasise [3,4].

Depending on cancer subtype, there are available several treatment strategies: i) surgery (mastectomy or lumpectomy), ii) hormone therapy, iii) radiotherapy, iv) antibody-based therapy and v) drug therapy (chemotherapy) [5]. Surgery and radiotherapy are usually used for treating primary breast tumoursand loco-regional lesions, and drug therapy to reduce tumour burden and to control and eradicate metastasis [6]. Although new antitumor agents have been investigated in the last decades and introduced in therapeutics with good results, chemotherapy (necessary treatment in high aggressive tumours) still showsa limited efficacy due to the difficulty to reach cancer cells.Although not all breast carcinomas required the use of chemotherapy, this is necessary in certain circumstances like before (neoadjuvant chemotherapy) or after surgery (adjuvant chemotherapy), or in advance tumours where chemotherapy could be the main cancer treatment strategy.The used drugs depend on cancer stage and cancer subtype. However, most of the chemotherapeutic regimens include anthracyclines like doxorubicin (Dox), taxanes like paclitaxel (PTX), 5-fluorouracil and/or cyclophosphamide.

The deployment of targeted chemotherapymay overcome the handicaps of the chemotherapeutic treatments, increasing their efficacy and decreasing their toxicity. In this way, the development of nanotechnology-based anticancer drugs can be a good approach to get their selective location at tumour mass level (Figure 1) [7,8].

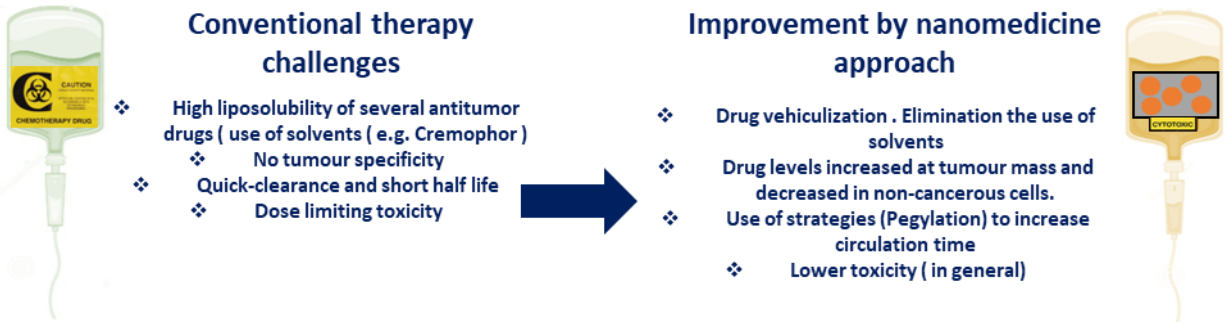

Figure 1: Improvement of conventional chemotherapy challenges by nanomedicines approach.

Currently there are several nanomedicines approved for the treatment of cancer, including several for the treatment of breast tumours. (Table 1). This review is focused on the nanoformulations for breast cancer therapy that have already been approved or are in clinical trials.

Brand name	Drug	Nanocarrier	Cancer type
Abraxane®	Paclitaxel	Albumin nanoparticles	Metastatic breast cancer
Genexol®	Paclitaxel	Polymeric micelles	Breast, lung and pancreatic carcinomas
Myocet®	Doxorubicin	Liposomes	Breast cancer
Doxil/Caelyx®	Doxorubicin	Pegylated-liposome	Metastatic breast cancer, ovarian cancer and Kaposi sarcoma
Daunoxome®	Daunorubicin	Liposome	Kaposi sarcoma
Onco-TCS®	Vincristine	Liposome	Non-Hodgkin lymphoma
Marquibo®	Vincristine	Liposome	Leukaemia
Mepact®	Mifamurtide	Liposome	Osteosarcoma
Onyvide®	Irinotecan	Liposome	Metastatic pancreas cancer
Lipusu®	Paclitaxel	Liposome	Non-small lung cancer, breast cancer
PICN®	Paclitaxel	Liposome	Metastatic breast cancer
Transdrug®	Doxorubicin	Nanoparticle	Hepatocarcinoma
Oncaspar®	Asparraginase	Polymeric conjugate	Acute lymphoblastic leukaemia

Table 1: Approved nanomedicines for cancer therapy

2. Targeted chemotherapy strategies

There are several available strategies to get this targeted therapy: i) passive targeting, ii) active targeting and iii) stimuli responsive drug release.

Passive targeting in cancer chempotherapy is mainly based on enhanced permeability and retention effect (EPR) characteristic of tumours. EPR effect is attributed to: i) the presence of hyperpermeable tumour vasculature, with a fenestrated endothelium with higher gaps (100-780 nm) compared to healthy tissues and ii) the impaired lymphatic drainage that limits the clearance of substances in this way. Due to EPR, intravenously administered nanomedicinestrend to accumulate at tumour site. As a consequence, the

drug biodistribution to healthy tissues is restricted and its toxicity decreased.

The active targeting strategy involves the binding of high-affinity moieties to the nanocarrier surface to get its "more selective" location at tumour site. This ligands have to be specifically recognised by cancer cells or tumour microenvironment (blood vessels or extracellular matrix). A high number of ligands have been investigated, including proteins, antibodies, peptides, carbohydrates and small molecules like folic acid. The election of the ligand depends on the targets overexpressed in each cancer subtype [9]. For example, folate receptor (FR) is overexpressed in 60-85% of breast cancers and is a promising target. In HER-2 positive tumours, the use of monoclonal antibodies against this receptor can be a good active targeting strategy. In fact, the first FDA-approvedactive targeting therapy (not involving nanomedicines) is trastuzumab (the anti-HER-2 antibody), which has become a standard treatment in this type of breast tumours [10]. In TNBC, epidermal-growth-factor receptor (EGFR) could be a therapeutic target, because it is overexpressed in more than 50% of the cases. Therefore, the use of anti-EGFR antibody coated nanocarriers can be a promising therapeutic tool.

Stimuli-responsive release strategy is based on the induction of drug release from the nanocarriers at cancer tissue, due to internal or external stimuli. The selective release of the drug at tumour site increases its efficacy and decreases its toxicity. Internal or external stimuli can trigger changes in the phase, structure or conformation of nanocarrier and, as consequence drug release. Regarding internal stimuli, differences between tumour and healthy tissue have been exploited, including changes in pH, redox, enzyme expression or temperature. For example, tumour have a more acidic pH than normal tissue (~ 6.7 vs 7.4 respectively) [11,12]. Therefore, the use of nanocarriers that release the drug in this acidic microenvironment may get a selective location of the drug at tumour mass. This is the mechanism that follow some micelle systems.The higher tumour temperature (~ 40ºC) could also be exploited. The application of external stimuli, including temperature, light, ultrasound, magnetic force and electric field; has also been investigated. It could be applied locally at tumour site, without damaging normal tissue.

3. Approved nanoformulations

Nowadays, the approved nanoformulations in breast cancer are based on the passive targeting strategy, to try to get a certain selective location of the drug at tumour mass, decreasing the toxicity of these treatments.

3.1Paclitaxel nanoformulations

Paclitaxel (PTX), an antimicrotubular agent belonging to taxane group, is largely used for the treatment of solid tumours, including breast cancer. In fact, a combination oftaxanes and anthracyclines remains the standard neo-adjuvant chemotherapy in breast cancer [13].

PTX is a poorly water-soluble drug. The available conventional formulation, named Taxol®, uses polyoxyethylated castor oil, known as Cremophor EL (Cre-EL) as solubilizing agent. Cre-EL is responsible for several adverse effects (including hypersensitivity reactions, neurotoxicity and nephrotoxicity). Therefore, it significantly contributes to the dose-limiting toxicity related to PTX conventional formulation. Patients need to be pre-treated with antihistamines and corticoids to prevent hypersensitivity reactions [14].Although Cre-El is also used to solubilise other hydrophobic drugs such as cyclosporine A, Taxol® contains much more quantity (around 26 ml per administration) [15]. The use of nanocarriers for PTX administration, eliminate the use of Cre-EL as solubilising agent and as a consequence, the toxicity related to this agent. Moreover, it has been reported that Cre-EL inhibit the PTX binding to endothelial cells and to plasmatic albumin, limiting the uptake of PTX by tumour cells [16]. In this context, nanoformulations, may also increase PTX efficacy [17-19]. Nowadays, there are approved four nanoformulations containing PTX as anticancer for breast cancer therapy: i) Abraxane®, ii) Genexol® iii) Nanoxel® and iv) Lipusu® (Figure 2).

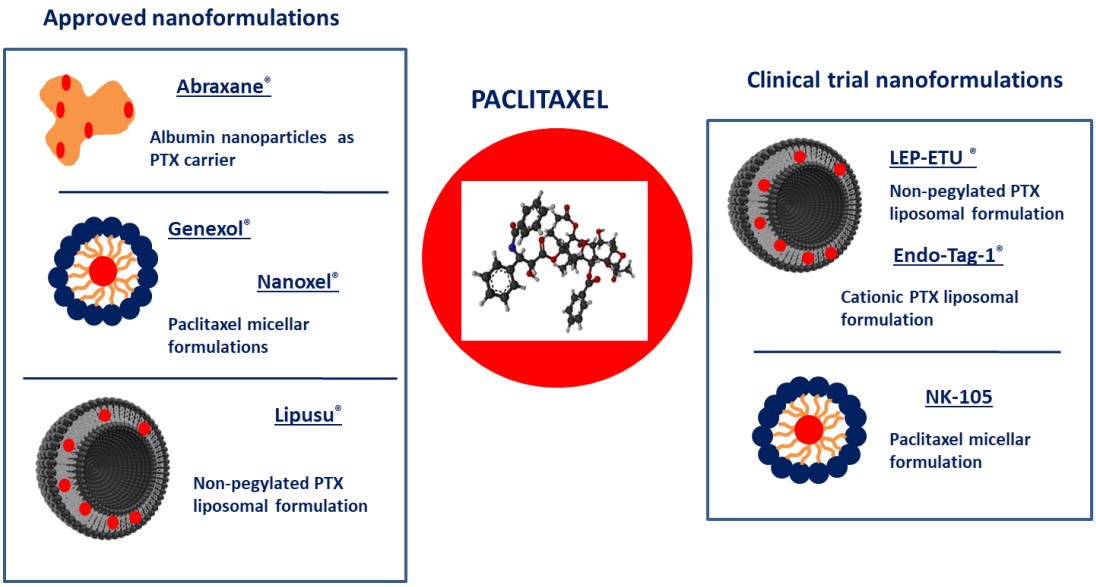

Figure 2: Paclitaxel nanoformulations that are approved or in clinical trials for the treatment of breast cancer

3.1.1 Abraxane®

Abraxane (nab-PTX) is a Cremophor-EL free nanoformulation that uses albumin nanoparticles (particle size around 130 nm) as PTX carrier. It was approved by FDA in 2005 for the treatment of metastatic breast cancer. Nab-PTX has a lower toxicity than conventional PTX formulations, showing a minimal risk of hypersensitivity reactions and allowing the elimination of corticoid premedication. While conventional PTX has a maximum tolerated dose (MTD) of 175mg/m^2, nab-PTX reported a higher value of 260mg/m^2(Table 2). It also showed a higher antitumor efficacy, due to an increased intratumoral concentration of PTX (around 33% of increase) [20].

Several mechanisms are involved in “albumin nanoparticle based targeting”. On the one hand, drug delivery involves glyprotein-60 (gp-60) receptors of vascular endothelium and that are also known as albondin receptors. Albumin shows high affinity for gp-60 receptors and their binding induces the activation of caveolin-1 pathway (an intracellular protein), triggering the invagination of cell membrane, the formation of vesicular structures called caveolae and consequently the internalization of PTX- albumin bounded to tumour interstice. On the other hand, albumin tends to accumulate at tumour site due to SPARC proteins; which are located in tumour interstice and on the surface of cancer cells and have been found overexpressed in the 50-60% of breast carcinomas and

have been associated with a poor prognosis [21]. Albumin nanoparticles bind to these proteins and are accumulated at tumour site [22-24].

Nowadays, nab-PTX is approved for the treatment of breast cancer (second line treatment when combination chemotherapy fails or cancer relapses within 6 months of adjuvant chemotherapy), metastatic pancreatic tumours (first line treatment in combination with gemcitabine) and in metastatic or locally advanced non-small cell lung cancer (first line treatment in combination with carboplatin when the use of curative surgery or radiotherapy is not possible). Several clinical trials are also ongoing for the treatment of: i) breast cancer in combination with epirubicin (NCT03647280), ii) early breast cancer in combination with capecitabine (NCT03647514), iii) HER-2 negative breast cancer in combination with PU-H17 (NCT03166085), iv) metastatic or advanced breast cancer in combination with phenelzinesulfate (NCT03505528)and v) in TNBC in combination with pembrolizumab, epirubicin and cyclophosphamide (NCT03289819).

3.1.2 Genexol®

Micelles has gained a deal of interest as chemotherapeutic agent nanocarriers. Genexol® is a PTX polymeric micellar formulation (with a particle size around 20-50 nm), formed by monomethoxy-poly(ethylene-glycol)-block-poly(D,L-lactide) di-block copolymer and that devoid of Cre-EL as solubilising PTX [25].

Several clinical studies have evaluated the efficacy and toxicity of this formulation in patients with metastatic breast cancer. For example, Genexol® was effective as chemotherapeutic agent, with an acceptable toxicity, in patients with metastatic breast cancer who failed to respond to standard chemotherapy. 37.5% of patients showed a partial response to this formulation [26]. In another clinical trial, a higher response rate (58.5% of overall response rate) was detected, with complete response rate of 12% [27]. The combination of this formulation with epirubicin was also effective. An open-label phase I study reported a good antitumor efficacy in patients with locally breast cancer receiving Genexol® (175 mg/m^2) and epirubicin (60 mg/m^2) for four cycles as neoadyuvant chemotherapy, showing a cumulative 5-year disease-free survival rate of 70.0% in patients with complete or partial remission [28].Genexol® did not show a lower efficacy than conventional PTX formulation (containing Cre EL), even it was higher (with

values of objective response rate of 39.1% and 24.3% in Genexol® and conventional PTX respectively), showing an acceptable safety profile. With respect to its safety profile, neutropenia rate was more common in micellar formulation (66.8%) than in conventional PTX (40.2%) [29] . Nevertheless, in general the safety profile of this micellar PTX formulation is better than conventional PTX, with a higher MTD (around 300-390mg/m^2) (Table 2).

	Maximum Tolerated Dose (mg/m^2)
Taxol®	175
Abraxane®	260-300
Genexol®	300-390
Nanoxel®	220-300
LEP-ETU	325
NK-105	180

Table 2: Maximum tolerated doses of PTX formulations.

Nowadays, Genexol® is approved for the treatment of metastatic breast cancer (first line treatment) and metastatic or locally advanced non-small cell lung cancer in combination with cisplatin. Several clinical trials are also ongoing for the treatment of advanced breast cancer in combination with doxorubicin (NCT01784120), for gynaecological cancer (NCT02739529), for recurrent pancreatic cancer in combination with gemcitabine (NCT02739633) and for hepatocellular carcinoma after failure of sorafenib (NCT03008512).

3.1.3 Nanoxel®

Nanoxel® is another PTX micellar formulation marketed in India since 2006. Nowadays, it is approved for the treatment of metastatic breast cancer, non-small cell lung carcinoma and Kaposi's sarcoma [30]. This formulation consists of polymeric micelles, with a particle size around 80-100 nm [31], formed by a pH sensitive co-polymer of N-isopropyl acrylamide and vinylpyrrolidone monomers. At physiological pH, the micelles are stable and PTX is entrapped in the formulation, but at acid pH values

the polymer is degraded and PTX is released. Thus, the acidic microenvironment of tumours triggers PTX release [32].

It is also a cremophor-EL free formulation, avoiding the adverse reactions related to this solvent. Although this micellar formulation reported similar adverse effects compared to conventional PTX formulations (including anorexia, nausea, vomiting, diarrhoea, stomatitis, alopecia, and myelosupression), the incidence and severity were lower, showing in general a better safety profile (MTD=220-300mg/m^2) [33,30]. In terms of efficacy, the PTX entrapped in this formulation maintains its antitumor efficacy, showing a similar activity than Taxol® [34].

3.1.4 Lipusu®

Lipusu® is a non-pegylated liposomal formulation approved in Chine since 2006 for the treatment of ovarian cancer as first line chemotherapy, breast cancer as follow-up treatment in patients receiving radiotherapy or doxorubicin based chemotherapy, lung cancer in combination with cisplatin and gastric cancers (Table 1). It is constituted by vesicles with a bilayer of phospholipids (mainly phosphatidylcholine) and cholesterol, in which PTX is entrapped, and with a particle size around 400 nm [30]. This liposomal formulation resolves PTX solubility challenges, eliminating the need of Cre-EL as PTX solvent and decreasing overall side effects [35]. In fact, similar anticancer efficacy [35,36] and a lower toxicity has been reported compared to conventional PTX formulation. For example while anaphylaxis and peripheral nerve toxicity are common adverse effectsin patients treated with Taxol®, the liposomal formulation considerable decreases hypersensitivity risk and peripheral nerve toxicity. In this way, the maximum tolerated dose of liposomal PTX is higher (200 mg/kg) than conventional PTX solution (30mg/kg), letting the increase of the dose and consequently the antitumor effects [35,37] (Table 2).

Nowadays, this liposomal formulation has only been approved in China. A clinical study to evaluate the efficacy and safety of Lipusu® plus carboplatin compared to gemcitabine plus carboplatin as first-line treatment is being undertaken in patients with advanced squamous cell carcinoma of lung (NCT02996214).

3.2 Doxorubicin nanoformulations

Doxorubicin (or adryamicin) is the most used anthracycline in chemotherapeutic regimens of a vast number of cancer types due to its high antitumor efficacy. In the case of breast carcinomas, it is one of the most efficacious drugs in both early- and late- stage tumours. Although dox interacts and interferes in several cell processes, the main accepted mechanism responsible for dox cytotoxicity, is the inhibition of DNA topoisomerase-II; with the inhibition of the progression of the S-cell cycle phase [38]. Dox also interacts with mitochondria inducing the overproduction ofreactive oxygen species (ROS) [39].

Despite dox anticancer efficacy, its clinical use is limited by the risk of both acute and chronic cardiotoxicity. Acute cardiotoxicity, with an incidence around 11%, is often reversible and occurs during and 2-3 days after dox therapy. On the contrary, chronic cardiotoxicity, although is less prevalent, with an incidence of 1.7%, is currently irreversible and shows a bad prognosis (one year mortality rate around 50%) [40]. Cardiotoxicity has been attributed to ROS production and to an increase in oxidative stress. In fact, cardiomiocytes are much more sensitive to dox oxidative stress than tumour cells, and the generation of ROS is enhanced by the intracellular non-chelated iron. Dox interaction with cardiomyocyte topoisomerase II-β is also considered a central mechanism responsible for cardiomyocyte damage, thus mice lacking this enzyme are resistant to Dox damage [39].

The use nanocarriers for the administration of doxorubicin, may led a more selective location of the drug at tumour mass, overcoming drug associated cardiotoxicity. In this way, several nanoformulations containing this anthracycline are currently approved. In the case of breast carcinoma, there are two dox liposomal formulations in the market: i) non-pegylated liposomal doxorubicin (LDox) (known as Myocet®) and ii) pegylated liposomal doxorubicin (PLDox) (known as Doxil®, Caelyx® or its FDA-approved generic formulation Lipodox®) (Figure 3).

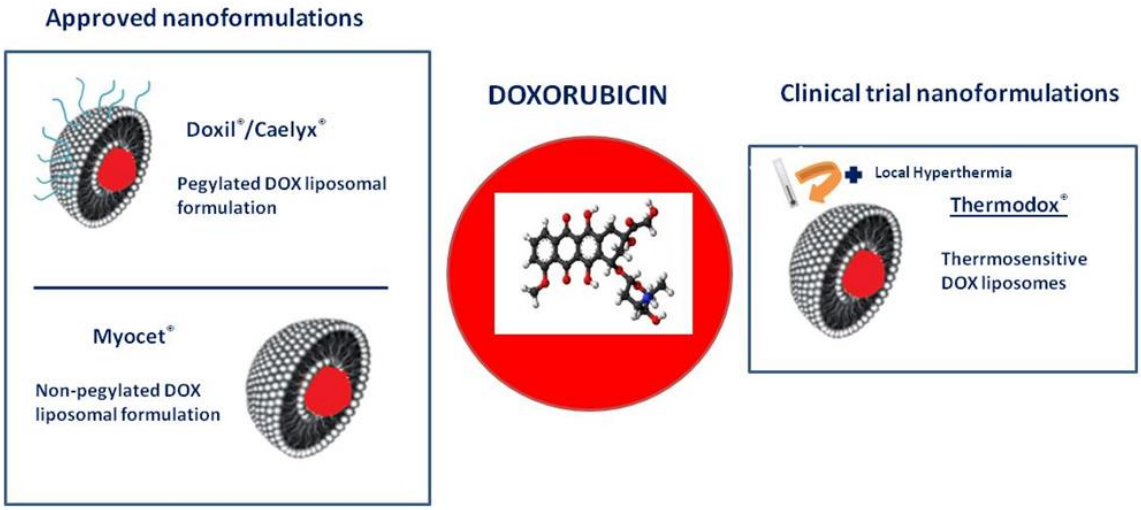

Figure 3: Doxorubicin nanoformulations that are approved or in clinical trials for the treatment of breast cancer.

3.2.1Doxil®/Caelyx®

Pegylated liposomal doxorubicin (Doxil®in EEUU and Caelyx®in EU) was the first FDA-approved nanoformulation for cancer treatment in 1995. Nowadays, it is indicated for the treatment of Kaposi's sarcoma, advanced ovarian cancer, metastatic breast cancer, and multiple myeloma.

This formulation consists of lamellar vesicles with a particle size around 80-90 nm. The external lipid bilayer is formed by fully hydrogenated soy phosphatidylcholine, cholesterol and PEG-modified phosphatidyl-ethanolamine, at a molar ratio of 55:40:5. The doxorubicin is encapsulated into the aqueous core [41]. Pegylation provides a hydrophilic cover to liposomes (known as stealth liposomes), increasing their stability in bloodstream and diminishing the uptake by macrophages. This is why the ratio of free dox circulating in plasma is reduced [42].

The main advantage of PLDox compared to free doxorubicin formulation is its lower cardiotoxicity risk (around 3 fold reduction) [43]. In metastatic breast cancer patients PLDox showed a comparable efficacy to the free doxorubicin formulation. However, the cardiotoxicity was significantly lower in PLDox treated patients. The incidence of myelosupression, vomiting and alopecia was also decreased [44]. PLDox also demonstrated a lower cardiotoxicity and a similar efficacy than epirubicin (another

anthracycline) in patients with invasive breast cancer who received this drug as neo-adyuvant chemotherapy [45]. In spite of pegylated liposomal formulation decreases doxorubicin overall toxicity, two adverse events that are not typical for free doxorubicin, was observed in PLDox treated patients: Palmar-Plantar Erythrodysthesia (PPE), also known as "hand-foot syndrome", and a complement activation-related pseudo-allergy (CARPA). Hand-foot syndrome, characterized by tenderness, redness and peeling of the skin; is probably the major shortcoming in clinic of this formulation. There is no a complete solution for this effect, but the increase of the treatment intervals decreases its incidence and severity [46]. CARPA is an acute hypersensitive reaction that usually appears with the first use of this formulation, and includes facial and neck flushing, dyspnoea, and, less commonly, back pain, cyanosis, and hypotension. It is not a classical hypersensitivity reaction, and it is attributed to complement activation via the alternate pathway. This complement protein activation triggers the stimulation of inflammatory mediators [47]. It is also known as "infusion reaction", because it seems to be related to fast infusion rates of PLDox. In fact, it can be reduced using slow infusion rates and the administration of pre-medication [48]. Although, overall, PLDox toxicity is well tolerated, these adverse events limit the clinical use of this formulation. PLDox is only approved as monotherapy. However, a marginal progression free survival improvement was detected in patients with platinum sensitive ovarian carcinomas that received carboplatin plus PLD compared to whose not received liposomal doxorubicin, as second line therapy [49].

Due to the lower cardiotoxicity of PLDox compared to free doxorubicin, this liposomal formulation could be administered in combination with trastuzumab or lapatinib (anti-HER-2 antibodies) [43]. In fact, a clinical study in patients with HER-2 positive breast cancer reported that the concomitant administration of PLDox, cyclophosphamide and trastuzumab had a significantly lower cardiotoxicity compared to the administration of free doxorubicin and cyclophosphamide [50]. The co-administration of PLDox and lapatinib was also effective in this kind of carcinoma, reporting a tolerated safety profile [51]. In women with locally advanced breast cancer, especially in patients with inflammatory breast carcinoma, the combination of PLDox, cisplatin and 5-fluoracil was also useful [52].

PLDox could also be useful in elderly patients, where anthracyclines are not commonly used due to their cardiotoxicity. In a study undertaken in patients (aged > 65) with stage II-IIb breast carcinomas the treatment with the combination of PLDox and paclitaxel was feasible and reported a well-tolerated profile [53]. Nevertheless, in patients with operable and locally advanced breast cancerreceiving low doses of PLDox (biweekly) and paclitaxel (weekly), the hand-food syndrome was a major side effect, and even in some patients (4 of 35) the last dose of PLDox was suspended due to severity of this syndrome [54]. In another study, a "feasible tolerated" safety profile was detected in patients with locally advanced breast cancer receiving low doses of PLDox plus cyclophosphamide, but a limited activity was observed[55].On the contrary, in patients with metastatic breast cancer, the combinations of PLDox and gemcitabine, or PLDox and cyclophosphamide were useful, showing an acceptable safety profile. Hand-food syndrome was detected in the 14% of PLDox-gemcitabine treated patients [56].

In summary, it could be concluded that although the hand-food syndrome could limit the clinical use of PLDox, the significantly lower cardiotoxicity compared to free doxorubicin is a great advantage, and makes liposomal formulation useful in patients in which doxorubicin could be a good option but it is not used due to the cardiac risk (e.g elderly people), even in combination with other anticancer drugs. Nowadays, several clinical studies are still evaluating the safety and efficacy of PLDox in breast cancer patients as monotherapy or in combination with cisplatin, docetaxel, cyclophosphamide, and/or atezolizumab (NCT03712956, NCT03123770, NCT03164993).

3.2.2 Myocet®

This formulation consists of lamellar vesicles in which lipid bilayer is formed by phosphatidylcholine and cholesterol. Compared to stealth liposomes of Doxil®, Myocet® has a larger particle size (around 190 vs 80-90 nm) and a much faster dox release rate. Consequently, the half-life is shorter (6.7 vs 45 hours) and doxorubicin distribution volume is higher (18.8 vs 4.1 litres). Probably, the major advantage of this formulation is the reduction of doxorubicin cardiotoxicity without presenting the side effects related to PLDox, specifically the hand-foot syndrome which limit the clinical use of pegylated nanoformulation [57]. The absence of this adverse effect in non-pegylated formulation could be attributed to PEG absence, in such a way that PEG could be the responsible for

the lower particle size of PLDox and consequently for its higher accumulation at skin level, triggering hand-food syndrome.

Nowadays, the non-pegylated liposomal formulation of Dox is approved for the treatment of metastatic breast cancer as monotherapy in Europe and Canada. Moreover, several clinical studies have evaluated its therapeutic potential in combination with other drugs. In this sense, in combination with cyclophosphamide this liposomal formulation has reported a similar antitumor efficacy and a lower cardiotoxicity than free doxorubicin in patients with metastatic breast cancer, becoming a promising chemotherapeutic regimen [58]. Similar results were obtained in elderly patients with breast cancer, where the combination of non-pegylated liposomal formulation and cyclophosphamide resulted in a low rate of cardiotoxicity [59]. Other clinical trial undertaken in patients with metastatic breast cancer not previously treated with chemotherapy reported that the combination of LDox and cyclophosphamide was more efficient than the combination of LDox and vinorelbine. No clinical evidence of cardiotoxicity was appreciated in both LDox plus cyclophosphamide and LDoxplus vinorelbine treated patients [61].

Several clinical studies also showed that the co-administration of LDox and docetaxel, with or without trastuzumab was beneficial and well tolerated in metastatic carcinomas, showing an acceptable cardiac toxicity [62-64]. On the contrary, other study reported that the combination of LDox and docetaxel in patients with metastatic breast cancer, although efficient, produced an unexpected cardiac dysfunction in the 15% of treated patients [65]. Although a deeper investigation should be undertaken, these data suggest than LDox could be used instead of doxorubicin injectable solution in chemotherapeutic standard regimens, in combination with docetaxel and trastuzumab, with an acceptable cardiac toxicity. This combination seems to be useful in patients with metastatic breast cancer not previously treated with chemotherapy, and could be particularly interesting in people with cardiac risk (e.g. elderly people).

4. Nanoformulations in clinical trial.

Numerous clinical trials are ongoing to evaluate the safety and efficacy of nanoformulations of antitumor drugs in breast cancer therapy. Most of these formulations are also based on passive targeting strategy. However, some of them go further and rely on a drug release stimulation at tumour mass due to internal or external stimuli (Figure 2 and 3).

4.1 Paclitaxel nanoformulations

4.1.1 LEP-ETU ®

LEP-ETU®(liposome-entrapped paclitaxel easy to use), with a particle size around 150nm, is composed by egg phosphatidylcholine, cardiolipin, cholesterol and D-α-tocopheryl acid succinate, with a molar lipid ratio (1,2-Dioleoyl-sn-glycero-3-phosphocholine: cardiolipin:cholesterol) of 90:5:5; and loaded with paclitaxel at a lipid:drug ratio of 33:1[66,67].

Due to the absence of Cre-El, this nanoformulation shows, in general, a lower toxicity than conventional PTX. A phase I study undertaken in patients with several types of carcinoma (breast and ovarian tumours were the most common) who had received prior chemotherapy in the 99% of the cases, reported that liposomal formulation could be administered safely at higher doses compared to Taxol®. While the maximum tolerated dose of conventional PTX was 175mg/m^2, liposomal formulation reported almost a 2 fold increase tolerated dose (325 mg/m^2) and a dose limiting toxicity of 375mg/m^2 (Table 2); being febrile neutropenia and neuropathy the associated adverse events. The 23% of the liposome treated patients suffered infusion related reactions. Their severity was similar to conventional paclitaxel [68]. Other study undertaken in patients with advanced cancer (including breast tumours) showed similar results, reporting that liposomal formulation was well tolerated, and also bioequivalent with Taxol®[66]. These data suggest that LEP-ETU could be an alternative to conventional PTX.

Nowadays, this nanoformulation is under a phase II clinical trial to evaluate its efficacy and safety in patients with metastatic breast cancer who have received prior chemotherapy (NCT01190982).

4.1.2 Endo-Tag-1®

This is another liposomal entrapped paclitaxel formulation, also known as MBT-0206 or Lipopac®. It is a cationic liposomal formulation composed by 1,2-Dioleoyl-3-Trimethylammonium Propane (DOTAP), dioleolylphosphatidyl choline and paclitaxel at a molar ratio of 50:45:5, showing a particle size of 180-200 nm and a positive zeta potential [69]. Due to this positive surface charge, this formulation could target at tumour mass blood vessels. The endothelial cell lining tumour vascularization tend to have a negative charge, and positive charge complexes tend to be uptaken by these cells, triggering microvascular damage at tumour mass and an increase in tumour permeability [70,71]. Thus, the use of cationic liposomes as PTX carriers could improve antitumor efficacy.

A phase II clinical trial undertaken in patients with advanced triple negative breast cancer, reported that the administration of Endo-Tag-1® (44mg/m^2), Endo-Tag-1®(22mg/m^2) plus Taxol® (70mg/m^2) or Taxol® (90mg/m^2) show a similar efficacy. The median overall survival did not differ significantly for all treatments (13.0, 11.9 and 13.1 respectively). Although infusion related reaction were more common in patients receiving liposomal formulation, their severity was classified as mild to moderate. Finally, in combination treated patients a higher rates of 3/4 neutropenia reactions were detected [72]. Similar results were found in patients with HER-2 negative breast cancer who receiving Endo-Tag-1® (22mg/m^2) plus Taxol® (70mg/m^2) for 12 cycles, followed by 3 cycles of a combination of fluorouracil, cyclophosphamide and doxorubicin as neo-adjuvant chemotherapy. In terms of efficacy, the combination of cationic liposomal and conventional paclitaxel formulations were useful, showing an 81% reduction in tumour mass. In terms of safety, infusion related hypersensitive reactions to Endo-Tag-1® (grade 3) were observed, and 4 of 15 patients required permanent discontinuation of the Endo-Tag-1® due to these adverse events. The study was finally suspended due to these hypersensitivity reactions [73].

Nowadays, a clinical trial in patients with Visceral Metastatic Triple Negative Breast cancer is evaluating the safety and efficacy of Endo-Tag-1® in combination with paclitaxel and gemcitabine versus paclitaxel in combination with gemcitabine (NCT03002103).

4.1.3 NK-105

This is a micellar formulation of PTX with a particle size of 85 nm. It is composed by polyethyleneglycol and modified polyaspartate as hydrophobic block and contains 23% (w/w) of PTX [74].

A phase II clinical trial undertaken in patients with several kind of carcinomas (not breast cancer patients were included) reported that NK-105 micelles were in general well tolerated, showing a maximum tolerated dose of 180mg/m^2, that is similar to Taxol®(175 mg/m^2) [75]. In another phase II study it was reduced up to 150mg/m^2 with a faster infusion rate(30 minutes) [76]. The main advantage of this formulation compared to conventional PTX is the elimination of Cre-El.

Compared to Genexol®, another PTX micellar formulation, NK-105 shows higher AUC and C_{max} values, probably due to a superior plasma micellar stability [77]; and a lower MTD (Genexol® shows a MTD around 390 mg/m2) (Table 2) [78]. In another clinical trial (phase I study) undertaken in patients with several carcinomas (including breast cancer), weekly doses of 80 mg/m^2 were recommended (doses from 50-100 mg/m^2 were evaluated with infusion rates of 30 minutes). It was also reported an objective response rate of 60% in patients treated with this recommended dose, concluding that NK-105 showed desirable antitumor effects. In terms of safety, this micellar formulation was in general well tolerated, being haematological toxicity manifestations (neutropenia and leucopoenia) the most common adverse events [79].

Finally, a phase III clinical trial in patients with metastatic or recurrent breast cancer, receiving NK-105 (65mg/m^2, lower dose than recommended previously) or PTX (80mg/m^2) reported a similar antitumor efficacy in terms of progression free survival (8.4 and 8.5 months respectively), and also similar safety profile. However, some major adverse events like peripheral sensory neuropathy showed a lower incidence in patients treated with micellar formulation (1.4 vs 7.5% ≥ grade3) [80].

All these studies support the idea that NK-105 nanoformulation shows a similar antitumor efficacy compared to Taxol®, but eliminates the need of Cre-El and allow the

use of faster infusion rates (30 minutes).

4.2 Doxorubicin nanoformulation: Thermodox®

Thermodox® is the first thermosensitive formulation to reach clinic. They consist of liposomal formulation containing doxorubicin whose lipid bilayer is formed by of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphatidylcholine (a lysolipid component) and N (carbonyl-methoxypolyethyleneglycol2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (mPEG2000-DSPE), at a molar ratio of 86:10:4.Due to the presence of this lysolipid, above its transition temperature the release of the drug is really fast. In this way, at 42ºC almost 80% of doxorubicin is released in 20s. To get this temperature and trigger drug release at tumour mass, a heat system like microwave hyperthermia or High Intensity Focused Ultrasound (HIFU) is "locally" applied prior or immediately after formulation administration [59,81].

In a phase I study undertaken in patients with inoperable hepatocarcinoma, the combination of Thermodox® and radiofrequency ablation reported a significant dose-response effect. In this way, patients receiving the maximum tolerated dose (50 mg/m2) showed a median time to treatment failure of 374 days, while in patients that received lower doses was 80 days [82]. However, in a phase III study in patients with intermediate liver tumours (3-7 cm), this combination did not enhance the antitumor activity of radiofrequency ablation [83].

In breast cancer therapy, there are two clinical trials in patients with loco regional breast cancer: i) a phase I/II completed study to evaluate the maximum tolerated dose, bioequivalence, pharmacokinetics and efficacy of Thermodox® in combination with microwave hyperthermia (NCT00826085) and ii) a phase I study to evaluate the efficacy and safety of Thermodox® in combination with local hyperthermia (induced by Magnetic Resonance guided High Intensity Focused Ultrasound) and cyclophosphamide (NCT03749850).

4.3 Other anticancer drugs

4.3.1 CRL-X101

This nanoformulation consist of cyclodextrin polymeric nanoparticles containing camptothecin, a potent topoisomerase I inhibitor that shows a high anticancer activity in a broad range of tumours. However, this drug is highly toxic (producing haemorrhagic cystitis and myelotoxicity) and unstable at physiological pH, resulting its withdrawal in clinical trials [84]. In this new formulation, camptothecin is covalently bounded to a linear co-polymer of cyclodextrin and polyethylene glycol, which self-assemble forming nanoparticles. This nanoformulation have reported a good tolerability and antitumor efficacy in preclinical models of a broad range of tumours, including triple negative breast cancer [85]. It was also effective in combination with bevacizumab [86].

A phase I/II clinical study undertaken in patients with solid advanced tumours (including breast carcinomas) reported a maximum tolerated dose of 15mg/m^2bi-weekly. The most common adverse effects related to the treatment were fatigue, anaemia and neutropenia. In general, at 15mg/m^2 biweekly, this formulation reported a promising safety profile. In terms of efficacy, encouraging activity was also detected showing a median progression free survival of 3.7 months. This was higher (4.4 months) in patients with non-small lung cell carcinoma [87]. Despite these promising results, these are preliminary data and further studies are necessary.

4.3.2 NK-012

Irinotecan is a camptothecin derivate that shows high anticancer efficacy in a broad range of tumours, especially in colorectal cancer. Its active metabolite, called SN-38 is almost 100-1000 fold more potent than irinotecan. However, it shows a low aqueous solubility that difficult its administration. To overcome this, polymer-drug conjugates of SN-38 has been developed [88].

NK-102 is a nanoformulation formed by a copolymer of polyethylene glycol and poly-glutamic acid conjugated to SN-38. Due to the EPR effect, this nanocomplex trends to accumulate at tumour site, releasing the drug gradually. A phase I study undertaken in patients with several solid tumours, including breast, ovarian and colon carcinomas

among others, reported a maximum tolerated dose of 28mg/m^2every 21 or 28 days at an infusion rate of 30 minutes, being myelosupression the dose-limiting toxicity adverse effect. Patients with triple negative breast cancer reported a partial antitumor response, even at doses of 10.5 mg/$m^2$for 8 cycles [89]. Due to these promising results, a phase II trial of NK012 efficacy was conducted in women with metastatic triple negative breast cancer who had been treated previously with one or two regimens that included a taxane (NCT00951054).

4.3.3 LE-DT®

LE-DT® is a liposomal nanoformulation containing docetaxel, a taxane group anticancer drug used in a wide variety of solid tumours, including breast, prostate, gastric, non-small-cell-lung, neck and head carcinomas. As in the case of PTX, docetaxel shows a low aqueous solubility and needs the use of certain solvents (like ethanol) to its administration, which triggers several adverse events that limit the used dose [90,91]. For example, a dose of 100 mg/m^2produces severe neutropenia (grade 4) in the vast majority of the patients (75-86%). The encapsulation of docetaxel could overcome solubility challenge, no need to use organic solvents and improving the safety profile. The clinical efficacy could also be increased. Docetaxel liposomes composition is similar to LEP-ETU® nanoformulation: Dioleoyl-sn-glycero-3-phosphocholinecholesterol, cardiolipin andα-tocopheryl acid succinate [92].

A phase I study undertaken in patients with solid tumours (gastric carcinomas were the most common malignance, but the study also included patients with breast and non-small-cell lung carcinomas among others), reported that LE-DT®was well tolerated at doses in the range of 50-110 mg/m^2. No dose limiting experiences were appreciated even at the highest dose (110 mg/m^2), which is higher than that of free docetaxel. Regarding therapeutic efficacy; a clinical benefit, in terms of partial response (41%) and stable disease, was observed, recommending the administration of 85 mg/m^2or 110 mg/m^2in combination with a granulocyte colony-stimulating factor to solve neutropenia related to this dose [92]. Another phase I clinical trial undertaken in patients with solid breast, ovarian, pancreatic and non-small-cell lung cancers among others, evaluated the administration of liposomal docetaxel (15-110 mg/m^2) as neo-adjuvant chemotherapy. Although, in general, a good safety profile was reported, at dose of 110 mg/m2 two of six

patients experienced stomatitis and neutropenia as dose limiting events of , supporting the previous recommendation of doses of 85 mg/m2. In terms of efficacy, a stable disease was observed in the 75% of treated patients.

5. Nanoformulations in research

As we mention previously, the approved or in clinical trials nanoformulations are based on the concept of passive targeting to get a more selective location of the drug compared to conventional formulations. In research, the trend is to develop "more selective nanoformulations", using the active targeting approach.

For the treatment of breast cancer, the first approved active-targeting system is trastuzumab that is currently used in combination with paclitaxel in HER-2 positive metastatic breast cancer patients [93]. This antibody has been used as a ligand to decorate several chemotherapeutic nanoformulations of in order to increase their anticancer efficacy. For example, polymer nanoparticles loaded with PTX and coated with trastuzumab have reported *in vitro* a higher anticancer efficacy in HER-2 positive breast cancer cells [94]. Similar results have been obtained with lipid nanocarriers decorated with trastuzumab and loaded with several anticancer drugs including docetaxel and gemcitabine [95-97].

Other ligands, like folic acid or transferrin are also being evaluated. For example lipid-protein-nanocomplexes coated with folic acid as PTX carriers have showed an increased antitumor in mice models of triple negative breast cancers [98]. Transferrin conjugated polymeric nanoparticles enhanced the anticancer activity of doxorubicin, being effective even in dox resistant cells [99]. This support the idea of using nanocarriers as a tool to overcome drug resistances. The development of dual targeted nanoparticles are also being investigated. For example, redox responsive polymeric nanoparticles loaded with doxorubicin and coated with trastuzumab and folic acid have reported *in vitro* and in mice tumour models, a higher anticancer activity compared to free doxorubicin [100].

6. Concluding remarks

The nanoformulations on antitumor drugs that are currently approved or under clinical trials are based, with few exceptions, on passive targeting strategies. Their main advantage is their lower toxicity; showing, in general, a better safety profile compared to conventional formulations. The lower toxicity of nanoformulations, could be attributed, at least in part, to the elimination of the organic solvents that are necessary in conventional formulations to solubilize some anticancer agents. This is the case of PTX whose standard formulation (Taxol®) contains Cre-El, which produces several adverse effects (hypersensitivity reactions, neurotoxicity and nephrotoxicity among others), limiting the clinical doses of the drug product. The lower toxicity, could also be related to a "particular biodistribution profile" of nanoformulations, which tend to accumulate at tumour site decreasing the systemic exposure of free drug. In fact, the maximum tolerated doses of nanosystems are, in general, higher than those of conventional formulations, allowing the increase of clinical doses and as a consequence, the antitumor efficacy, the use in combination with other anticancer drugs, or the use in certain groups of population in which standards formulations are not indicated. This is the case of doxorubicin. On the hand, due to the higher cardiotoxicity of free doxorubicin, it cannot be concomitant administrated with certain drugs (e.g trastuzumab). However, this combination is possible with dox-nanomedicines. On the other hand, these nanomedicines can be used in elderly people, where the administration of free dox is not recommended due to their higher cardiotoxicity risk. It has to take into account, that several adverse reactions could also be attributed to these nanodevices, like in pegylated liposomes containing doxorubicin, where their different biodistribution triggers an undesirable and limiting reaction (the Palmar-Plantar Erythrodysesthesia). The elimination of polyethylene glycol coating resolved it. Hypersensitive reactions relate to the administration by infusion have also be associated with certain nanocarriers (especially liposomes). Interestingly, this adverse effect improves with the use of lower infusion rates. Despite the more selective location of the currently approved nanoformulations, and as a consequence of the anticancer drug, at tumour mass level, an increase of antitumor efficacy (at the same drug concentrations than conventional formulations) is, in the most cases, not detected. Probably, the use of active targeting nanocarriers could lead a higher accumulation of the drug at tumour cell level, thus increasing the antitumor effect. This improvement has been detected in research *in vitro* and in animal models. However, further studies are necessary to get this

new nanomedicines to clinic.

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OBJETIVOS/AIMS

A pesar de los avances llevados a cabo en terapia antitumoral, el cáncer de mama y ovario constituyen dos de las neoplasias más importantes en la población femenina. Por un lado, el cáncer de mama es el tumor más frecuente, y una de las principales causas de mortalidad por cáncer en mujeres. Por otro lado, el cáncer de ovario, aunque menos frecuente, es altamente letal. En ambas neoplasias, la quimioterapia constituye una importante estrategia de tratamiento, principalmente en estados avanzados de la enfermedad. No obstante, los tratamientos quimioterápicos disponibles hoy en día son altamente tóxicos, presentando numerosos efectos adversos importantes que limitan la dosis utilizada. En este sentido, hay una intensa investigación en la búsqueda de nuevos agentes con actividad antitumoral que presenten una baja toxicidad y que potencien el efecto de los quimioterápicos convencionales, permitiendo reducir su dosis y, por consiguiente, sus efectos adversos.

En las últimas décadas los cannabinoides han surgido como potenciales activos antitumorales. Uno de los cannabinoides más prometedores es el cannabidiol (CBD), fitocannabinoide que carece de propiedades psicoactivas. Su actividad anticancerígena, principalmente en monoterapia, ha sido evaluada en diversas neoplasias, tales como cáncer de mama, cáncer de próstata, tumores gastrointestinales, glioma y leucemia. Sin embargo, no se ha evaluado el potencial antitumoral de este activo en cáncer de ovario.

No obstante, a pesar del potencial terapéutico del CBD, este compuesto presenta una baja hidrosolubilidad y una elevada inestabilidad, lo que dificulta su manipulación y compromete el diseño de formulaciones eficaces. Esto es especialmente crítico en terapia antitumoral, donde se recurre habitualmente a la administración parenteral. En este sentido, la micro y nanoencapsulación del CBD constituirían eficaces estrategias para solventar estos problemas y administrar este activo por estas vías.

Por todo ello, el **principal objetivo** de la presente tesis doctoral es diseñar, desarrollar y evaluar sistemas de liberación modificada para aumentar la eficacia antitumoral del cannabidiol en el tratamiento de cáncer de mama y de cáncer de ovario, en monoterapia y en combinación con agentes antineoplásicos convencionales. De manera específica se trabaja con las siguientes formulaciones:

- **Micropartículas poliméricas de CBD** diseñadas para conseguir una actividad antitumoral prolongada, con una única administración, durante el mayor tiempo posible en el tratamiento de cáncer de ovario y de mama.

- **Nanopartículas poliméricas de CBD no funcionalizadas y funcionalizadas con ácido fólico** diseñadas para ser administradas vía intraperitoneal buscando una localización selectiva del activo a nivel de células tumorales en el tratamiento de cáncer de ovario.

Para alcanzar el objetivo general de la presente tesis doctoral, se abordan los siguientes **objetivos parciales**:

1. **Evaluar el potencial antitumoral del CBD en solución en monoterapia y en combinación con agentes antineoplásicos convencionales en cultivos celulares de cáncer de ovario.** Inicialmente se evalua la actividad antiproliferativa del CBD en solución en monoterapia en diversas líneas celulares de cáncer de ovario (SKOV-3, IGROV-1 y OAW-42). A continuación, se selecciona la línea celular SKOV-3, debido a su alto grado de invasividad y dificultad de tratamiento, para evaluar la actividad del CBD en combinación con paclitaxel, doxorrubicina, y cisplatino, fármacos quimioterápicos convencionales utilizados en el tratamiento de cáncer de ovario. Los resultados derivados de la consecución de este objetivo quedan recogidos en la **publicación 6** de la presente tesis doctoral.

Cabe destacar que parte de estos estudios se han llevado a cabo en colaboración con el grupo de investigación de " Terapias moleculares" del " Istituto Nazionale dei Tumori" (Milán, Italia), centrado en el desarrollo de nuevas terapias para el tratamiento de cáncer de ovario, mediante una estancia de investigación pre-doctoral (Abril-Junio 2016).

2. **Evaluar el potencial antitumoral del CBD en solución en combinación con agentes antineoplásicos convencionales en cultivos celulares de cáncer de mama.** Se han seleccionardo las líneas celulares MCF-7, como modelo de cáncer de mama positivo a los receptores hormonales, y MDA-MB-231, como modelo de cáncer de mama triple negativo. En ambas líneas, se evalua la actividad antitumoral del CBD en solución en combinación con doxorrubicina y paclitaxel. Los resultados derivados de la consecución de este objetivo quedan recogidos en la **publicación 7** de la presente tesis doctoral.

3. **Evaluar la estabilidad del CBD en solución frente a diferentes factores: temperatura, luz y oxígeno.** Los cannabinoides son, en general, compuestos altamente inestables. Algunos autores han publicado datos de estabilidad, frente a la temperatura principalmente, de extractos acuosos y oleosos de cannabinoides que contenían CBD. Sin

embargo, no hay datos sobre la estabilidad del CBD aislado. Por ello, en la presente tesis se evalua la estabilidad del CBD en solución. Previamente, seprocede a validar un procedimiento analítico por HPLC en fase reversa para la cuantificación del CBD en los estudios de estabilidad y en los estudios de caracterización de las formulaciones desarrolladas. Los resultados derivados de la consecución de este objetivo quedan recogidos en la **publicación 5** de la presente tesis doctoral.

4. **Diseñar, desarrollar y caracterizar micropartículas poliméricas de PLGA cargadas con CBD de administración parenteral.** Inicialmente, se optimiza el protocolo de obtención de las micropartículas cargadas con CBD evaluando el efecto de diferentes resomeros de PLGA con distinto peso molecular, concretamente PLGA-RG®-502 y PLGA-RG®- 504. Una vez optimizado el protocolo de elaboración se evalua el uso de diferentes ratios CBD: polímero. Las micropartículas desarrolladas se caracterizan mediante las técnicas habituales (difracción de rayos láser, SEM, DSC, contenido en activo y cesión in vitro). Debido a que la esterilidad es un requisito imprescindible en medicamentos de administración parenteral, se evalua el efecto de la esterilización por radiación sobre las características de las formulaciones desarrolladas. Así mismo, debido a la inestabilidad del CBD se llevan a cabo estudios de estabilidad física y química de las formulaciones de micropartículas. Los resultados derivados de la consecución de este objetivo quedan recogidos en la **publicaciones 6 y 7** de la presente tesis doctoral.

5. **Evaluar la actividad antitumoral de las micropartículas de CBD desarrolladas en monoterapia y en combinación con paclitaxel y doxorrubicina en cultivos celulares de cáncer de ovario.** Estos estudios se llevan a cabo en la línea celular SKOV-3. Los resultados derivados de la consecución de este objetivo quedan recogidos en la **publicación 6** de la presente tesis doctoral.

6. **Evaluar la actividad antitumoral de las micropartículas de CBD desarrolladas en monoterapia y en combinación con paclitaxel en modelos *in ovo* de cáncer de ovario.** Estos estudios se llevan a cabo sobre tumores de cáncer de ovario, derivados de la línea celular SKOV-3, que se desarrollan sobre la membrana corioalantoidea de embriones de pollo. Esta parte de la presente tesis doctoral se ha realizado en colaboración con el Dpto. de Tecnología Farmacéutica y el Dpto. de Ginecología y Obstetricia de la Universidad de Ginebra mediante una estancia de investigación pre-doctoral (Abril-Junio 2017). Los resultados derivados de la

consecución de este objetivo quedan recogidos en la **publicación 6** de la presente tesis doctoral.

7. **Evaluar la actividad antitumoral de las micropartículas de CBD desarrolladas en monoterapia y en combinación con paclitaxel y doxorrubicina en cultivos celulares de cáncer de mama.** Estos estudios se llevan a cabo en la líneas celulares MCF-7, como modelo de cáncer de mama positivo a los receptores hormonales y MDA-MB-231 como modelo de cáncer de mama triple negativo. Los resultados derivados de la consecución de este objetivo quedan recogidos en la **publicación 7** de la presente tesis doctoral.

8. Diseñar, desarrollar y caracterizar nanopartículas poliméricas de PLGA cargadas con CBD de administración intraperitoneal. Inicialmente se avalua el método más adecuado para la obtención de las nanopartículas cargadas con CBD Las nanopartículas desarrolladas se caracterizan mediante las técnicas habituales (DLS, potencial Z, SEM, DSC, contenido en activo y cesión in vitro). Los resultados derivados de la consecución de este objetivo quedan recogidos en la **publicación 8 y en la discusión** de la presente tesis doctoral.

9. Diseñar, desarrollar y caracterizar nanopartículas poliméricas de PLGA de administración intraperitoneal funcionalizadas con ácido fólico y cargadas con CBD. Con el objetivo de conseguir una distribución selectiva del activo en las células de cáncer de ovario, se desarrollan estas formulaciones. El ácido fólico se incorpora sobre la superficie de las nanopartículas ya preparadas. Las nanopartículas desarrolladas se caracterizan mediante las técnicas habituales, descritas previamente Los resultados derivados de la consecución de este objetivo quedan recogidos en la **discusión** de la presente tesis doctoral.

10. Evaluar la actividad antitumoral *in vitro* e *in ovo* en monoterapia de las formulaciones de nanopartículas desarrolladas en cáncer ovario. Estos estudios se llevan a cabo en la línea celular SKOV-3 y en los tumores desarrollados sobre la membrana corioalantoidea de embriones de pollo. El trabajo se realiza en colaboración con el Dpto. de Tecnología Farmacéutica y el Dpto. de Ginecología y Obstetricia de la Universidad de Ginebra mediante una estancia de investigación pre-doctoral (Mayo-Julio 2018). Los resultados derivados de la consecución de este objetivo quedan recogidos en la **publicación 8** de la presente tesis doctoral.

Breast and ovarian carcinomas are major health problems in female population. Breast cancer is the most frequent tumour in women worldwide, and one of the leading causes of cancer death in this group. Ovarian cancer, although shows a lower frequency, is a highly lethal disease. In both types of tumours, chemotherapy represents an essential treatment strategy, especially in advanced stages of the disease. Nevertheless, chemotherapeutic agents are really toxic, which limits their dose. In this way, the search of novel antitumor agents that shows a lower toxicity and potentiate the activity of conventional chemotherapy is highly desirable.

In the last decades, cannabinoids have emerged as potential anticancer drugs, and cannbidiol (CBD) that lacks of psychoactive properties, is one of the most promising cannabinoids. The anticancer efficacy of CBD, especially in monotherapy, has been evaluated in a broad range of tumours, including breast and prostates carcinomas, gastrointestinal neoplasms, glioma and leukaemia. Nevertheless, CBD antitumor activity has not been tested in ovarian cancer.

Despite the potential therapeutic use of CBD, its shows a high lipophilicity and several stability problems, hampering its manipulation and the development of effective formulations. This is especially critical in antitumor therapy, where parenteral administration is usually used. Micro and nanoencapsulation could be a good technological strategy to resolve these challenges and administer CBD by this route.

Against this background, the main aim of this thesis is to design, develop and characterise controlled release systems of cannabidiol to improve its antitumor efficacy in breast and ovarian cancer, administer as monotherapy or in combination with conventional chemotherapeutics. Specifically, it will be developed the following formulations:

- **Polymeric microparticles loaded with CBD** designed to get an extended antitumor activity, after a single administration, in the treatment of ovarian and breast cancer.
- **Non-coated and folic acid-coated polymeric nanoparticles loaded with CBD** designed to be administered intraperitoneally, getting a selective location at tumour cells in ovarian cancer treatment.

This global aim can be broken down into three specific aims:

1. **To evaluate the antitumor potential of CBD in solution as monotherapy or when combined with conventional antineoplastic agents in ovarian cancer cells**. Firstly, the antiproliferative activity of CBD was evaluated in IGROV-1, OAW-42 and SKOV-3 ovarian cancer cells. Then, SKOV-3 cell line was selected for combination experiments due to its high invasiveness. Paclitaxel, doxorubicin and cisplatin were selected as conventional chemotherapeutics in ovarian cancer treatment. **Publication 6** tackles this specific aim.

Part of this specific aim has been achived in collaboration with " Molecular Therapies" group of "Istituto Nazionale dei Tumori" (Milán, Italia), that is focused on the development of novel therapies of ovarian cancer treatment, thanks to a pre-doctoral research stay (April-June 2016).

2. **To evaluate the antitumor potential of CBD in solution when combined with conventional antineoplastic agents in breast cancer cells**. MCF-7 and MDA-MB-231 cells were used as model of hormone receptor positive and triple negative breast tumours respectively. Paclitaxel and doxorubicin were selected as conventional chemotherapeutics in breast cancer treatment. **Publication 7** tackles this specific aim.

3. **To evaluate the stability of CBD against several factors: temperature, light and oxygen.** Cannabinoids are highly unstable compounds, and several authors have reported stability data of cannabis extract containing CBD. Nevertheless, not stability studies have been published in respect of pure CBD. An HPLC analytical method was previously validated for CBD quantification. **Publication 5** tackles this specific aim.

4. **To design, develop and characterise parenteral PLGA polymeric microparticles loaded with CBD.** Microencapsulation protocol was previously optimised by evaluating the effect of PLGA molecular weight (specifically PLGA-RG®-502 y PLGA-RG®- 504) and different drug: polymer ratios was also evaluated. Obtained formulations were characterized by laser diffraction, SEM and DSC. Drug loading and in vitro drug release were also determined. Due to the fact that sterilization is required for parenteral formulation, the effect of γ irradiation on microparticles characteristics was

also evaluated. Finally, due to the instability of CBD, the stability of CBD loaded-microparticles was also studied. **Publications 6 and 7** tackle this specific aim.

5. **To evaluate the antitumor activity of CBD microparticles as monotherapy or in combination with paclitaxel and doxorubicin in ovarian cancer cells. SKOV-3 cell line was used as model of ovarian cancer. Publication 6** tackles this specific aim.

6. **To evaluate the antitumor activity of CBD microparticles as monotherapy or in combination with paclitaxel in an "*in ovo* model" of ovarian cancer.** For this studies, SKOV-3 derived tumours were formed on the chorioallantoic membrane of chick embryo. **Publication 6** tackles this specific aim.

This specific aim has been achieved in collaboration with the Department of Pharmaceutics and the Department of Gynaecology and Obstetrics of Geneva University, thanks to a pre-doctoral research stay (April-June 2017).

7. **To evaluate the antitumor activity of CBD microparticles as monotherapy or in combination with paclitaxel and doxorubicin in breast cancer cells.** MCF-7 and MDA-MB-231 cells were used as model of hormone receptor positive and triple negative breast tumours respectively. **Publication 7** tackles this specific aim

8. To develop, design and characterised PLGA polymeric nanoparticles loaded with CBD for intraperitoneal administration. The elaboration protocol was optimised and developed nanoparticles were characterised by DLS, zeta potential, SEM, DSC, drug loading and in vitro drug release. **Publication 8 and discussion** tackle this specific aim

9. To develop, design and characterised folic acid coated PLGA polymeric nanoparticles loaded with CBD for intraperitoneal administration. This formulation was developed to get a selective location of CBD in ovarian cancer cells. Folic acid was incorporated on nanoparticle surface. This formulation was characterised by the techniques described previously**. Discussion** tackles this specific aim.

10. To evaluate the *in vitro* and *in ovo* antitumor activity of CBD nanoparticles as monotherapy in ovarian cancer. These studies were undertaken using SKOV-3 cells and in SKOV-3 derived tumours that were formed on chrorioallantoic membrane of chick embryo. **Publication 8** tackles this specific aim.

RESULTADOS/ RESULTS

En las últimas décadas los cannabinoides han ganado un importante potencial terapéutico en numerosas patologías, existiendo cada vez más formulaciones comercializadas a base de estos compuestos. De entre todos los cannabinoides, el Δ^9-Tetrahidrocannabinol (Δ^9-THC) y el cannabidiol (CBD), son los más prometedores. Sin embargo, los efectos psicoactivos característicos del Δ^9-THC limitan su uso terapéutico. El CBD no sólo no es psicoactivo, sino que contribuye a disminuir los efectos psicótropos del Δ^9-THC [1-3].

A pesar del importante potencial terapéutico que presentan los cannabinoides en general y el CBD en particular, su elevada liposolubilidad [log P(CBD) = 6.33, solubilidad en agua ≈10μg/ml] dificulta su manipulación y su dosificación. Este hecho, junto con su intenso metabolismo dificulta el desarrollo de formulaciones farmacéuticas con una biodisponibilidad adecuada. Por ejemplo, la mayoría de las preparaciones disponibles son de administración oral (cápsulas, soluciones oleosas y comprimidos). Sin embargo, por esta vía la biodisponibilidad de los cannabinoides es muy baja (6-13%) y errática. Esta errática biodisponibilidad explicaría las diferencias en la sensibilidad de los sujetos (en cuanto a efectos terapéuticos y adversos) a los cannabinoides. El uso de formulaciones de administración bucal (hay disponible un aerosol comercializado a este nivel) evita el efecto de primer paso e incrementa, aunque ligeramente, su biodisponibilidad (10-25%). Por vía inhalatoria la biodisponibilidad de los cannabinoides es también mayor pero altamente variable (2-56%) [1, 4, 5].

Para el uso de los cannabinoides como agentes quimioterápicos, se precisará el desarrollo de formulaciones de administración parenteral, lo cual supondría un problema dada la elevada liposolubilidad del CBD. Al igual que ocurre con otros antitumorales, el caso más típico es el del paclitaxel, para la elaboración de soluciones acuosas de CBD se requiere el uso de co-solventes orgánicos como el etanol y/o de agentes dipersantes como el Cremophor® o el Tween® para su administración [6-9]. Estos compuestos incrementan la toxicidad de las formulaciones. De hecho, las formulaciones convencionales de paclitaxel utilizan una mezcla de etanol: Cremophor® como solubilizante, presentando una toxicidad elevada y limitando la dosis terapéutica [10, 11].

Además, los cannabinoides son moléculas que presentan una elevada inestabilidad frente a numerosas factores como temperatura, luz y oxidación [12, 13]. Esta elevada inestabilidad dificulta aún más el desarrollo de formulaciones. Es importante destacar que la mayoría de los estudios de estabilidad realizados hasta la fecha se centran en el Δ^9-THC. También hay datos sobre la estabilidad de diversos extractos oleosos e infusiones de la planta de cannabis [14, 15]. Sin embargo, apenas existen estudios sobre la estabilidad del CBD puro, y los que hay se centran más en su reactividad química que en aspectos cinéticos. Precisamente uno de los objetivos de la presente tesis doctoral es la evaluación de las características de estabilidad de disoluciones de CBD puro con el fin de garantizar su correcta manipulación, dosificación y evaluación tanto *in vitro* como *in vivo*, y el desarrollo de formas farmacéuticas en las que la estabilidad del principio activo no se vea comprometida (resultados recogidos en la **publicación 5** de la presente tesis doctoral).

Una de las estrategias tecnológicas disponibles para resolver esos problemas de solubilidad e inestabilidad del CBD que dificultan el desarrollo de formulaciones farmacéuticas efectivas por vía parenteral es la microencapsulación. En la presente tesis doctoral se plantea el desarrollo de micropartículas poliméricas como transportadores de CBD. Por un lado, estos sistemas evitarían el uso de solventes orgánicos y/o agentes dispersantes, ya que se pueden administrar por vía parenteral en forma de suspensión en soluciones fisiológicas. Por otro, podrían proteger al CBD e incrementar su estabilidad. Además, estos sistemas permiten obtener una liberación prolongada del principio activo con una única administración [16, 17], especialmente importante cuando se recurre a la vía parenteral para tratamientos prolongados como en terapia antitumoral. De hecho, estudios previos en nuestro grupo de investigación con micropartículas poliméricas cargadas con Δ^9-THC han demostrado una eficacia antitumoral prolongada en un modelo xenográfico de glioblastoma humano inducido en ratones [18].

De entre todos los polímeros disponibles, el co-polímero del ácido poli-láctico-co-glicólico (PLGA) es uno de los más utilizados en la microencapsulación de sustancias bioactivas, debido a su alta biocompatibilidad y su biodegradabilidad. De hecho, está aprobado por la FDA, existiendo comercializadas diversas formulaciones que lo contienen. Además, desde el punto de vista tecnológico, presenta una gran versatilidad,

al haber disponibles numerosos co-polímeros con distinto peso molecular e hidrofilia que permiten modificar las características de los sistemas desarrollados (como tamaño, carga y liberación del activo) y obtener los sistemas que mejor se adapten a nuestros objetivos [19-21].

Por ello, en la presente tesis doctoral se plantea el desarrollo de micropartículas de PLGA como transportadores de CBD, que permitan un efecto antitumoral prolongado con una única administración para mejorar los tratamientos quimioterápicos convencionales de cáncer de ovario (resultados recogidos en la **publicación 6**) y de cáncer de mama (resultados recogidos en la **publicación 7**).

Por otro lado, la nanoencapsulación sería otra buena estrategia tecnológica para resolver los problemas de administración del CBD. En el caso de cáncer de ovario, uno de los principales problemas derivados de la reserción quirúrgica del tumor es el riesgo de diseminación de las células tumorales por la cavidad peritoneal, por lo que el desarrollo de nanopartículas de administración intraperitoneal sería una interesante estrategia de tratamiento. El uso de nanosistemas como transportadores de antitumorales incrementarían la retención del fármaco en la cavidad peritoneal comparado con la administración del fármaco libre [22, 23]. Por ello, en la presente tesis también se plantea el desarrollo de nanopartículas poliméricas de PLGA de administración intraperitoneal cargadas con CBD para el tratamiento de cáncer de ovario (resultados descritos en la **publicación 8**).

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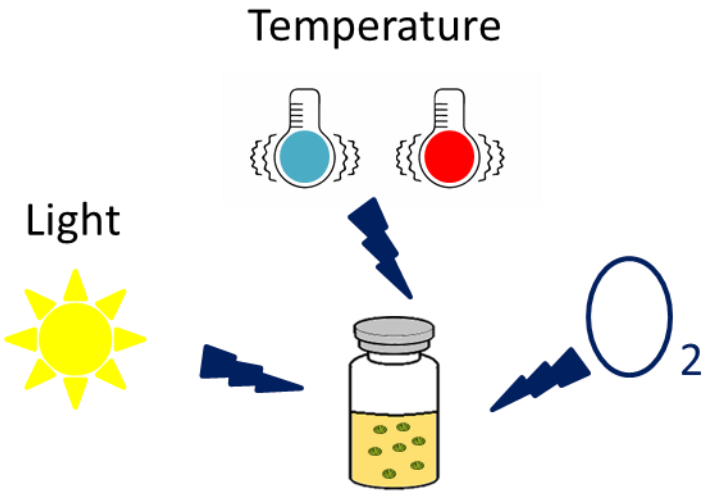
PUBLICACIÓN 5

Stability characteristics of CBD of interest for the design of pharmacological, biochemical and pharmaceutical studies



Stability characteristics of CBD of interest for the design of pharmacological, biochemical and pharmaceutical studies

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ABSTRACT

Cannabidiol (CBD) is one of the most promising cannabinoids in therapeutics. Nevertheless, no stability testing has been reported of pure CBD. The aim of this work was to evaluate the stability of pure CBD in solution. An HPLC analytical method, with CBD in ethanol, was previously verified as suitable for these studies. The resulted method was lineal and proportional in a range of concentrations of 1-150 μg CBD/ml and precise. It also resulted suitable to quantify CBD in aqueous medium (water with Tween at 0.5%) as reported accuracy studies. The stability of CBD was influenced by multiple factors. Temperature was one of the most critical parameters, with an activation of 92.19KJ/mol. At room temperature, CBD was high unstable (t_{95}=117.13days). However, at 5ºC it was stable for at least 12 months. CBD was also sensible to oxidation, with a short t_{95} of 1.77 daysin oxidizing environments, and to light. The photolytic reaction seemed to be oxidative. The solvent influences CBD stability, being CBD more stable in ethanol than in aqueous medium. In fact, in simulated physiological conditions (pH 7.4 and 37ºC) 10% of CBD was degraded within 24 hours. These studies indicate that CBD is highly unstable, what should be taken into account in the design of in vitro and in vivo studies and in the pharmaceutical development of dosage forms.

Keywords: Cannabidiol, Degradation, Stability, Shelf-life, Oxidation.

1. Introduction

Stability testing is a critical part of the pharmaceutical development. It is essential to evaluate the environmental and formulation factors (like temperature, pH, humidity and light) that could influence on the physical, chemical, microbiological, toxicological and therapeutic stability of a drug product in order to establish the optimal materials and processes during pharmaceutical development and to determine the best storage conditions [1].

In the last decades, cannabinoids, the active compounds of "Cannabis plant", have garnered a great deal of interest as potential new therapeutic agents for the treatment of different pathological conditions including neurodegenerative disorders (e.g. multiple sclerosis and epilepsy), pain, nausea, vomiting, anxiety or cancer [2-6].In fact, several formulations (tablets, capsules, oro-mucosal spray and creams) are avalaible for the management of several conditions including loss of appettite, nausea and vomiting related to chemotherapy, convulsions, and pain [7]. Among all cannabinoids, cannabidiol (CBD) is one of the most promissing candidates.

Regarding cannabinoids stability, these compounds have shown, in general, a high instablity. However, most of the studies have been focused on Δ^9-tetrahydrocannabinol (Δ^9-THC), the main cannabis constituent and the major responsible for cannabis psychedelic properties of cannabis plant preparations. Δ^9-THC have demonstrated to be thermolabile [8], sensitive to oxidation [9], unstable in acid solutions [10] and photolabile [11]. As the best of our knowledge, not stability studies have been published in respect of pure CBD.

Therefore, the aim of this work wastoevalute the stability of pure CBD in solution. For this, the effect of temperature, oxigen, radiations and physiological conditions on dissolutions of CBD was evaluated and a HPLCanalytical method was verified as suitable for these stability studies.

2. Materials and methods

2.1 Chemicals

Cannabidiol (CBD) was purchased from THC-Pharma (Frannkfort, Germany). Methanol and acetonitrile HPLC grade were obtained from Fisher Scientific (Madrid, Spain). Hydrochloric acid, sodium hydroxide, ethanol absolute and Tween®-80 were provided by Panreac (Barcelona Spain). Distilled demineralized Milli-Q® water (Millipore, Spain) was used. All chemicals and reagents were used as received.

In order to avoid cannabidiol glass and plastic binding, all material was pre-treated with Sigmacote® (Sigma, Missouri, USA).

2.2 Analytical method

CBD samples were analyzed by HPLC using anAgilent 1200 series Liquid Chromatgraph with Agilent Chemstation software (Agilent technologies, California, USA).

The chromatographic conditions used were based on the analytical method described by Munjal et. al [8]. As stationary phase a Mediterranea®-Sea C18 (150 x 4.6 mm i.d., 5 μm) column was used (Teknokroma, Barcelona, Spain) and the mobile phase consisted of a mixture of methanol, acetonitrile and water (52:30:18 v/v) adjusted to pH of 4.5 with acetic acid. It was filtered through a Millipore® membrane filter (47mm membrane, 0.45μm pore size, nylon) and degassed in an ultrasonic bath before use. An isocratic analysis, at a flow rate of 1.8 mL/min and at room temperature was performed. The injection volume was 20 μL and the detection wavelength was set at 228 nm. The quantification of CBD was carried by measuring the peak-area count.

CBD stock solution in ethanol at a concentration of 1mg/ml, was prepared and stored at -20ºC. Validation characteristics were determined according to the ICH Q2 (R1) guidelines [12].

Linearity was evaluated using three freshly prepared calibration curves (covering the range of 1-150μg/mL) prepared from three CBD stock solutions (1mg/mL).

The limit of detection (LOD) and limit of quantification (LOQ) were determined using the following equations:

$$LOD: 3.3SD/b \text{ and } LOQ= 10\ SD/b$$

where SD and b are the standard deviation of the response (γ-intercept standard deviation was used) and the slope respectively of a calibration curve performed with 5 concentrations close to LOQ (1-10 μg/mL).

The precision of the method was evaluated as intra-day variability (repeatability) and inter-day variability (intermediate precision). Repeatability was determined by measuring CBD concentrations of 5,50 and 100 μg/mL(standard solution), 3 replicates of each, on the same day. The intermediate precision was determined measuring three CBD concentrations (5,50 and 100 μg/mL), 3 replicates of each, on three consecutive days. The mean values and variation coefficient (CV) was calculated in both cases.

Accuracy was determined with three concentrations of CBD in demineralised water with Tween 80 at 0.5% (v/v) (as CBD solubilising agent), 3 replicates of each, prepared from three stock solutions (1mg/mL) in the same solvent.

Specificity was determined from the analysis of the chromatograms of samples of the different stability studies covering degradation products from light, heat and oxidation. Purity of the peak corresponding to CBD was determined by diode array.

System suitability was also evaluated to confirm that the equipment was adequate for the analysis to be performed. This test was carried out by making replicate injections of the standard solution (100 μg/mL of CBD) and determining the retention time, column efficiency as number of theoretical plates (N) and symmetry factor.

2.3 Temperature-dependent stability

Briefly, a standard solution of CBD in ethanol (100 μg/ml) was prepared and disposed into glass ampoules that were closed and stored in refrigerator at 5±3ºC and in thermostatic chambers (Memmert UN30, Germany) at 25, 40, 50, 60 and 70ºC±2ºC. All samples were maintained in dark. At predetermined time intervals, 4 ampoules of each condition (n=4) were analysed by HPLC. The degradation rate constant (K) was

calculated for each storage temperature and the relationship between K and the temperature (T) was established by means Arrhenius equation:

$$\ln K = \ln A - E_a/RT$$

where E_a is the activation energy, A and R are a frequency factor and gas constant respectively and T the absolute temperature (ºK).

The time interval required for the degradation of 5% of the drug in the solution (t_{95}) was extrapolated at 5ºC and at 25ºC from Arrhenius equation. These values were compared with those calculated from data of the stability study at both temperatures.

2.4 Oxygen-dependent stability

To evaluate the stability of CBD against oxidation three CBD solutions (100µg/ml) were prepared using as vehicle:

- Demineralised water with Tween 80 at 0.5% (v/v): samples (O±);
- Demineralised water with Tween 80 at 0.5% (v/v) and H_2O_2 at 3% (v/v): samples (O+);
- Demineralised water with Tween 80 at 0.5% (v/v) and flushing with nitrogen to displace the dissolved oxygen: samples (O-).

CBD solutions were disposed into glass ampoules, which were properly closed and placed into a stability chamber at 25ºC±2ºC. At predetermined time intervals four samples (n=4) of each solution were analysed by HPLC.

2.5 Photo-stability studies

The stability of CBD against light was evaluated following ICHQ1B.

A standard solution of CBD in ethanol (100 µg/ml) was prepared and disposed into glass ampoules that were closed and placed into a photo-stability chamber (CCI: Xenoterm 1500-RF, CCI, Barcelona, Spain)equipped with a D65/ID65 xenon lamp for 72 hours in order to guarantee an overall illumination greater than 1.2 million of lux h and an integrated near ultraviolet (above 320 nm) energy greater than 200 W h/m^2 [13]. Ampoules covered with aluminium foil and stored in the same chamber were used as controls. At each time point, 4 ampoules were analysed by HPLC. These studies were

undertaken at 5 and 25ºC.

Ampoules flushed with nitrogen before closing were also stored in the photo-stability chamber at 5ºC±3ºC.

2.6 Stability in simulated physiological conditions

To simulate physiological conditions, a CBD solution (100 µg/ml) was prepared in phosphate buffer solution (pH 7.4) containing Tween 80 at 0.5% (w/v). This solution was degasified in an ultrasonic bath for 40 minutes to eliminate the oxygen and incubated at 37ºC±0.5ºC in a thermostatic sacking bath (agitation of 100 rpm) for 10 days. At predetermined time points, samples were extracted, filtered and analysed by HPLC. 4 samples (n=4) were analysed at each time point.

2.7 Analysis of data

All the data are presented as the mean ± standard deviation. The fitting of the stability profiles was carried out by the least squares in such a way that the slope of the curves directly corresponds to the degradation rate constant. Statistically analysis was realized using Statgraphics Centurion XVIII (Statgraphics Technologies, Virginia, USA). All the graphs were done using Origin 2017 software (Origin lab, Massachusetts, USA).

3. Results

3.1 Analytical method

As illustrated in figure 1A, the chromatogram of the standard CBD solution showed a sharp large peak separated from the solvent front with a retention time of 4.72 minutes.

The linearity results (summarized in table 1) indicated a strong correlation between the two variables in the range of 1-150 µg/ml, so more than 99.87% of the variation observed in the peak area was explained by the variation in the CBD concentration (figure 1B).The F-lack of fit ANOVA ($p > 0.05$) showed that the two variables (area and concentration) fitted a linear model. Moreover, the analytical method

was proportional, with a p value> 0.05 in the t-test for the intercept. The LOD and LOQ were estimated to be 246.78 and 747.84 ng/ml respectively.

Regarding precision, the CV values obtained for intra-day and inter-day variability are shown in table 2. All of them were lower than 2%. With these results, three replicates were enough to maintain a precision in the results below ±2%.

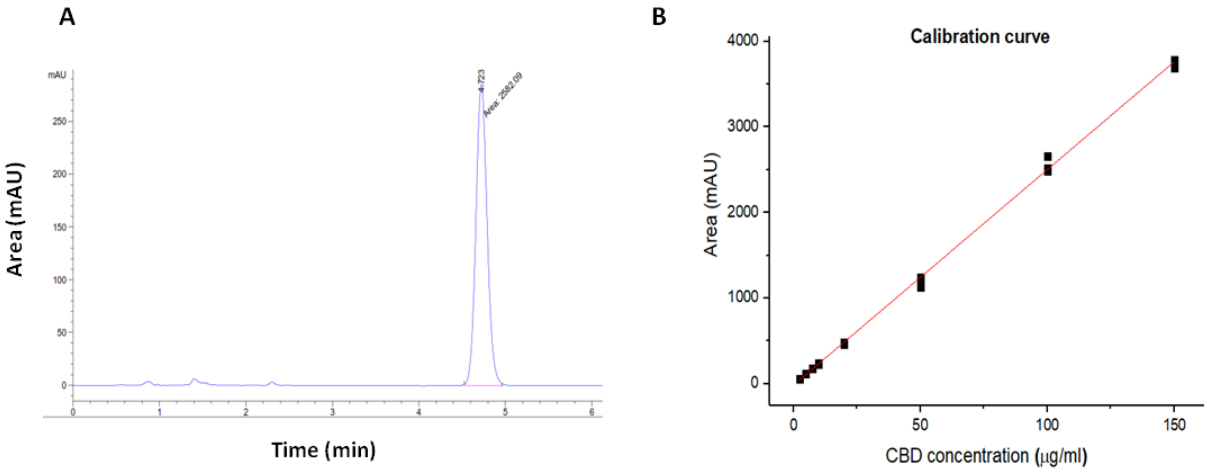
Figure 2B shows the results obtained in the study of accuracy carried out on samples of CBD dissolved in water with 0.5% Tween 80 and using the calibration curve in ethanol. All the recovery percentages were in the interval of 99.2-101.6%, with, a mean recovery close to 100% (100.4 ± 1.20%). Moreover, the plotting of estimated concentration versus actual value was fitted to a linear model with a slope close to 1 and an intercept statistically equal to 0 (figure 2A).

The system suitability parameters that guarantee the right working of the HPLC system are depicted in figure 1C.

Parameter	**Result**
Linearity range (µg/ml)	1-150
Correlation coefficient (r)	0.9994
Determination coefficient (r^2)	0.9987
Slope "b" (mUA·ml·µg^{-1})	25.2095
SD slope (mUA·ml·µg^{-1})	0.1932
Intercept (mUA)	-17.9863
SD intercept (mUA)	13.1666
" F " ANOVA test for regression (p)	17015.510 (0.0000)*
" F " ANOVA test for lineal model(p)	1.5186 (0.2385)
"t" Student's test for proportionality (p)	-1.3660 (0.1863)

*Statistical significance (p value<0.05)

Table 1: Linearity statistical parameters



C

System suitability

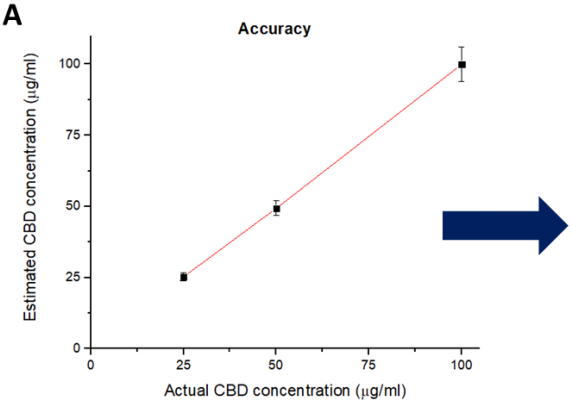
Retention time (min)	4.723
Theoretical plate number (N)	15862
Symmetry factor	0.892

Figure 1: Chromatogram of CBD solution of 100 µg/ml (A), calibration curve of CBD (1-150 µg/mL) (B) and system suitability parameters (C).

CBD concentration (µg/ml)	**Intra-dayvariability (CV, %)**	**Inter-dayvariability (CV, %)**
5	0.796	1.295
50	1.700	1.865
100	1.332	1.723

Table 2: Precision data for HPLC method validation.

A



B

PARAMETER	RESULT
R (%)	100.401
CV (%)	1.204
Correlation coefficient (r)	0.993
Determination coefficient (r^2)	98.694
Slope "b" ± SD	0.997± 0.028
Intercept ± SD	-0.003± 1.898
" F " ANOVA test for regression (p)	1208.82 (0.000)*
" F " ANOVA test for lineal model (p)	0.16 (0.609)
"t" Student's test for proportionality (p)	-0,00158 (0.998)

Figure 2: Graphical representation of actual drug concentration of CBD samples dissolved in water with Tween 80 (0.5%) versus the values obtained from calibration curve of linearity study (A) and statistical parameters of this study (B).*p value<0.05.

3.2 Temperature-dependent stability

Temperature is one of the critical factors that compromises cannabinoid stability. In this work, the effect of temperature was evaluated by storing CBD solutions in ethanol at different temperatures in the range of 5-70ºC. As shown in figures3A and 3Bthe higher the temperature the higher the degradation rate of CBD, except at 70ºC where no differences in the degradation profile with that obtained at 60ºC were found. The values of % remaining of CBD vs time for each storage temperature were fitted to first order kinetic, obtaining the parameters of table 4. A statistically significant correlation between both variables was detected for all temperatures except 5ºC. With data obtained at 25ºC a value of t_{95} of 117.13±17.66 days was obtained.

	Temperature (ºC)					
T (ºC)	**5**	**25**	**40**	**50**	**60**	**70**
r	0.3588	-0.8070	-0.9452	-0.9507	-0.9771	-0.9885
r^2(%)	12.88	65.12	89.35	90.39	95.49	97.71
$F_{regression}$	2.07*	31.74	76.62	123.17	165.57	382.96
s	---	0.03593	0.10141	0.05007	0.11716	0.07893
K (day^{-1})	---	4.38E-4	2.682E-3	6.722E-3	0.023600	0.022579
SD_K	---	7.77E-5	4.14E-4	8.95E-4	2.093E-3	1.410 E-3
t_{95} (days)		117.13±17.66				

Table 4: Degradation rate constants of CBD at different temperatures after fitting the data

to first order kinetic. r (correlation coefficient), r^2 (determination coefficient), F ("F ANOVA" for regression model), s (standard error), K (degradation constant), SD_k (standard deviation of degradation constant) t_{95} (time in which is degraded the 5% of the drug).*p >0.05.

Statistically significant differences were found among degradation rate constants (K) obtained at 25ºC; 40ºC; 50ºC and 60ºC, but not between K_{60} and K_{70}. Therefore, only K values obtained at 25ºC, 40ºC, 50ºC and 60ºC were fitted to Arrhenius equation obtaining the parameters shown in table 5. It is worth mentioning the high correlation between both variables, with a value of r =-0.9983, and the good fit to the proposed model ($F_{lack\ of\ fit}$=1.34; p= 0.3181). From the slope of the curve, a value of E_a= 92.19KJ/mol was calculated.

When the value of t_{95} at 25ºC was extrapolated from the parameters of the Arrhenius equation, 118.85days were obtained. At last, the value of t_{95} at 5ºC was also extrapolated from Arrhenius equation, being of 4.76 years.

As depicted in figure 3 C, numerous peaks, corresponding to CBD degradation products can be observed in the chromatograms of degraded samples, being the peaks that appeared around 2.8 and 5.6 minutes the most representative.

r	-0.9983	**K_{25}extrap. (d^{-1})**	4.316E-4
r^2(%)	99.66	**t_{95} (25ºC) (days)**	118.85
s	0.118941	**K_5extrap. (d^{-1})**	2.9546E-05
b (1/ºK)	-11107.4	**t_{95} (4ºC) (days)**	1736.03
SD_b(1/ºK)	455.311		
Ea (KJ/mol)	92.1914		
a	29.5251		
SD_a	1.4411		
A (d^{-1})	6.64642E+12		
$F_{regression}$(P)	595.13 (0.0017)		
$F_{lack\ of\ fit}$(P)	1.34 (0.3181)		

Table 5: Parameters of Arrhenius equation. r (correlation coefficient), r^2 (determination coefficient), s (standard error), b (slope), SD_b(standard deviation of the slope), E_a

(activation energy), a (intercept), SDa (standard deviation of the intercept), A (frequency factor), $F_{regression}$("F ANOVA" for the regression model), $F_{lack\ of\ fit}$ ("F ANOVA" for the lack of fit test), K_x (degradation rate extrapolated at each temperature), t_{95} (time in which is degraded the 5% of the drug).

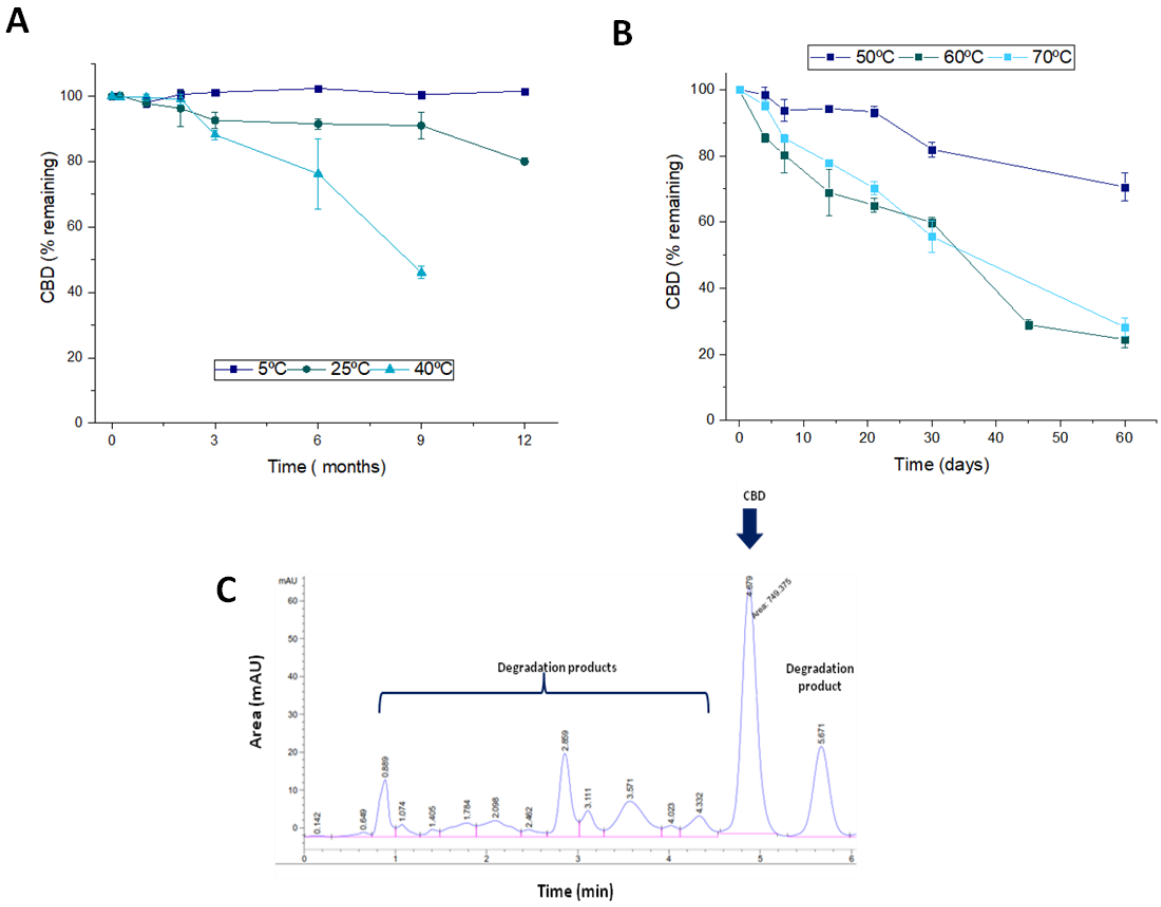


Figure 3: Stability studies of CBD in solution stored at 5, 25 and 40ªC (A) and 50, 70, and 60ºC (B). Representative chromatogram of a CBD solution of 100µg/ml after exposed to 40ºC for 12 months (C).

3.3 Oxygen-dependent stability

The results of the study of the stability of CBD against oxygen are depicted in figure 4A. In all the cases CBD degradation was detected, but more intensive when H_2O_2 was incorporated in CBD solution (samples O+), especially in the first days of the assay (20% of drug degradation was detected after 10 days of storage), and less intensive in

samples flushed with nitrogen (O-). These data were fitted to first order kinetic, obtaining the parameter showed in table 6. For the three CBD solutions statistically significant correlation between drug concentration and storage time was demonstrated. The values of degradation rate constant (K) allowed to calculated the values of t_{95} included in the table, according to which 5% of the drug is degraded in less than 2 days in oxidizing environment while this degradation percentage is not reached until 20 days in an oxygen-free solution.

	O+	**O+/-**	**O-**
r	-0.873	-0.8158	-0.8850
r^2(%)	76.22	66.56	78.32
$F_{regression}$ (p)	60.90 (0.00)	29.86 (0.0001)	72.26 (0.00)
$F_{lak\ of\ fit}$ (p)	0.49 (0.8423)	3.50 (0.050)	1.90 (0.1505)
s	0.26646	0.08831	0.022871
K ($days^{-1}$)	0.02901	0.006752	0.002605
SD_k($days^{-1}$)	0.003717	0.001236	0.000303
t_{95} (days)	1.77	7.60	19.69

Table 6: Parameters obtained from the oxygen dependent study fitting to a fist order kinetic. r (correlation coefficient), r^2 (determination coefficient), $F_{regression}$ ("F ANOVA" for the regression model), $F_{lack\ of\ fit}$ ("F ANOVA" for the lack of fit test), s (standard error), K_x (degradation rate), SD_k (standard deviation of degradation constant), t_{95} (time in which is degraded the 5% of the drug).

As depicted in the chromatograms of figure 4C and 4D, the main degradation products of CBD appeared around 2.9 and 5.8 minutes. Moreover, in the N_2-flushed solution (figure 4B), these degradation products were also detected, although their percentage in the sample was significantly lower than in non-oxygen-free solutions.

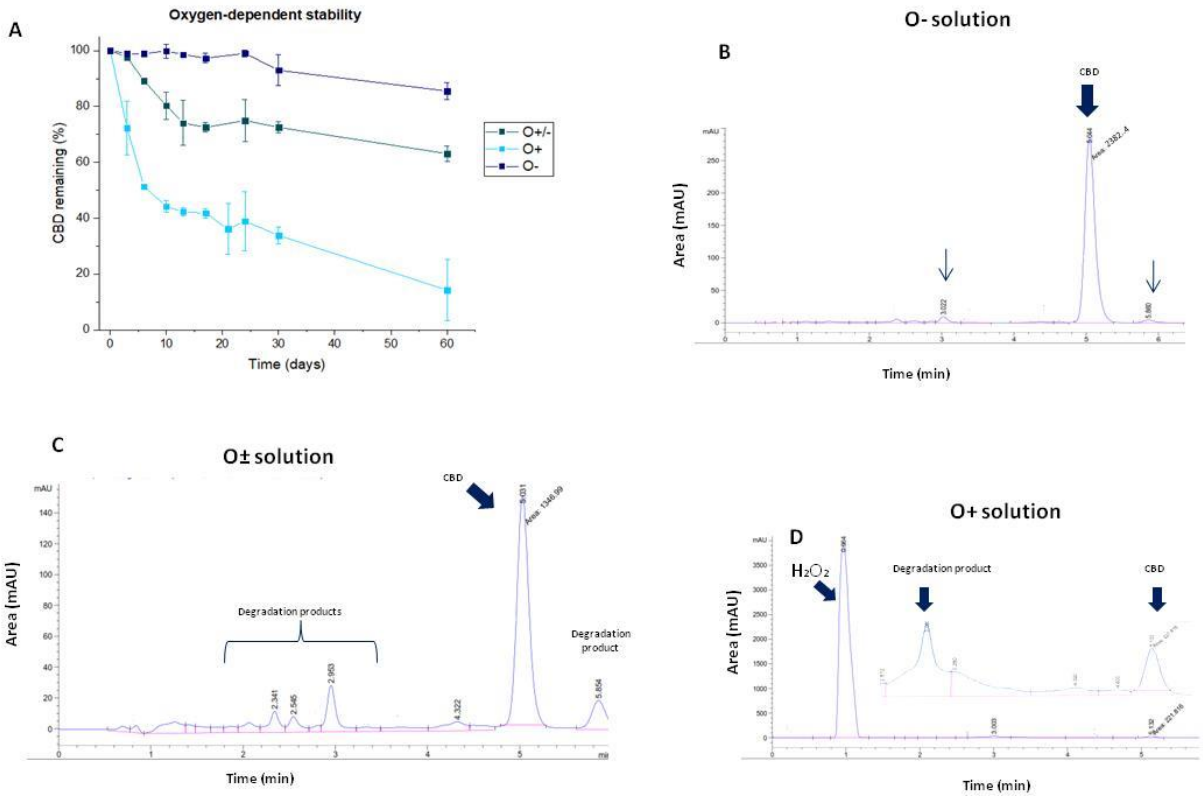
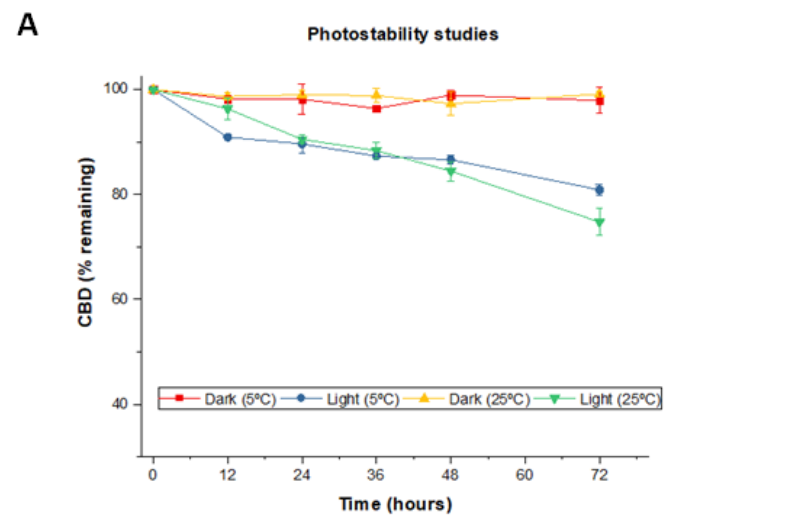


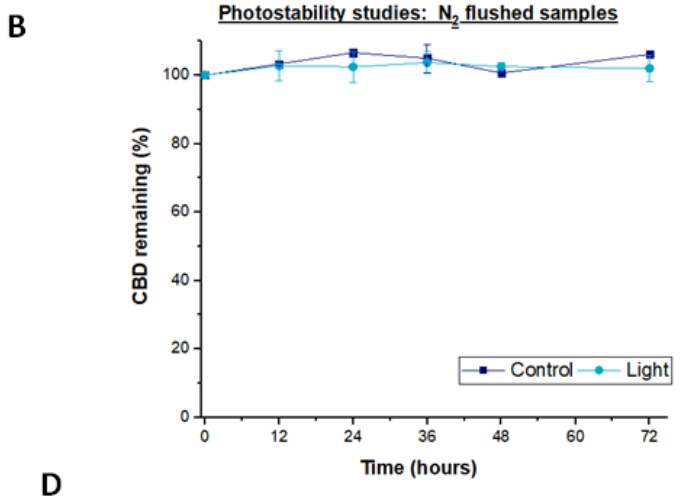
Figure 4: Oxygen dependent stability studies of CBD in solution (A). Representative chromatograms of CBD in solution (100µg/ml) from oxygen degradation studies (2 month samples) (B-D).

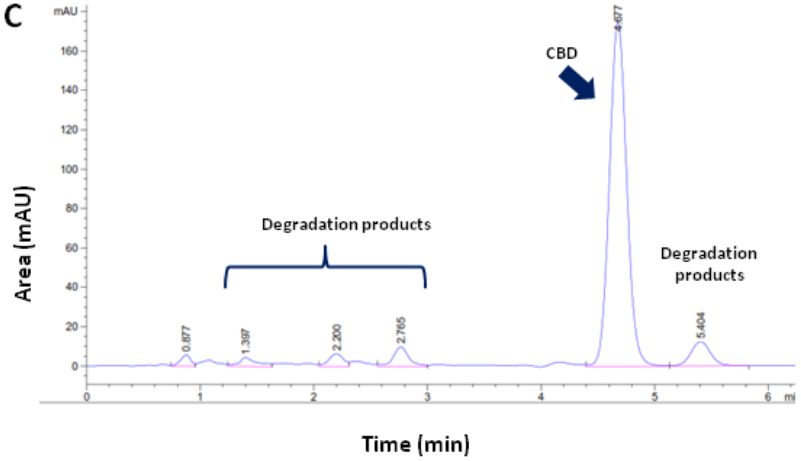
3.4. Photo-stability studies

As it is observed in figure5A, while CBD solutions stored in darkness remained stable during all the experiment, samples exposed to light exhibited a CBD degradation, lightly more intensive at 25ºC with a loss of 25% of drug. In effect, when the data were statistically analysed (figure 5D), no correlation between CBD concentration and storage time higher than 25% was found in samples stored in darkness, while in samples irradiated, correlation was higher than 87%.

Due to the influence of oxygen on CBD stability, to determine the participation in light instability of CBD, nitrogen flushed solutions was also analysed at 4ºC. As can be seen in figure 5B, no CBD degradation was appreciated in light exposed samples within the 72 hours of the study, and no statistically significant correlation was found between CBD concentration and exposure time.







D

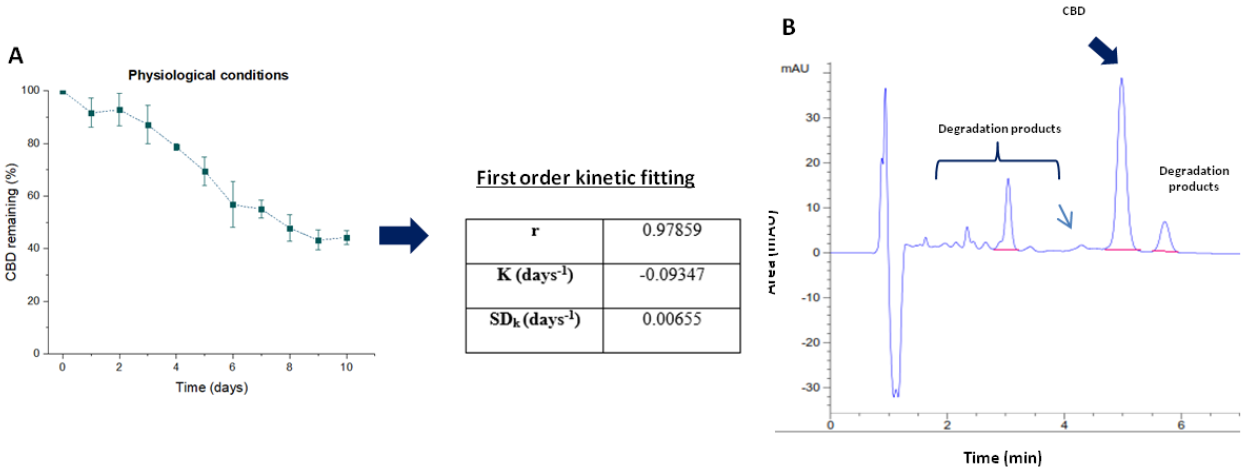
	25°C		5°C			
	Light	Dark	Light	Dark	Light-N_2	Dark- N_2
r	-0.9716	-0.3929**	-0.9369	-0.3996**	-0.2448***	-0.4958***
K (h^{-1})	-0.003962	-	-0.002547	-	-	-
SD_K(h^{-1})	0.000241	-	0,000237	-	-	-

Figure 5: Photo-stability studies of CBD in solution: non-nitrogen flushed (A) and nitrogen flushed (B). Representative chromatogram of CBD in solution exposed to light at 25ºC for 72 hours (C). Statistical analysis ofdata (D). r (correlation coefficient), K(degradation rate constant), SD_k (standard deviation of degradation rate constant). ** with the best fit ($Y^2/\sqrt{X}$), *** with the best fit (1/Y-$\sqrt{X}$).

3.5 Stability at physiological conditions

As depicted in figure 6, the CBD solution incubated at simulated physiological conditions (pH 7.4 and 37±0.5ºC) was highly unstable, and 10% of the drug was degraded within the first day of the study. The degradation profile was fitted to a first order kinetic.

In this study, the main degradation products appeared around 2.9 and 5.8 minutes as illustrated in figure 7B.



r	0.97859
K (days^{-1})	-0.09347
SD_k (days^{-1})	0.00655

Figure 6: Stability of CBD in simulated physiological conditions and first order degradation kinetic fitting (A). Representative chromatogram of CBD in phosphate buffer solution (pH 7.4) incubated at 37ºC for 10 days (B).

4. Discussion

Stability studies provide information about the environmental conditions that could trigger drug degradation and the mechanisms responsible for these processes. These studies conduct to the determination of the shelf life of a formulation in the best storage conditions. But stability studies are also essential during the pharmaceutical development of a new drug product, to guarantee the right handle and formulation of the drug substance, and to avoid mistakes in the evaluation, both in vitro and in vivo, of the activity or bioavailability due to dosing problems or drug instability in physiological conditions. The first requirement to perform any stability study is to have a valid analytical method. HPLC is one of the most useful techniques in stability testing, to detect and quantify the drug and degradation products.

In this work, we validated an isocratic reverse phase HPLC procedure to evaluate the stability of CBD in solution against different factors (temperature, oxygen and light). This procedure provided an efficient separation of CBD from its main degradation products. The retention time of CBD (4.72min) allowed rapid analysis of a large number of samples. The method resulted lineal and proportional in a range of 1-150 µg CBD/ml ethanol, in such a way that a standard was enough, instead of a complete calibration curve, to quantify the CBD in problem samples. The inter day variability was higher than the intra-day variability along the CBD concentration range of 5-100µg/ml, but both CV values were lower than 2%, indicating the adequate precision of the analytical method. Three determinations are enough to research a result with a precision of ± 2%. Finally, the method was also valid to quantify CBD in aqueous solution with 0.5% Tween 80,showing an accuracy according to USP specifications (a slope close to 1 and an intercept statistically equal to 0 for the curve estimated concentrations vs actual values) [17] what discarded proportional and constant errors. The suitability parameters guaranteed the correct performance of the chromatographic system. In fact, the number of theoretical plates was around 16000, and symmetry factor was 0.892, in accordance to the limits (N >2000 and T<2) of FDA [14].

The reported study results showed that CBD stability is influenced by multiple factors, such as temperature, light and oxygen presence. Temperature is one of the most important factors, since CBD degradation was detected in the study carried out on CBD in ethanol solution stored at 25ºC, 40ºC, 50ºC, 60ºC and 70ºC. The higher the storage temperature, the higher the degradation rate constant (K), except at 70ºC, probably due to a change in the degradation pathway. For this reason, data at 70ºC were not used to establish the relationship between degradation rate and temperature using the Arrhenius equation.It is worth highlighting the good adjustment of the experimental data to this equation, with a determination coefficient (r^2) of 99.66%. The Ea value obtained (92.19KJ/mol) is characteristic of degradation of drugs in solution (usually between 40 and 125KJ/mol), and reflects a high sensitivity of CBD against temperature changes. By K_{25} value extrapolated from the Arrhenius equation a t_{95} value (time at 25ºC for the degradation of 5% of CBD) of 119 days was calculated. This value was statistically equal to that interpolated from data on real time of the study at 25ºC (117.13±17.66 days), what validates the Arrhenius equation to estimate the stability of the CBD in solution at different temperatures. The high instability of the solutions of CBD at room temperature

(25ºC) requires its storage in a refrigerator. Since in the stability study at 5ºC no significant degradation was detected after 12 months of storage (the correlation between the variables CBD concentration vs storage time was not statistically significant), the shelf life (t_{95}) of CBD solutions stored in refrigerator was estimated from the Arrhenius equation, obtaining a value of 4.76 years, that allows the storage of ethanol solutions of CBD in these conditions.

Other authors have also reported the stability of CBD methanol solutions in refrigerator at 4ºC [15]. However, stability studies carried out on cannabinoid aqueous solutions (obtained from cannabis flowers) reported high cannabinoid instability after 7 days of storage even at 5ºC, with a CBD loss around 60%. Oil preparations stored in the same conditions were also unstable, although a lower CBD degradation was detected [16]. Nevertheless, a more recent study demonstrated that cannabis oil preparations also showed a high thermal stability with a slight (although significant) loss of CBD content of around 12% after one year of storage in darkness at 4ºC [17].

Our study also showed that pure CBD is more unstable in aqueous solvents than in ethanol, with a t_{95} value of 7 days at 25ºC when water with 0.5% Tween 80 was used as solvent. This instability was due, in part, to the presence of dissolved oxygen, more reactive than atmospheric oxygen. Therefore, when the aqueous solution of CBD was saturated with an inert gas such as nitrogen, which displaced the oxygen, stability increased obtaining a t_{95} value of 16.7 days; although it is still a value far removed from that obtained when using ethanol as solvent. On the other hand, when H_2O_2 was incorporated into the aqueous solution of CBD creating a strongly oxidizing environment, rapid degradation of CBD occurred (t_{95} of 1.77 days), confirming that CBD is an easily oxidizable substance.

Like other cannabinoids, the sensitivity of CBD to oxidation has been previously reported [18], and the exposure of this cannabinoid at oxidizing environment caused a significant drug degradation even at short exposure times (after 3 days a CBD loss around 30% was detected in the solution containing H_2O_2). In fact, CBD is considered as a potent antioxidant [19].

Cannabinoid molecules have also showed to be sensible to light, and storage in darkness is required. In our study, CBD has demonstrated to be sensible to light when incubated at both 5 and 25ºC in a photostability chamber according ICH. Due to the influence of oxygen on CBD stability, to determine its participation in the light instability of CBD, nitrogen flushed solutions was also analysed at 5ºC. In fact, the photo-degradative reactions can be either oxidative or non-oxidative (e.g. isomerisation, rearrangements, alkylation, de-alkylation or decarboxilation). In photo-oxidative reactions, singlet oxygen can react with the unsaturated bonds presents in polynuclear aromatic rings of the drug structure, as is the case of CBD; or triplet oxygen can also react with drug free radicals and form peroxides [20]. In this work, when the air of the ampoules containing CBD solution was replaced by an inert gas (nitrogen), the degradation of CBD exposed to light was avoided. This suggests that the photolytic reaction of CBD is oxidative.

In simulated physiological conditions (pH 7.4 and 37ºC) CBD was highly unstable with 10% of the drug degraded in 24h. Comparing CBD aqueous solution (O±) incubated at 25ºC, a higher degradation was appreciated. While at 25ºC the estimated half-life (50% of degradation) was 19 days, the half-life estimated from data obtained at 37ºC in PBS was 7 days. This difference could be attributed not only to the increase in the degradation rate with the incubation temperature but also to the contact with the air of the dissolution during the assay in physiologic conditions.

Numerous chemistry reactions of CBD have been reported previously, leading to different products [21]. By oxidation reactions monomeric and dimeric hydroxyquinones or cannabelsoic acid type compounds can be formed. The last ones have also been identified in CBD samples in the presence of oxygen and under irradiation. As depicted in the table 7, the two main CBD degradation products detected in our study appeared at 2.8-2.9 min and 5.4-5.8min.These peaks were observed in all experimental conditions (temperature, oxygen and light) and could be related with oxidative reactions. When oxidation was accelerated (by H_2O_2, light or in simulated physiological conditions), a third peak was detected al 2.2-2.3min; while thermal decomposition of CBD leaded to a different degradation product at 3.5min.

		CBD DEGRADATION PRODUCTS: RETENTION TIME (min)			
		2.2-2.3	**2.8-2.9**	**3.5 min**	**5.4-5.8**
EXPERIMENTAL CONDITIONS	**Temperature**		✔	✔	✔
	Oxygen	✔	✔		✔
	Light	✔	✔		✔
	Simulated physiological conditions	✔	✔		✔

Table 7: Main CBD degradation products detected in stability studies

5. Conclusions

The present study provide data on degradation kinetic of CBD in solution under different environmental conditions. The solvent used has an important influence on the stability of the CBD, so that the ethanolic solutions are much more stable than the aqueous solutions. In spite of this, it is necessary to keep the ethanol solutions of CBD in the refrigerator in order to guarantee a shelf life of more than one year, since at room temperature the solutions only remain stable for 3.5 months. The Arrhenius equation (with Ea= 92.19KJ/mol and $A=6.65E+12d^{-1}$) is a validated tool to estimate the stability of CBD alcoholic solutions at different temperatures. CBD is an easily oxidizable substance, and its aqueous solutions have to be saturated with an inert gas like nitrogen in order to increase their stability from 7 to 19 days at room temperature. The light catalyses the oxidation of CBD in solution, being essential the protection of all solutions against light. The use of antioxidants in CBD formulations should be considered. At last, in the design of both in vitro studies simulating physiological conditions and in vivo studies, the instability of CBD in solution under the test conditions must be taken into account.

Acknowledgements

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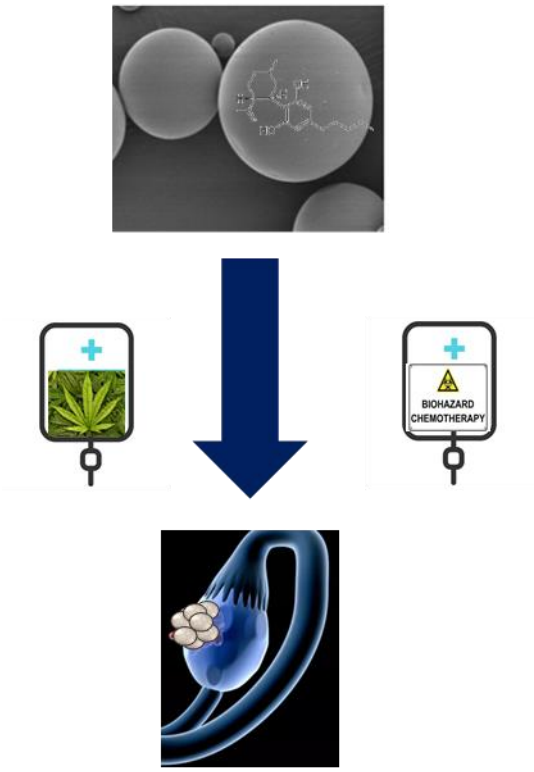
PUBLICACIÓN 6

Enhancing ovarian cancer conventional chemotherapy through the combination with cannabidiol loaded microparticles



Enhancing ovarian cancer conventional chemotherapy through the combination with cannabidiol loaded microparticles

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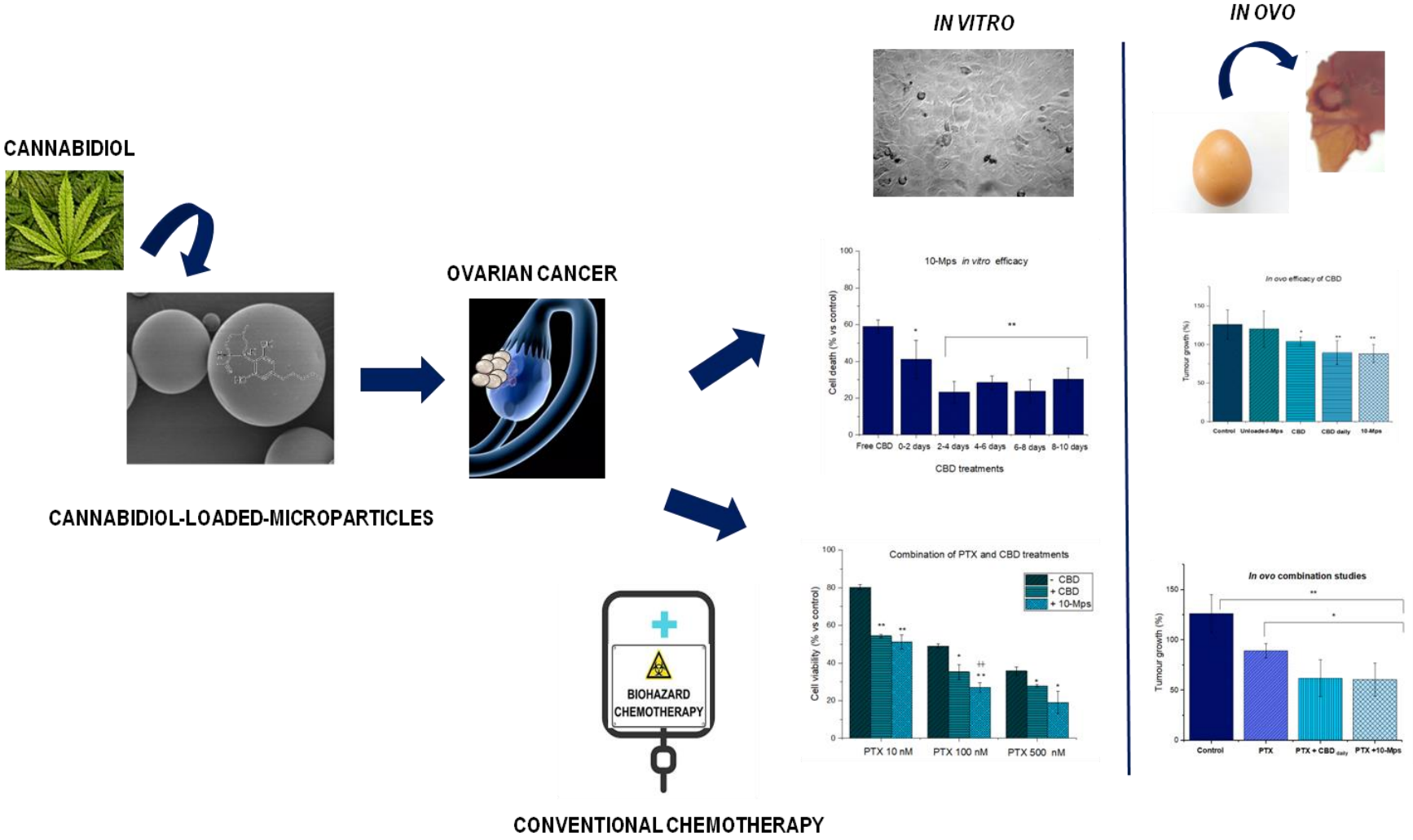
GRAPHICAL ABSTRACT



Abstract

Cannabidiol (CBD), a highly lipophilic drug, has emerged as a potential anticancer drug but it has not yet been evaluated in ovarian cancer, a highly lethal disease. In this work, CBD-loaded-PLGA-microparticles (Mps) has been developed as a novel CBD delivery system for ovarian cancer therapy. Spherical microparticles, with a mean particle size around 25 μm and high entrapment efficiency were obtained. Mps-formulation elaborated with a CBD: polymer ratio of 10: 100 was selected, due to the most suitable release profile, with a zero-order CBD release (14.13±0.17 μg/day/10 mg MPs) for 40 days. The inhibitory effect of CBD on the proliferation of ovarian cancer cells has been reported for the first time. CBD-loaded-microparticles were also effective, reporting an extended antitumoral activity for at least 10 days. The CBD antiproliferative activity was also demonstrated *in ovo*, where CBD-microparticles showed a comparable efficacy to that of CBD in solution daily administered (≈1.5-fold reduction in tumor growth vs control). The use of CBD in combination with paclitaxel (PTX) was really effective on ovarian cancer cells. Firstly, CBD pre-treatment (1 and 10μM) modulated the PTX effect, with a 1.2-2.3 fold reduction in IC_{50} values; and CBD-loaded microparticles were even more effective (≈ 5.5-fold reduction). Secondly, CBD plus PTX co-administration reported a synergistic effect, with a ≈7 fold decrease in PTX $IC_{50}$when combined with CBD at 15 and 20μM. The best treatment schedule was the pre+co-administration of CBD (at 10μM) in combination with PTX, achieving a ≈8- and 10- fold reduction in PTX IC_{50} when CBD was used in solution and in microparticles respectively. This protocol was also effective in *in ovo* models. While PTX conducted to a 1.5-fold tumour growth inhibition, its combination with both CBD in solution (daily administered) and CBD-microparticles (single administration) showed a 2-fold decrease. These results show the promising potential of CBD-Mps administered in combination with PTX for ovarian cancer treatment, since it would be possible to reduce the administered dose of this antineoplastic drug maintaining the same efficacy and, as a consequence, its adverse effects.

KEYWORDS: Antitumor, cannabinoids, combination therapy, drug delivery, gynaecological cancer, paclitaxel.

1. INTRODUCTION

In the last decades, cannabinoids (terpenophenolic compounds present in the plants from the genus Cannabis) [1, 2] have garnered an increasing interest in therapeutics for the treatment of a vast number of medical conditions, including neurodegenerative disorders, pain, nausea and vomiting, psychiatric diseases and cancer [3, 4]. Despite this therapeutic potential, the psychedelic properties of cannabis and Δ^9-tetrahydrocannabinol (Δ^9-THC), the main cannabis active component, limit their clinical use. In this way cannabidiol (CBD), the second in abundance and without psychedelic activity, is one of the most promising cannabinoids [5-8].

In the field of cancer disease, apart from palliating chemotherapy-related symptoms, cannabinoids have become in potential anticancer drugs in several kinds of tumours (gliomas, gastrointestinal, breast and prostate tumours among others).In fact, many authors have reported the ability of CBD to reduce *in vitro* and *in vivo* the proliferation, migration and invasion of tumour cells and to impair tumour angiogenesis [1, 2, 9, 10].Likewise, CBD has also been related to the overcoming anticancer therapy resistances, increasing the cytotoxicity of anticancer drugs [11, 12]. For example, it has been reported that CBD increases the sensitivity of vinblastine and vincristine in leukaemia cells [13, 14]and of temozolomide in glioma [15].The combination of CBD with common anticancer drugs is really attractive due to the low toxicity of this cannabinoid. Moreover, it has been reported that CBD improves the safety profile of conventional chemotherapeutics. For example, CBD palliates the neuropathy related to paclitaxel (PTX) [16-18]and exerts a cardioprotective effect against doxorubicin (DOX) [19, 20].In this way, the inclusion of CBD in conventional chemotherapeutic regimens could improve the efficacy of cancer treatments and minimize the side effects. In fact, several clinical trials are being performed to evaluate the safety and efficacy of CBD in combination with chemotherapy and radiation in the treatment of gliomas, multiple myeloma and gastrointestinal malignancies (NCT03246113, NCT0360764 and NCT03529448).

Ovarian cancer is one of the most common gynaecological malignancies, with 295.414 new cases and 184.799 deaths occurring in 2018 worldwide [21]. Due to its asymptomatic character, it is usually diagnosed at advanced stages (85% of the

diagnostics), hampering the treatment and substantially increasing the risk of death. In fact, the 5-year survival rate is around 45% [22, 23]. Ovarian cancer treatment includes a combination approach with surgery and platinum/taxane-based-chemotherapy. To improve the cytoreductive surgery and the survival rates, intraperitoneal chemotherapy is garnering interest [24, 25].

Although the cytotoxic activity of CBD has been demonstrated in a broad range of tumours, including glioma, breast and prostate carcinomas; to the best of our knowledge, no data in ovarian cancer cells have been reported. However, an overexpression of cannabinoid receptor type I has been found in ovarian cancer cells, and it has been related to tumour invasiveness; suggesting that endocannabinoid system could be a potential target for ovarian cancer treatment, and consequently cannabinoids would be potential anticancer drugs [26].

Despite the potential clinical use of cannabinoids, their high lipophilicity hampers the development of effective formulations, especially for their parenteral administration. In the case of ovarian cancer therapy, where intravenous or intraperitoneal administrations are required, the low aqueous solubility of CBD may constitute a major challenge. In fact, its administration to animals requires the use of non-aqueous solvents and solubilizing agents [27, 28]. In this context, the use of polymeric microparticles as CBD carriers could be a good strategy for CBD administration as parenteral aqueous suspensions. An extended CBD release after a single administration could also be achieved, which is particularly interesting in cancer disease, where long term treatments are necessary.

Among all the polymers available, poly-(lactide-co-glycolic acid (PLGA), FDA-approved, has been widely used to develop controlled release systems due to its biocompatibility and biodegradability [32-36].

Therefore, the main objective of this work was to develop a controlled release formulation of CBD that shows an extended and suitable antiproliferative activity against ovarian cancer and that can be used in combination with the conventional chemotherapeutic regimens. For this purpose: i) PLGA microparticles as CBD carriers have been developed, ii) the antitumor activity of cannabidiol as well as its combination

with conventional anticancer agents have been evaluated for the first time in ovarian cancer cells and iii) the antitumor efficacy of CBD microparticles developed alone or in combination with conventional chemotherapeutic agents using *in vitro* and *in ovo* models of ovarian carcinoma has been studied

2. Materials and methods

2.1 Materials

Cannabidiol (CBD) was purchased from THC-Pharma (Frannkfort, Germany). Poly-(lactide-co-glycolic acid-resomer RG® 502 (PLGA-502) (i.v. 0.16–0.24 dL/g) was obtained from Evonik® Industries (Essen,Germany). Doxorubicin hydrochloride, cisplatin and paclitaxel (Acros Organics) were obtained from Fisher Scientific (Madrid, Spain). Polyvinyl alcohol (PVA, Mw= 30,000–70,000), Sigmacote® and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Missouri, USA). Methanol, acetonitrile, dichloromethane (DCM) and dimethyl-sulfoxide (DMSO) HPLC grade were purchased from Fisher Scientific (Madrid, Spain). Potassium di-hydrogen phosphate (KH2PO4), disodium hydrogen phosphate dehydrate (Na2HPO4•2 H20) and Tween®-80 were provided by Panreac (Barcelona Spain). RPMI Medium 1640- GlutaMAX™-I, Gentamicin (10 mg/ml) Hank's Balanced Salt Solution (HBSS) and Geltrex®were supplied by Gibco (Life technologies, California, USA). Fetal bovine serum (FBS) was obtained from Biowest (Nuaillé, France). 24-well Inserts (3µm pore size) were supplied by Falcon (Corning, Nueva York, USA). Demineralized Milli-Q® water (Millipore, Spain) was used. All chemicals and reagents were used as received.

In order to prevent cannabidiol glass binding, all material was pre-treated with Sigmacote®.

2.2. Preparation of CBD loaded microparticles.

Microparticles with a drug: polymer ratio of 10:100 (10-Mps) and 20:100 (20-Mps) were elaborated by the oil-in-water (O/W) emulsion–solvent evaporation technique using PLGA-RG-502 as polymer. Briefly, PLGA (500 mg) and CBD were dissolved in 5

mL of DCM to obtain the organic phase. Subsequently, it was poured onto 250 mL of a 0.5% (w/v) PVA aqueous solution under stirring at 4000 (10-Mps) or 6000 (20-Mps) rpm for 6 min. With the aim of causing organic solvent removal and microparticle hardening, the resulting O/W emulsion was continuously stirred at 200 rpm in a turbine homogenizer for 3-4 h. Finally, the resulting microparticles were collected by filtration using a 5 μm PTFE membrane, washed with demineralised water to remove residual PVA and lyophilised for 18h at −50 °C and 0.2 mbar.

Unloaded microparticles (Unloaded-Mps) were prepared by the same protocol. The yield of the microencapsulation processes was calculated.

2.3. Microparticle characterization

2.3.1. Morphological studies and particle size

The shape and surface of microparticles were studied by scanning electron microscopy (SEM Jeol, JSM-6400, Tokyo, Japan). For SEM examination, Unloaded-Mps, 10-Mps and 20-Mps were placed onto aluminium stubs and coated with gold.
The particle size was measured by laser diffraction using Microtrac® S3500 Series Particle Size Analyzer (Pennsylvania, USA). The average particle size was expressed as volume diameter. Span values were calculated as polydispersion measurement as follows:

$$SPAN = D_{90}-D_{10}/D_{50}$$

where D_{10}, D_{50} and D_{90} were the diameters determined at the 10^{th}, 50^{th} and 90^{th}percentiles of the size distribution curve.

2.3.2. CBD quantification: analytical conditions

High performance liquid chromatography (HPLC, Agilent 1200 series, Agilent Technologies, California, USA) was used for the quantification of CBD in the microparticles and for release experiments. The analytical conditions were as follows: i) the mobile phase consisted on a mixture of methanol: acetonitrile: water at pH4.5 (52:30:18 v/v) at a flow rate of 1.8ml/min; ii) a reversed-phase Mediterranea® C18 (15×0.46 cm i.d.; 5 μm) (Teknokroma®) column was used, iii) injection volume of 20μL and iv) detection at 228nm.

2.3.3. Drug loading and encapsulation efficiency

To evaluate the amount of CBD encapsulated, 10 mg of microparticles were dissolved in 1 ml of DCM. Afterwards, 9 ml of mobile phase were added to precipitate the polymer. The samples were filtered using PTFE 0.45μm syringe filters and analysed by HPLC. The encapsulation efficiency (EE) was determined using the following equation:

$$EE\ (\%) = [(\text{CBD:PLGA ratio}_{Experimental})/ (\text{CBD:PLGA ratio}_{Initial})] \times 100$$

2.3.4. Drug physical state into microparticles

To determine CBD-polymer compatibility and CBD physical state into polymeric microparticles, differential scanning calorimetry (DSC) analyses were performed. Samples were place into sealed aluminium pans and heated from 20 to 100ºC, cooled from 100-20ºC and heated again up to 100ºC at a rate of 10ºC/min under an inert nitrogen atmosphere (70 mL/min). Samples of pure CBD, raw PLGA, CBD: PLGA physical mixtures, Unloaded-Mps, 10-Mps and 20-Mps were analysed. The value of glass transition of the polymer was calculated in the second heating cycle [37].

2.3.5. *In-vitro* release studies

Briefly, samples of 10 mg of microparticles suspended in 5 ml of PBS (pH 7.4) containing Tween® 80 at 0.5% (w/v), to maintain sink conditions during all the experiment, were placed in a thermostatic shaking water bath at 37 ±0.5 ºC and an agitation of 100 rpm. CBD released was determined indirectly from the data of CBD remaining in the microparticles. For this, at specific time points (2h,8h,1,2,4,7,10,14,21,28 and 31 days) the samples were removed, the supernatant was withdrawn, and CBD was extracted from microparticles as previously described (2.3.3) and analysed by HPLC.

Polymer matrix degradation during the release assay was also evaluated by SEM. Briefly, at pre-determined time points (equivalent to CBD release studies), samples were removed, the release medium was withdrawn, and microparticles were washed with

demineralised water and lyophilised for SEM examination as previously described (2.3.1).

2.3.6. Effect of gamma sterilization on microparticle characteristics

To evaluate the effect of sterilization on CBD-loaded microparticles, 10-Mps and 20-Mps were sterilized by gamma irradiation at a dose of 25kGy.Samples were place in a container with dry ice to prevent formulation heating. Afterwards, sterilized formulations were characterized in terms of morphology (SEM), particle size, DSC, drug loading and CBD release kinetic using the protocols previously described.

2.4. *In-vitro* antitumor activity of CBD

2.4.1. Cell culture

The human ovarian cancer cell line SKOV-3 was obtained from American Type Culture Collection. OAW-42 and IGROV-1 cells were provided by Dr. Mezzanzanica (Fondazione IRCSS, Milano, Italy). All the cells were grown in RPMI-1640 medium supplemented with Glutamine (200 mM), 10% (v/v) of FBS and 1% (v/v) of Gentamicin. They were incubated at 37 ±0.5ºC in a humidified atmosphere containing 5% of CO_2.
For cytotoxic studies, cells in exponentially growing phase were used.

2.4.2. Cell viability studies with free CBD

The ability of free CBD to inhibit cell proliferation was tested in IGROV-1, OAW-42 and SKOV-3 ovarian cancer cell lines. Cells were seeded at a density of 1.5 10^4 cells/cm^2 in a 96-well-plate. Twenty-four hours after seeding, the medium was discarded and the treatments were added. After 24 or48 hours all the treatments were removed and 100 µL of MTT solution (0.5 mg/ml) in complete RPMI medium were added to each well and incubated for 3.5 hours. Afterwards, the medium containing MTT was discarded and 100 µL of DMSO were added to each well. Plates were agitated for 10 minutes and read at 570 nm (Varioskan Flash, Thermo Scientific). RPMI medium and Triton-X at 1% were used as negative and positive control respectively. The inhibitory concentration 50 (IC_{50})

was calculated in each case for comparison purposes using CompuSyn Software (ComboSyn, Inc. NJ, USA.) All the experiments were performed in triplicate.

The highly invasive SKOV-3 cell line was selected for the following studies.

2.4.3. *In vitro* antitumor efficacy of CBD microparticles

SKOV-3 cells were seeded in 24-well plate at a density of 1.5 10^4 cells/cm^2.24 hours after seeding, the medium was removed and the treatments were added. For these experiments, release studies of CBD from 10-Mps were undertaken in parallel. At pre-determined time points (2,4,6 and 8 days) release medium was removed after centrifugation, and microparticles were collected, suspended in cell culture medium and positioned in the top of 3μm pore size inserts, which were placed in each well. The amount of microparticles was calculated according to in vitro CBD release. Cell viability was evaluated by MTT as aforementioned with some modifications 48 hours after treatments.

The results were compared with those obtained when an equivalent concentration of CBD_{sol} was administered. Unloaded microparticles were also tested.

2.4.4. Combination studies: modulatory effect of CBD

The ability of sub-optimal concentrations of free CBD (with a cell death lower than 10%) to increase the antiproliferative activity of conventional anticancer drugs: i) paclitaxel (PTX), ii) cisplatin (CIS) and iii) doxorubicin (DOX), was evaluated in SKOV-3 cells. CIS and DOX stock solutions were prepared in sterile physiological solution. However, PTX stock solutions were prepared in cell grade DMSO, with a final well concentration lower than 0.01% (v/v). Briefly, 24 hours after seeding (1.5$10^4$ cells/cm^2) cells were treated with free CBD at concentrations of 1and 10 μM for 24 hours. Then, the medium was discarded (eliminating CBD treatment) and PTX (10-500 nM), DOX (1-60μM) or CIS (5-100μM) was added. Cell viability was determined by MTT 48 hours later.

The activity of 10-Mps was also tested in 24-well plates (1.5 10^4 cells/cm^2). Microparticles (corresponding with a free CBD concentration of 10μM in 24 hours) were placed onto 3μm pore size inserts. After 24 hours, inserts were removed (eliminating CBD

treatment) and PTX (10-500 nM) or DOX (1-60) was added. Then cell viability was determined by MTT 48 hours later.

IC_{50} of PTX, DOX and CIS were calculated. The reduction in IC_{50}value when combined with CBD was considered as follows:

- Highly positive effect: statistically significant differences in IC_{50} at p value<0.01
- Positive effect: statistically significant differences in IC_{50} at 0.01<p <0.05
- No-effect: no statistically significant differences in IC_{50} (p value >0.05).

2.4.5. Combination studies: synergistic effect

The co-administration of CBD (at concentrations of 10, 15 and 20μM) with different concentrations of PTX (10-500 nM), DOX (1-120 μM) or CIS (5-100 μM) was also investigated. SKOV-3 cells (1.510^4 cells/cm^2) were seeded in 96-well plates and treated, 24 hours after seeding, with different drug pairs (CBD+PTX, CBD+DOX or CBD+CIS). Cell viability was then assessed after 48 hours of incubation by MTT. Combination index (CI) values were calculated using CompuSyn Software [39]. The CI values offer quantitative definition of the effect in drug combinations: antagonism (CI>1), additive effect (CI = 1) and synergism (CI < 1)[40].

2.4.6 Combination studies with CBD: pre+co-administration protocol

For this experiment, SKOV-3 cells were seeded in a 24 well-plate at a density of $1.5*10^4$ cells/cm^2. Briefly, 24 hours after seeding, CBD-Mps were added in the top of 3μm pore size inserts and placed in each well to achieve a daily administration of CBD of 10 μM. 24 hours later, PTX (10-500 nM) or DOX (0.1-20 μM) were added to cells (without removing the inserts). After 48h of incubation cell viability was determined by MTT as aforementioned. The activity of microparticles was compared to the daily administration of the equivalent amount of $CBD_{sol.}$

2.5. Chicken embryo chorioallantoic membrane (CAM) assay

2.5.1. Cytotoxicity of CBD and CBD-loaded-microparticles

An *in ovo* CAM assay was performed according to the method previously described by Dupertuois et. al. [41]. Briefly, fertilized chicken eggs were incubated in an automated incubator at 37°C and 47% humidity. Eggs were automatically rotated into the incubator. On incubation day4, a 3-mm window was drilled in the eggshell and sealed with adhesive tape to avoid desiccation. Eggs were placed again into incubator in a stationary mode. On incubation day 8, the window was enlarged and CAM membrane was gently scratched. Then, SKOV-3 cells suspended in Geltrex® solution were inoculated into eggs ($2*10^6$ cells/egg). On incubation day 11, the formation of the tumour was verified and it was surrounded with a silicone O-ring. Tumour area was measured by optical microscopy. Then, tumours were treated topically with: i) CBD in solution in a single administration, ii) CBD in solution daily administered, iii) Unloaded-Mps in a single administration and iv) 10-Mps microparticles in a single administration. CBD was always administered at a daily dose of 31.4µg/ml (100µM). The amount of microparticles that provide this daily dose of CBD was calculated using the CBD release data corresponding to the first 3 days of the *in vitro* release assay, since the total duration of the experiment was 60 hours. Eggs treated with RPMI medium served as control. At least 7 eggs per condition were analysed. After the treatments, the eggs were placed again into the incubator until incubation day 13.5.The tumour area of each egg was then measured. The tumour growth was evaluated as follows:

$$\text{Tumour growth (\%)} = [\text{Tumour area}]_{FINAL} * 100 / [\text{Tumour area}]_{INITIAL}$$

where $[\text{Tumour area}]_{INITIAL}$ is the are measured at incubation day 11 (before treatments) and $[\text{Tumour area}]_{FINAL}$ the measured at incubation day 13.5 (after treatments).

2.5.2. Combination studies

The potential use of PTX and CBD in combination was also evaluated in the SKOV-3 derived tumours formed on the chorioallantoic membrane using the protocol previously described. For these experiments, at incubation day 11 tumours were treated topically with CBD in solution at a concentration of 100µM or the amount of 10-Mps

equivalents to a daily free CBD concentration of 100µM. After 24 hours (incubation day 12) PTX in solution at a concentration of 100µM was added. While 10-Mps were single administered at incubation day 11, CBD_{sol} was added daily since incubation day 11 until 13.5 when the tumour area was measured. Eggs not treated with CBD were used as control.

Tumour growth was measured as aforementioned.

2.6. Statistical analysis

The data were reported as a mean ± S.D (standard deviation) of at least three experiments (n=3). Multiple groups were compared using one-way analysis of variance (ANOVA). Significant differences were reported as follows: $*p < 0.05$ and $**p < 0.01$. All the graphs and statistical analysis were performed using Origin 2017 software (Origin lab, Massachusetts, USA).

3. RESULST AND DISCUSSION

3.1. Microparticle characterization

3.1.1. Morphology and particle size

SEM images of unloaded-Mps, 10-Mps and 20-Mps are shown in figure 1A. In all cases, spherical microparticles with a smooth and slick surface were obtained. No morphological differences were appreciated between unloaded and CBD loaded formulations. The absence of CBD crystals (pure drug shows a crystalline structure) on the surface of both CBD formulations denoted that the drug was encapsulated into the polymeric matrix.

Microparticle size did not change significantly (p value >0.05) with CBD incorporation, so that all formulations showed an average particle size around 25µm which is suitable for intraperitoneal administration using a 21 G needle (Table 1)[42]. Although unimodal size distributions were appreciated in both unloaded and CBD loaded microparticles, the size distribution was not monodisperse, with span values around or above 2. A higher polydispersion was detected in 20-Mpscompared to unloaded- or 10-Mps microparticles (Table 1).

Formulation	Process Yield (%)	Size (µm)	SPAN	Tg (ºC)	Drug loading (mg CBD/100 mg Mps)	Encapsulation Efficiency (%)
Unloaded-Mps	89.25±1.012	24.03	1.98	40.95	----------	----------
10-Mps	87.36 ±2.77	24.17	2.02	37.45	8.601±0.42	94.62±4.62
20-Mps	81.05 ±5.33	25.14	2.36	34.79	14.97±0.20	89.86±10.33
Sterile 10-Mps	----------	23,42	1.95	37.28	7.42±0.126	----------
Sterile 20-Mps	----------	24,31	2.42	35.08	13.43±0.430	----------

Table 1: Process yield, size, Tg value, drug loading and encapsulation efficiency of unloaded and CBD loaded formulations.

3.1.2. Drug loading and encapsulation efficiency

The results of process yield, drug loading and encapsulation efficiency are shown in table 1. Process yield was pretty good and reproducible in all formulations, with values above 80%. Good results were also obtained in terms of drug loading and encapsulation efficiency in both 10-Mps and 20-Mps formulations. The high encapsulation efficiency (above 89%) is attributed to CBD lipophilicity that limited its diffusion to the external aqueous phase during microparticle hardening.

3.1.3. Drug physical state into microparticles

The thermal analysis of pure CBD showed a sharp endothermic peak at 69.68 ºC which corresponds to CBD melting point [43]. The corresponding fusion enthalpy value was ΔH_m= 75.60 ±0.95 J/g, similar to the value reported in the literature [31]. In the thermograms of physical mixtures of CBD and PLGA, this CBD characteristic peak was also observed, demonstrating that there were no chemical interactions between the drug and the polymer (Supplementary material). Nevertheless, in 10-Mps and 20-Mps the CBD melting point peak was not observed, indicating that the drug is dissolved or molecularly dispersed within the polymeric matrix.

Regarding glass transition temperature (Tg) of raw PLGA, a value of 40.93 ± 0.95 ºC was obtained. A pretty similar value (40.95 ± 0.89ºC) was appreciated in unloaded microparticles, indicating that the encapsulation process did not affect polymer glass transition. However, a decrease in polymer Tg was detected in both 10-Mps (37.45 ± 0.3ºC) and 20-Mps (34.79 ± 0.14 ºC). This reduction of Tg value indicated that CBD made the polymer be plastic. The DSC thermograms are depicted in supplementary material (Figure S1).

3.1.4. CBD release kinetic from microparticles

Both CBD formulations showed a controlled drug release for around 40 days, with more than 80% of the drug released in 35 days. A slight burst effect of 4 and 8% was detected for 10-Mps and 20-Mps respectively over the first 2 hours. This burst release has been usually related to "non-trapped drug", and could be attributed to the most superficial CBD that was not completely trapped into the polymeric matrix [44].

As depicted in figure 1B and 1C, in both formulations can be differentiated a tri-phasic release profile. In 10-Mps, a 1st faster phase was observed up to day 2, a 2nd slower phase from day 2 to 14, and a 3rd phase from day 14 to 42. In 20-Mps, the 1st faster phase was observed until day 1, the 2nd phase from day 1 to day 21 and the 3rd from day 21 until day 42. However, when release drug data were fitted to a kinetic model, a good fit to a zero order kinetic was obtained with data from day 2 to day 42 in case of 10-Mps(r^2=0.988, K_0= 14.48 µg/day/10 mg Mps), while in case of 20-Mps this was not possible and the two different kinetics were clearly identified: a first zero order release kinetic from day 1 to day 21 (r^2=0.981, K_0= 13.74 µg/day/10 mg Mps) and a second zero order kinetic from day 21 to day 42 (r^2=0.983, K_0= 44.41 µg/day/10 mg Mps). In 20-Mps the enormous difference between both rates of drug release, would hamper the adequate dosage of microparticles to achieve constant drug concentrations. On the contrary, with 10-MPs, the CBD controlled release obtained for 40 days will allow a properly dosage.

SEM images depicted in Figure 2reporte the polymer matrix degradation during release studies. In both 10-Mps and 20-Mps, no significant polymer degradation was detected during the first 7 days. However, at day 14 although the microparticles

maintained their spherical shape, signs of corrugations were appreciated on their surface, indicating that polymer degradation started to be more intense. These corrugations were more evident in 10-Mps. At day 21, a higher microparticle deformation was detected, with the appearance of craters and pores in both CBD microparticle formulations. Finally, at day 28 microparticles completely lost their shape and a high polymer degradation was appreciated.

The polymer degradation could be related to CBD release. 20- Mps showed a slower polymer erosion compared to 10-Mps. In 20-Mps a change in CBD release rate was appreciated at day 21, when intense polymer erosion was detected. However, in 10-Mps the erosion seems to start earlier, which could explain the more constant CBD release from this formulation.The slower polymer degradation of 20-Mps could be attributed to the higher CBD content. CBD shows a high lipophilicity (log P=6.33), and it is known that hydrophobic drugs difficult the entrance of water into the microparticles, so that the hydrolytic degradation of the polymer matrix is delayed [45]. In 10-Mps, the water entrance is easier compared to 20-Mps. For this reason, 10-Mps showed a faster drug release.

The differences in release kinetics of a drug from polymeric particles have also been related to the polymer Tg values. In general, at temperatures above Tg values a faster drug release has been reported due to the higher polymer chains mobility that leads to an increase in drug diffusion coefficient [45-47]. In this way, a faster drug release could be expected for 20-Mps, that showed a Tg below the temperature used for drug release studies (37±0.5ºC) and lower than 10-Mps (Table 1). Nevertheless, in this work CBD release up to day 21 was slightly slower in 20-Mps.

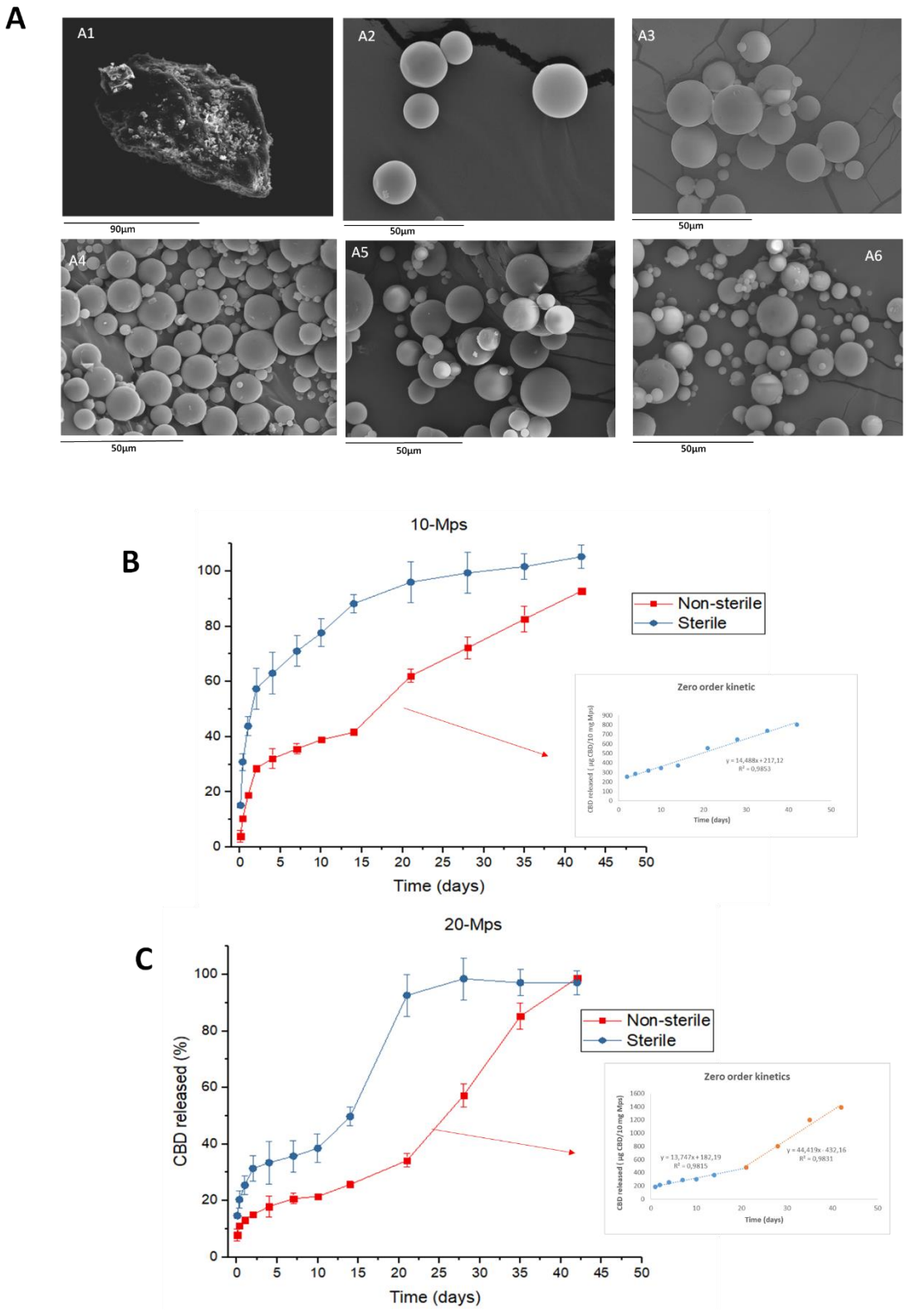


Figure 1: A) SEM images of pure CBD (A1), Unloaded-Mps (A2), 10-Mps (A3), 20- Mps (A4), Sterile 10-Mps (A5) and Sterile 20-Mps (A6). B) Release profile of 10-Mps. C) Release profile of 20-Mps.

3.1.5. Effect of gamma sterilization on microparticle characteristics

Ionizing radiation has been proposed by numerous authors as a useful sterilizing agent for PLGA microparticle. In our study no changes on particle morphology were detected after sterilization. SEM images reported than sterile microparticles were not deformed, maintaining their spherical shape and smooth surface (Figure 2). No significant differences in particle size were appreciated either (p value >0.05). Moreover, DSC thermograms showed the absence of CBD endothermic peak in both 10-Mps and 20-Mps sterile formulations, indicating that CBD was still dissolved into the polymeric matrix and that no crystallization processes occurred during sterilization. Similar Tg values, compared to non-sterile formulations, were also appreciated (Table 1).

However, a CBD degradation in both sterile 10-Mps and 20-Mps was appreciated, with statistically significant differences in CBD content (P value<0.05) between sterile and non-sterile formulations. A slight higher degradation was appreciated in 10-Mps (with 13.75 % of degraded CBD) compared to 20-Mps (10.28% of degraded CBD). The degradation of CBD could be attributed the heating of the samples during sterilization process, although dry ice was used to prevent this.

Regarding drug release profile, a much faster CBD release was detected in both sterile formulations compared to non-sterile counterparts. In sterile 10-Mps more than 90% of the drug was released in 15 days. This sterile formulation exhibited a bi-phasic release profile with a high burst effect (≈ 15% of the drug was released within the first two hours). A1st faster phase was observed up to day 2 with the 60% of CBD released, followed by a slower 2nd phase from day 2 to day 21. Sterile 20-Mps reported a similar burst effect, compared to sterile-10 Mps, with ≈15% of the drug released in the first 2 hours, and a more prolonged CBD release during 21 days. In contrast to sterile 10-Mps, a tri-phasic profile was detected in 20-Mps. A1st faster phase was observed up to day 2, followed by a slower 2nd phase from day 2 to 10 and a faster 3rdphase up to day 21. The faster release of CBD in sterile formulation compared to non-sterile counterparts could be attributed to a decrease in polymer molecular weight.

As depicted in figure 2, higher polymer erosion was denoted in both sterile formulations compared to non-sterile ones, which could be correlated with the faster

release of sterile formulations. No significant differences between non-sterile and sterile formulations were appreciated at day 7. However, at day 14 higher polymer erosion was observed in both, sterile-10-Mps and sterile-20-Mps. At this point, microparticles started to loss their shape, exhibiting a high pore formation. At day 21 both formulations were completely deformed. Indeed, this point matches with the final time of the last phase of the release profile, with more than 90% of the CBD released. This faster polymer erosion would be related to the loss of molecular weight during sterilization process. In fact, faster polymer erosion, with the loss of microparticle shape and the appearance of corrugation, has been reported in particles elaborated with low molecular weight PLGA resomers compared to high molecular weight PLGA microparticles [46].

Similar results in γ-irradiated PLGA microparticles loaded with several actives have been found by other authors; reporting that this sterilization technique does not affect physicochemical properties of particles (size and morphology), but usually accelerates drug release due to a decrease of polymer molecular weight[47-49]. Nevertheless, De Oliverira et al reported no differences in drug release in PLGA microparticles loaded with methotrexate, even at higher irradiation doses [50].

Due to the CBD degradation during sterilization process and the acceleration of the release of this active from PLGA microparticles, the sterility of the microparticles will be achieved by the previous sterilization of the aqueous and the organic phases followed by the microparticle elaboration in aseptic conditions.

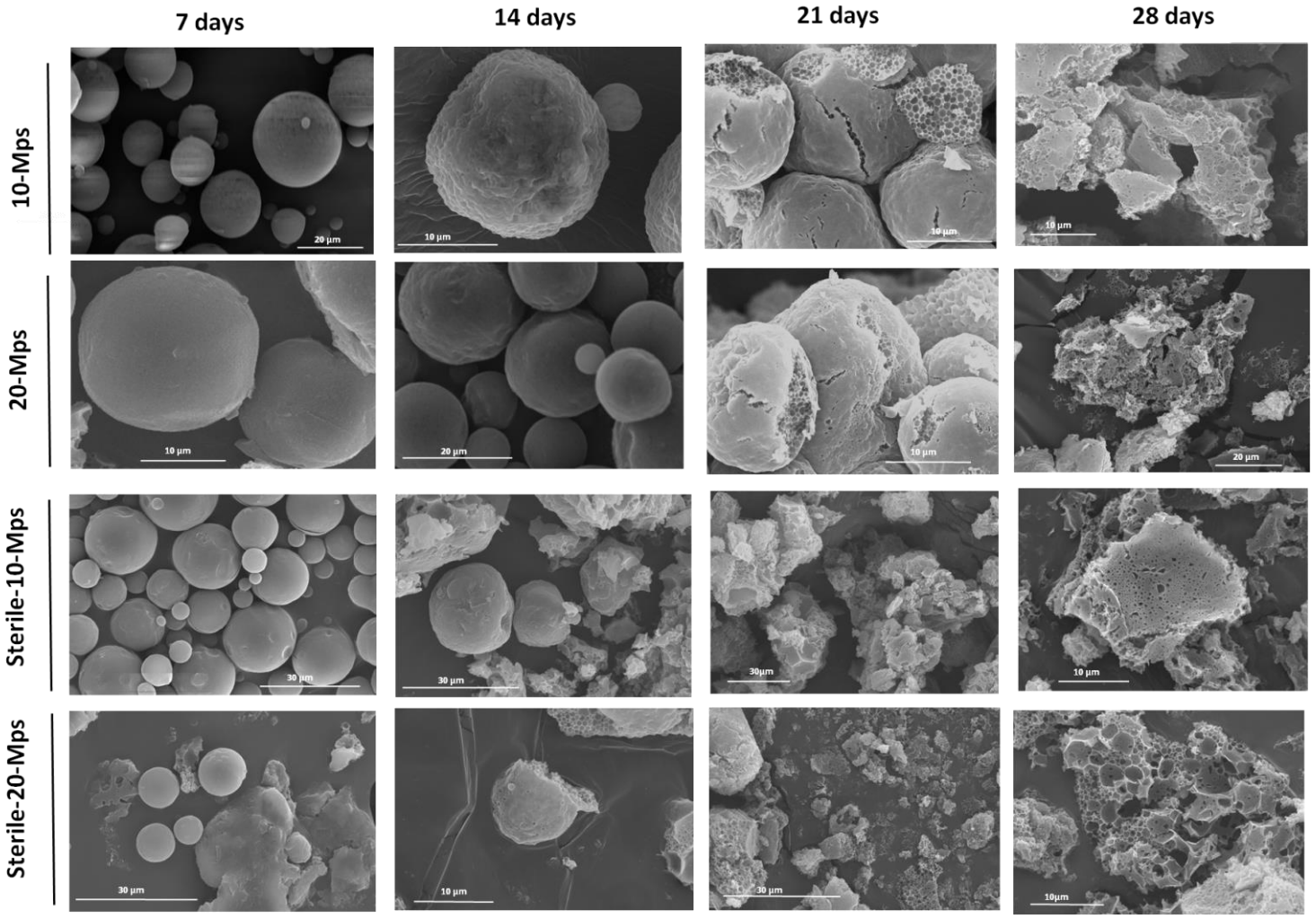


Figure 2: SEM images of non-sterile and sterile CBD loaded microparticles during release studies (7,14,21 and 28 days).

In summary, in spite of 20-Mps showed a high drug loading so that this formulation would let the administration of a lower amount of microparticles to get the same CBD plasmatic concentration, this formulation exhibited a clearly biphasic drug release, with a very low release rate until day 21 (around 35% of the dose was released). Nevertheless, 10-Mps exhibited a constant zero order drug release until day 40, with around 60% of drug released in the first 21 days. For this reason, 10-Mps, with a more suitable profile, was selected for efficacy experiments.

3.2. *In-vitro* antitumor efficacy

3.2.1. Cell viability studies with free CBD

Interestingly, although the cytotoxic activity of CBD has been demonstrated in a broad range of tumours, as the best of our knowledge, no data in ovarian cancer cells have been reported. However, an overexpression of cannabinoid receptor type I have been found in ovarian cancer cells and it was related to tumour invasiveness. This suggests that endocannabinoid system could be a potential target for ovarian cancer treatment and, as a consequence, CBD could be effective against this type of tumour [26].

As depicted in figures 3A and 3B**,** CBD has demonstrated to inhibit the proliferation of all tested ovarian cancer cell lines, being OAW-42 cells the least sensitive to CBD activity (the images of ovarian cancer cells treated with CBD are illustrate din supplementary material figure S2). While IGROV-1 and SKOV-3 cells exhibited a more similar IC_{50} after 48 hours of treatment, with values of 18.57±2.27 and 22.73±1.18μM respectively; OAW-42 showed a higher $IC_{50,}$ with values of 31.80±1.01μM. These IC_{50} values are in the range of those reported for CBD in breast and prostate carcinomas. Differences in the activity of conventional anticancer drugs have also been reported between these ovarian cancer cells. For example, while OAW-42 cells are resistant to taxanes, IGROV-1 and SKOV-3 are sensitive [52].

Interestingly, some authors have reported a dual activity of cannabinoids in cancer cell proliferation, underlying that while at concentrations in the micromolar range they inhibit cancer cell proliferation, cannabinoids may stimulate tumour cell proliferation when administered concentrations in the nanomolar range [53-55]. The

ability of CBD to stimulate ovarian cancer cell proliferation has been tested in SKOV-3 cell line, which was selected for the following experiments due to its high invasiveness grade and sensitivity to CBD. CBD did not increase significantly the proliferation of SKOV-3 cells when administered in a range of concentrations of 0.1-1μM (Figure 3C).

3.2.2. *In vitro* antitumor efficacy of 10-Mps

Despite the antiproliferative activity of CBD in ovarian carcinoma, its low aqueous solubility hampers its administration. The microparticle formulation developed would resolve this challenge.

The antitumor efficacy of CBD-loaded microparticles was evaluated in SKOV-3 cells. While unloaded-Mps were no toxic (up to the evaluated concentration of 1.95 mg/ml), 10-Mps showed an antiproliferative activity that was maintained throughout the 10 days of the assay, as depicted in figure 3 D. In this experiment 2.92 mg of microparticles (containing 0.251 mg of CBD) were administered. The amount of microparticles used was calculated from the data of the CBD release assay fitted to zero order kinetic (from day 2-42). During the first 2 days of the experiment (0-2 sample) a higher cell death (≈ 90%) was achieved with 10-Mps compared to free CBD (≈40% of cell viability inhibition). This higher antiproliferative effect is attributed to the faster CBD release during the first 48 hours. In this period, a free CBD concentration above 100μM was achieved with the microparticles, producing a higher cell death. Nevertheless, from the second day, CBD was released from 10-Mps at a constant rate of ≈9μM/day and, as consequence, the cell death detected decreased (≈30%), being slightly lower to that obtained with CBD in solution (Figure 3D). From day 2, the inhibition of cell viability detected with 10-Mps remained constant.

These data show that encapsulated CBD maintains its antiproliferative activity and that microparticles could be a good strategy to achieve an extended anticancer activity of CBD after a single administration.

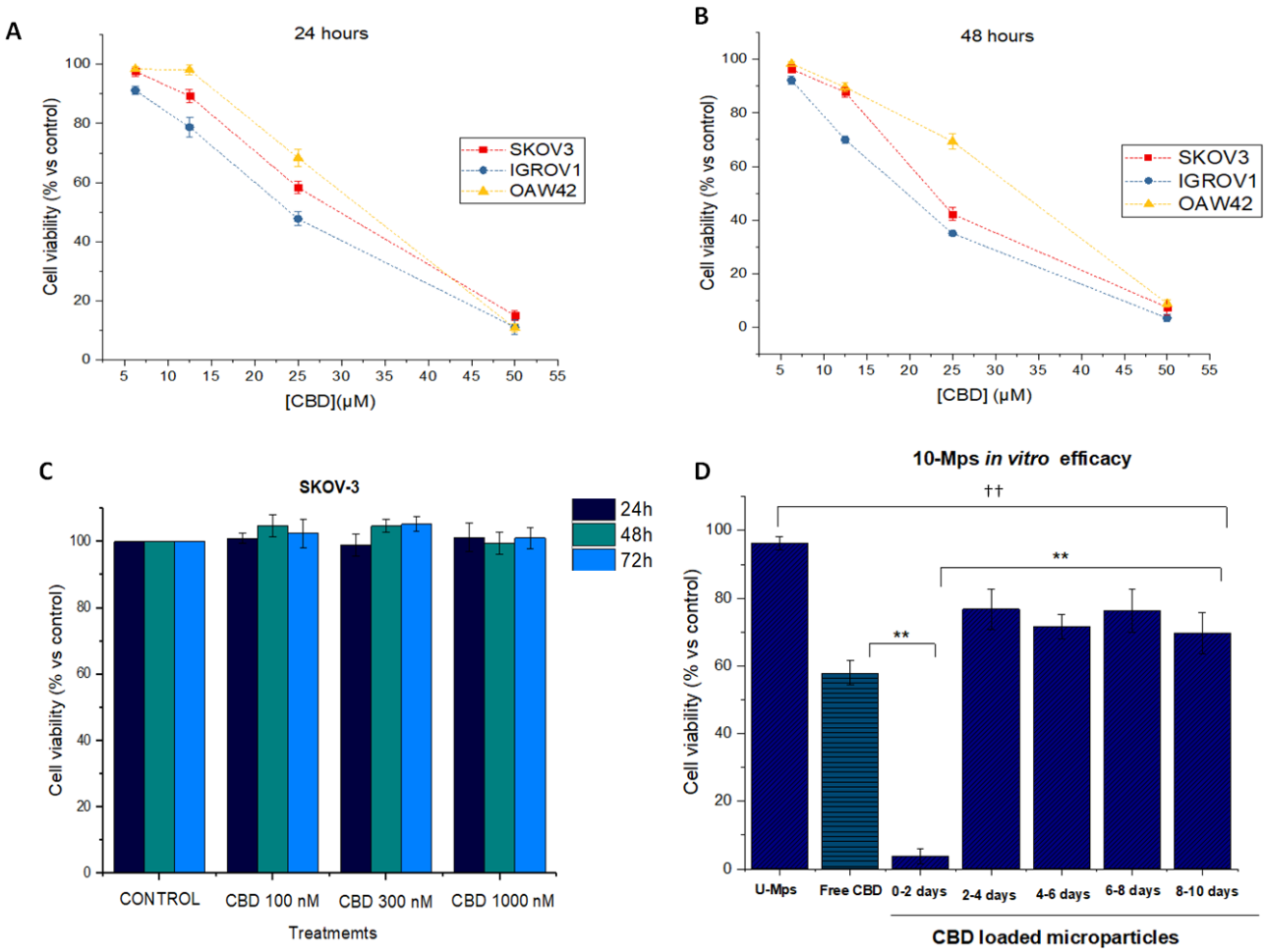


Figure 3: Cell viability studies of CBD in solution with SKOV3, IGROV-1 and OAW-42 cell lines after 24 (A) and 48 (B) hours of incubation. Effect of low concentrations of CBD (nanomolar range) in SKOV-3 cells (C). Cell viability studies of 10-Mps in SKOV-3 cells (D). ** significant differences with 0-2 days sample. †† significant differences with unloaded microparticles

3.2.3 Combination studies with free CBD and 10-Mps

The ability of cannabinoids to module the activity of several anticancer drugs and to improve the chemotherapeutic regimens has been reported in *in vitro* and *in vivo* models of several tumours (e.g. leukaemia, glioma and breast cancer) with promising results [14, 56, 57]. In this work the potential use of CBD to improve the effect of

conventional anticancer drugs in ovarian cancer treatments (paclitaxel, doxorubicin and cisplatin) has been evaluated.

Firstly, the pre-treatment of SKOV-3 cells with sub-optimal concentrations of CBD in solution (1 and 10μM) (1^{st} step: only CBD for 24 hours, 2^{nd} step: only PTX for 48 hours) reported a sensitization of the cells to PTX (Figure 4A). A positive effect was detected when CBD at 10 μM was used, with a ≈ 2.5-fold reduction in IC_{50} values. However, no effect was found when CBD was used at 1μM (Table 2). Secondly, as depicted in figure 4B, the co-administration of CBD in solution and PTX (CBD +PTX for 48 hours) also showed a positive effect for all combinations tested. In fact, combination indexes in the range of 0.70-1.01 were obtained in all cases (supplementary material, figure S5 and table S1), indicating that a moderate synergism or additive effect between CBD and PTX occurred. This suggests that the combination of both drugs could be really attractive, even at the lowest CBD concentration tested (10μM). For example, the co-administration of CBD 10μM plus PTX 100 nM produced a similar cell death (≈60%) than the administration of single PTX at 500 nM.

The pre-treatment with 10-Mps (1^{st} step: only 10-Mps for 24 hours, 2^{nd} step: only PTX for 48 hours) at a free CBD concentration of 10μM (0.675 mg of 10-Mps were administered) also reported a positive effect (Figure 4C), with a 5-fold reduction in PTX IC_{50} (Table 2).

All these results suggest that the administration of CBD prior to the administration of PTX as well as the co-administration of CBD+PTX could considerably reduce the doses of PTX necessary to achieve the same antiproliferative effect. In this sense, the use of microparticles could really be a good strategy, due to extended release of CBD after a single administration and, as consequence, their extended antitumor activity. In fact, the pre-treatment of SKOV-3 cells with CBD in solution (daily administered) or 10-Mps (in a single administration) at a free CBD concentration of 10μM (the images of treated cells are depicted in supplementary material, figure S3), followed by the co-administration of PTX (1^{st} step: CBD treatments for 24 hours, 2^{nd} step: addition to PTX and incubation for 48 hours) reported a significant increase of cell death (Figure 4D). Moreover, 10-Mps formulation was more effective than CBD in solution with 10-fold reduction in IC_{50}

values for encapsulated CBD compared to an almost 8-fold reduction for CBD in solution (Table 4).

The combination of CBD and PTX could be really interesting. Firstly, due to the fact that CBD is a non-psychedelic cannabinoid and that it shows, in general, a lower toxicity, no safety problems could be related to CBD use. Secondly, an improvement in PTX toxicity could also be achieved. The entourage effect on PTX may let a decrease of taxane dose and, as a consequence, of chemotherapy related toxicity. In this work, it has been reported that the combination of 10-Mps, previously administered, and PTX at a concentration of 10 nM, achieved a similar efficacy than the single administration of PTX 100 nM. Moreover, CBD has reported to palliate some adverse events (e.g. neuropathic pain) related to this taxane making CBD and PTX combination even more interesting [16-18].

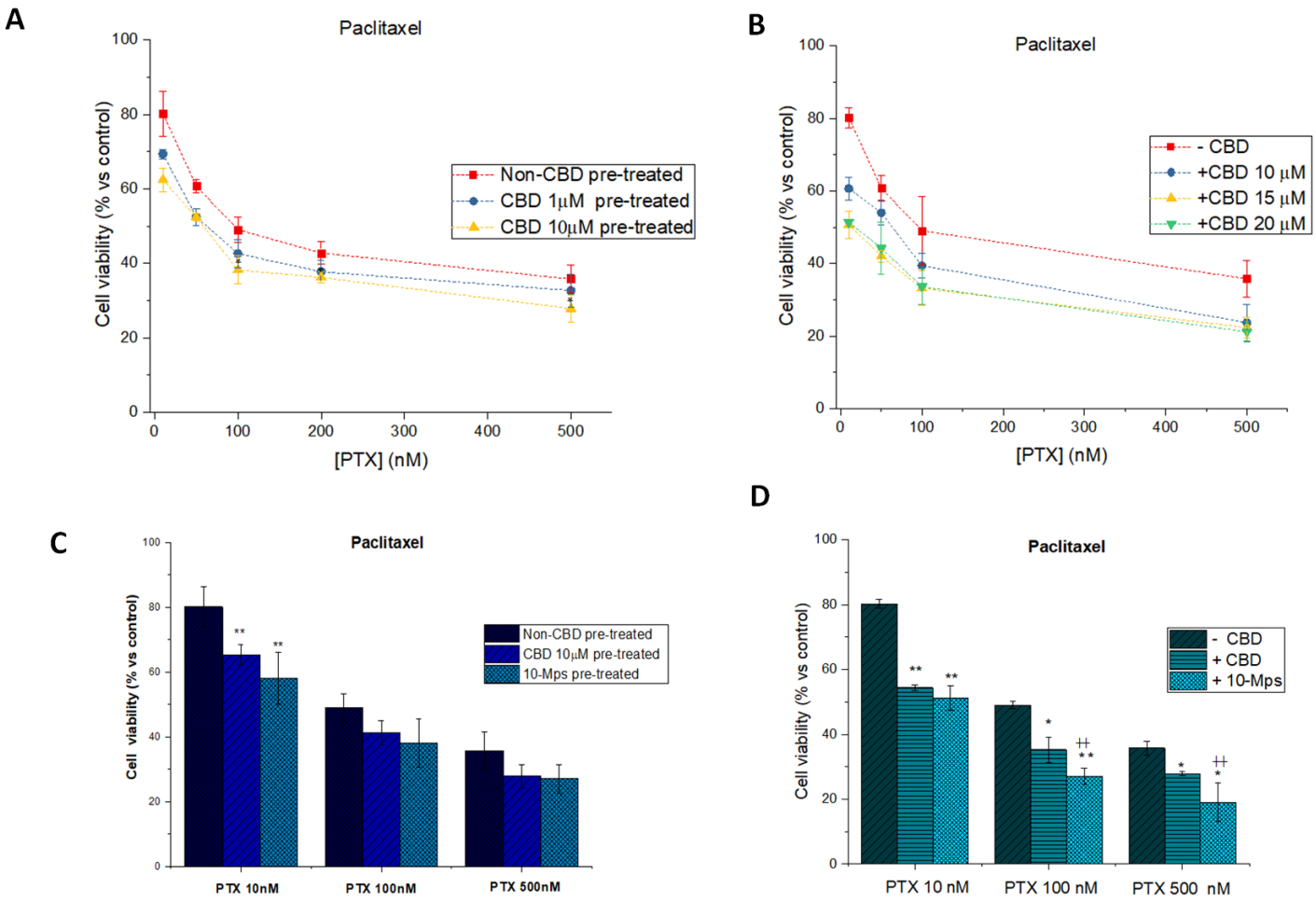


Figure 4: Cell viability of SKOV-3 cells: pre-treated with CBD_{sol} for 24 hours followed by the administration of PTX (A); treated with CBD_{sol}+PTX (B); pre-treated

with 10-Mpsfor 24 hours followed by the administration of PTX (C); pre+cotreated with CBD (in solution or encapsulated into polymeric microparticles) and PTX (D). Incubation time after the last treatment: 48h.

Regarding the combination with doxorubicin, it has been reported that CBD intraperitoneally administered in animal models exert a cardioprotective effect against DOX cardiotoxicity [19, 58], the mayor problem associate to DOX treatments [59]. Nevertheless, with respect to the antiproliferative effect of this combination, no-modulator effect was detected with CBD in solution neither with 10-Mps (1º step: CBD or 10-Mps for 24 hours, 2º step: single DOX for 48 hours), because although a reduction in DOX IC_{50} was found with the pre-treatment with CBD (Table 2), differences with no pre-treatment value were not statistically significant (Figures5.A and 5C). Neither synergistic effect was appreciated when CBD was co-administered with DOX (1º step: CBD for 24 hours, 2º step: CBD + DOX for 48 hours), as illustrated in figure 5B. In fact, combination indexes below 1 were only obtained at the lowest DOX concentration co-administered with CBD 10 and 15μM, and, on the contrary, an antagonism effect was appreciated (Supplementary material, figure S6 and table S2). Nevertheless, when the pre+co administration of CBD with DOX were tested (1º step: CBD in solution or 10-Mps for 24 hours, 2º step: CBD in solution or 10-Mps + DOX for 48 hours) (the images of treated cells are depicted in supplementary material, figure S4), a highly positive effect was appreciated with both CBD in solution and 10-Mps, with around 3-fold reduction in IC_{50} (Figure 5D). No significant differences between CBD (daily administered) and 10-Mps (single administration) were detected.

Finally, CIS and CBD combination was not effective neither modulatory nor synergistic effect studies. CBD did not sensitize the cells to CIS activity (Figure 5D) and similar IC_{50} values were obtained in non- CBD and CBD pre-treated cells. Likewise, the co-administration of CBD and CIS only reported a slightly decrease in IC_{50} values of CIS at concentrations of 15 and 20μM. Moreover, combinations indexes values above 1were obtained for CIS+CBD combinations (supplementary material, figure S7 and table S3).

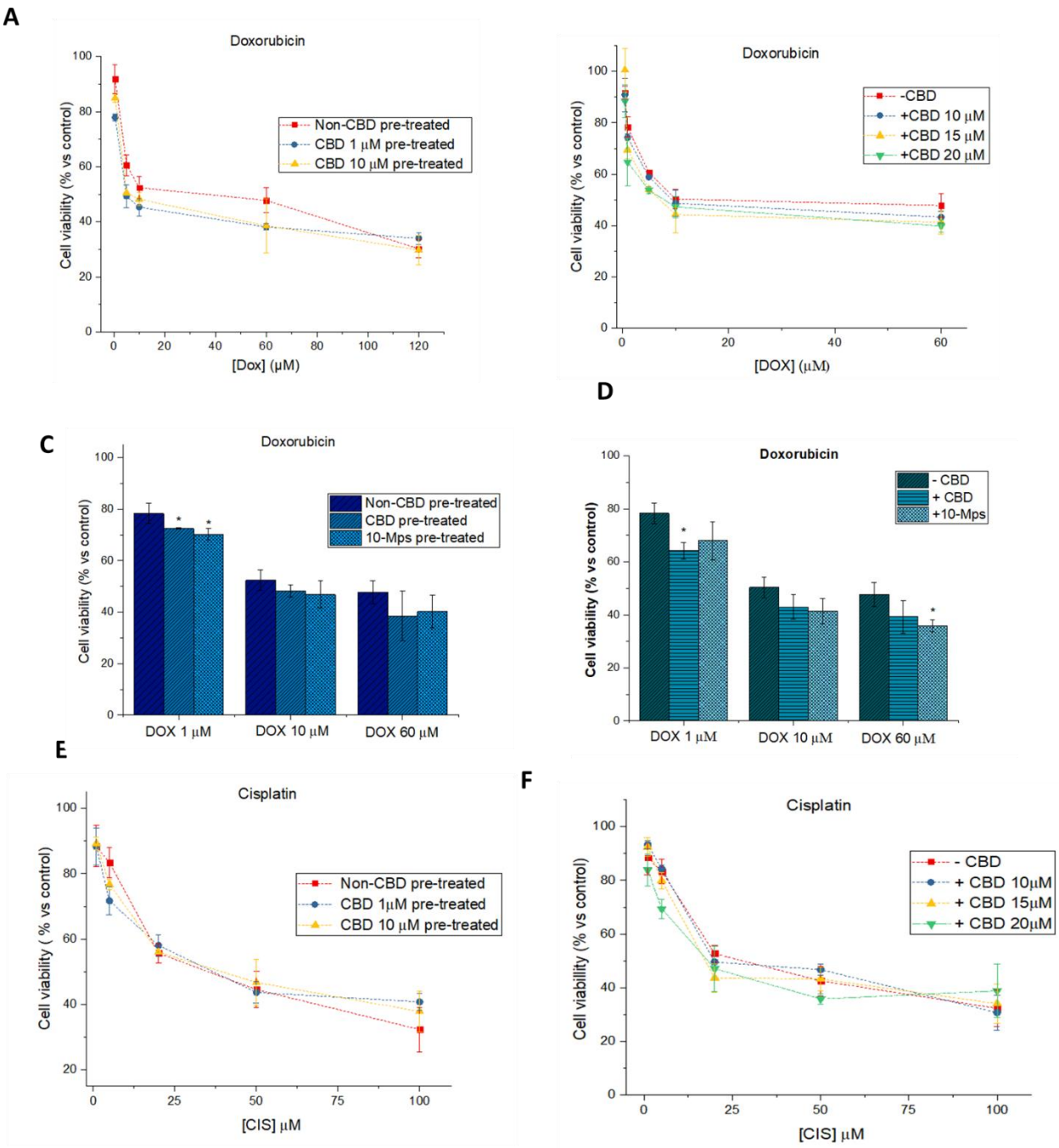


Figure 5: Cell viability of SKOV-3 cells: pre-treated with CBD_{sol} for 24 hours followed by the administration of DOX (A); treated with CBD_{sol}+DOX (B); pre-treated with 10-Mps for 24 hours followed by the administration of DOX (C); pre+co treated with CBD (in solution or encapsulated into polymeric microparticles) and DOX(D); pre-treated with $CBD_{sol}$for 24 hours followed by the administration of CIS (E); treated with CBD_{sol}+CIS (F). Incubation time after the last treatment: 48h.

Antiproliferative activity of doxorubicin in non pre-treated and CBD-pre-treated SKOV3 cells (A). Combination studies of different concentrations of CBD with doxorubicin (B). Antiproliferative activity of SKOV-3 cells pre-treated with 10-Mps (at a CBD concentration of 10 µM) followed by single doxorubicin (C). Antiproliferative activity of SKOV-3 cells pre-treated with 10-Mps (at a CBD concentration of 10 µM) followed by 10-Mps plus doxorubicin (D). Antiproliferative activity of cisplatin in non pre-treated and CBD-pre-treated SKOV3 cells (E). Combination studies of different concentrations of CBD with cisplatin (F).

	PTX (nM)	DOX (µM)	CIS (µM)
Single drug	102.52±22.32	18.65±4.25	38.71±6.85
Pre-CBD 1µM	72.94±8.03	17.82±2.58	39.76±6.05
Pre-CBD 10µM	49.13±17.35**	12.26±3.16	40.50±5.71
Pre 10-Mps (CBD 10µM)	20.65±0.38**	12.75±2.54	--------

Table 2: IC_{50} values of PTX, DOX and CIS in SKOV-3 cells pre-treated with CBD or 10-Mps for 24 hours and then with PTX, DOX or CIS for 48 hours.

	PTX (nM)	DOX (µM)	CIS (µM)
-CBD	102.52±22.32	18.65 ±4.25	38.71±6.85
+ CBD 10 µM	39.53±1.68**	15.16±2.78	36.13±2.97
+CBD 15 µM	13.14±1.50**	12.58±5.96	28.47±3.53
+ CBD 20 µM	14.05±3.88**	11.29±5.30	26.97±5.30

Table 3: IC_{50} values of PTX, DOX and CIS in SKOV-3 cells treated with CBD+PTX, CBD+DOX and CBD+CIS for 48 hours.

	PTX (nM)	DOX (μM)
Single drug	102.52±22.32	18.65 ±4.25
Pre-CBD+CBD co-administration 10μM	14.69±5.34**	7.12±2.42**
Pre-10-Mps+10-Mps co-administration 10μM	11.61±3.08**	6.77±2.13**

Table 4: IC_{50} values of PTX and DOX in SKOV-3 cells pre-treated with CBD or 10-Mps for 24 hours and then with CBD treatments plus PTX or DOX for 48 hours (Schedule 3).

3.3. *In -ovo* anticancer studies

In recent years, CAM model has been proposed as an interesting technique in cancer research due to the feasibility to induce tumour formation. This is a simple and low-cost model. Moreover, the chick embryo is not immunocompetent and transplantation from different tissues and species could be undertaken [60, 61], making it in a great model to evaluate tumour growth. In fact, this model has been reported as a good technique to test novel anticancer therapies in ovarian tumours as an alternative to murine in vivo studies [62].

In figure 6A, an example of SKOV-3 derived tumour obtained in CAM model is displayed. Its histophatological analysis with haematoxylin and eosin staining (Figure 6B) showed the invasion of CAM membrane by tumours cells, and the formation of new blood vessels. This indicated that a "real tumour" was formed and that it was not a cell agglomeration.

When the antitumor efficacy of CBD in solution daily administered was evaluated with this model, a significant inhibition (p value<0.05) of the growth of SKOV-3 derived tumours was detected with CBD concentration of 100 μM (31.47 μg/ml) (Figure 5C). When unloaded microparticles were evaluated, no significant differences in tumour size (p value > 0.05) with respect to controls were detected (at a concentration of 3.65 mg/ml). Finally, when CBD-Mps (3.65 mg/ml of microparticles containing 0.313 mg of CBD and with a daily CBD release of 31.3 μg) were administered a significant reduction in tumour size (1.5 fold reduction) was detected. It is noteworthy that the inhibition of tumour growth obtained with 10-Mps in a single administration was similar to the produced by the daily administration of the CBD in solution.

Due to the promising *in vitro* results of CBD and PTX combination, their efficacy was also tested *in ovo*. As depicted in figure 5D, the combination of PTX and CBD treatments also reported a higher and statistically significant tumour growth inhibition compared to paclitaxel alone. While the administration of PTX alone showed around a 1.5-fold reduction in tumour growth compared to the controls, its combination with CBD in solution (daily administered) or 10-Mps (single administration) produced around 2-fold decrease. No significant differences were detected in tumour growth between CBD_{sol} daily administered and CBD-Mps.

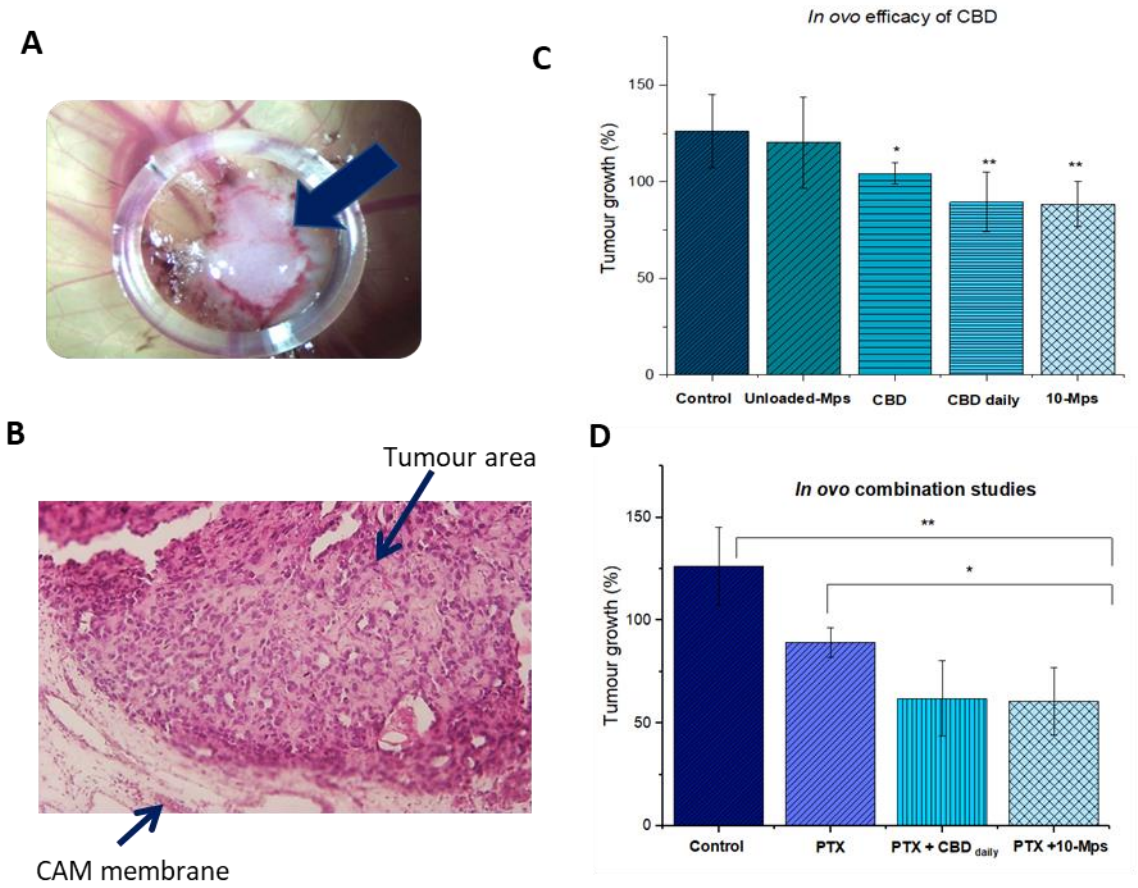


Figure 6: Images of SKOV-3 derived tumour formed on CAM membrane (A) and its haematoxylin-eosin staining (B). *In ovo* antitumor efficacy of CBD in solution and encapsulated into microparticles (10-Mps) (C) and their combination with paclitaxel (D).

4. CONCLUSIONS

Microparticles elaborated with a CBD:polymer ratio of 10: 100 (10-Mps) allow the suitable administration of this drug, showing a constant release since day 2 to day 42. CBD in solution demonstrated *in vitro* antiproliferative activity in all tested ovarian cancer cell lines (SKOV-3, IGROV-1, OAW-42). The single administration of CBD loaded microparticles (10-MPs) reported an antitumor efficacy comparable with the daily administration of equivalent doses of CBD in solution in both *in vitro* and *in ovo* models of ovarian cancer. 10-Mps show as a useful formulation for CBD administration, improving its dosing interval.

CBD treatments were also useful as combination therapy for ovarian cancer treatment. The combination of CBD and PTX showed a synergistic activity. This allow the reduction of PTX doses, with the consequently decrease of the toxicity related to this drug. The protocol that involve the previous plus co administration of CBD with paclitaxel was the most effective. Nevertheless, the combination of CBD with doxorubicin and cisplatin were no effective.

Although further studies in animal models are required, this work provides by the first time, the attractive use of CBD in combination with paclitaxel for ovarian cancer therapy. The combination of both drugs would allow to increase the efficacy of the treatment without an increase of the adverse effects associated with the taxane. The administration of CBD formulated in microparticles, would be especially interesting, because this formulation would resolve the dosage problems related to the lipophylicity of CBD at the same time that would reduce the number of administrations of the cannabinoid, prolonging its effect.

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SUPPLEMENTRY MATERIAL

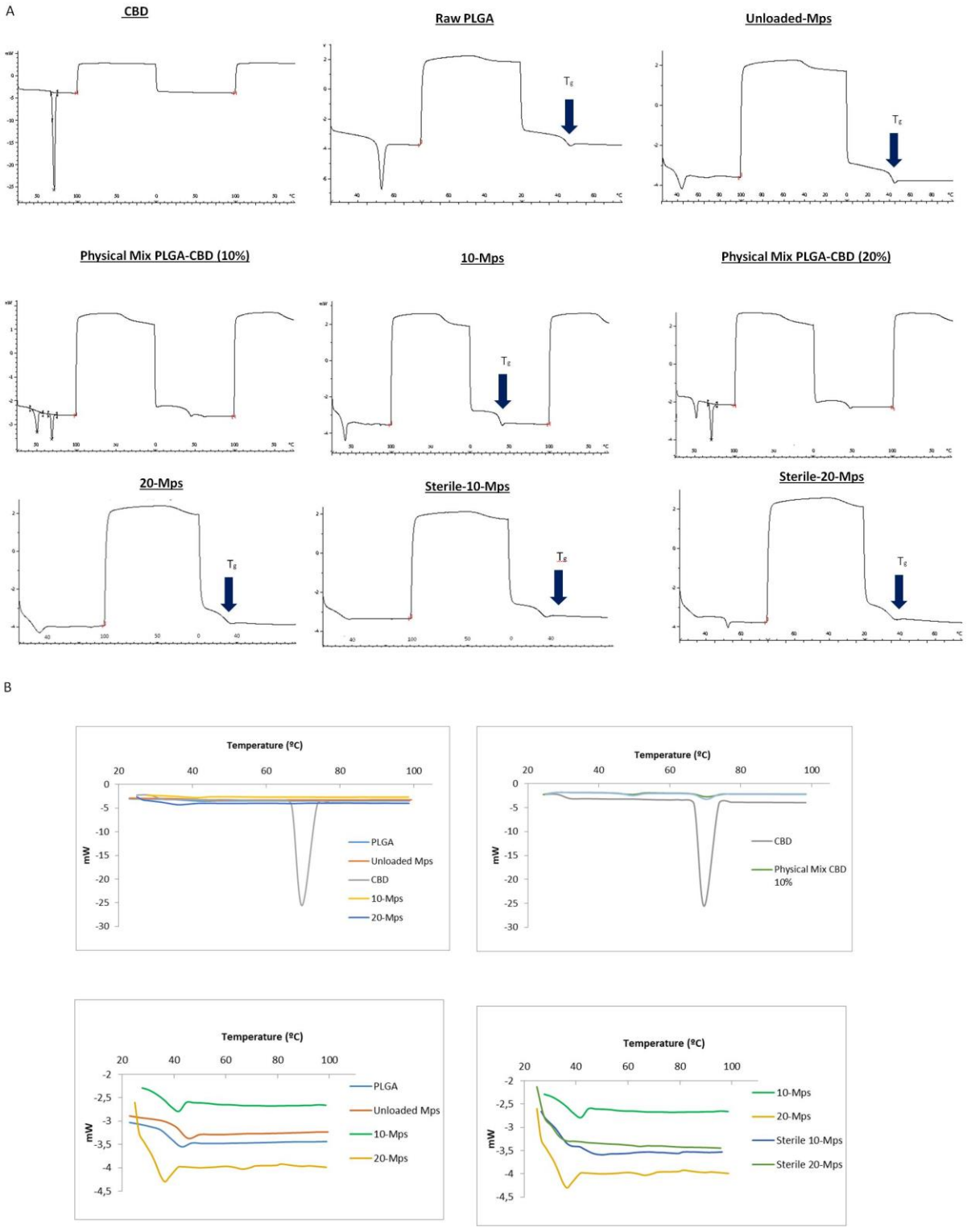


Figure S1: DSC thermograms of CBD, PLGA, their physical mixtures and all developed formulations(A). Graphs of DSC studies (B).

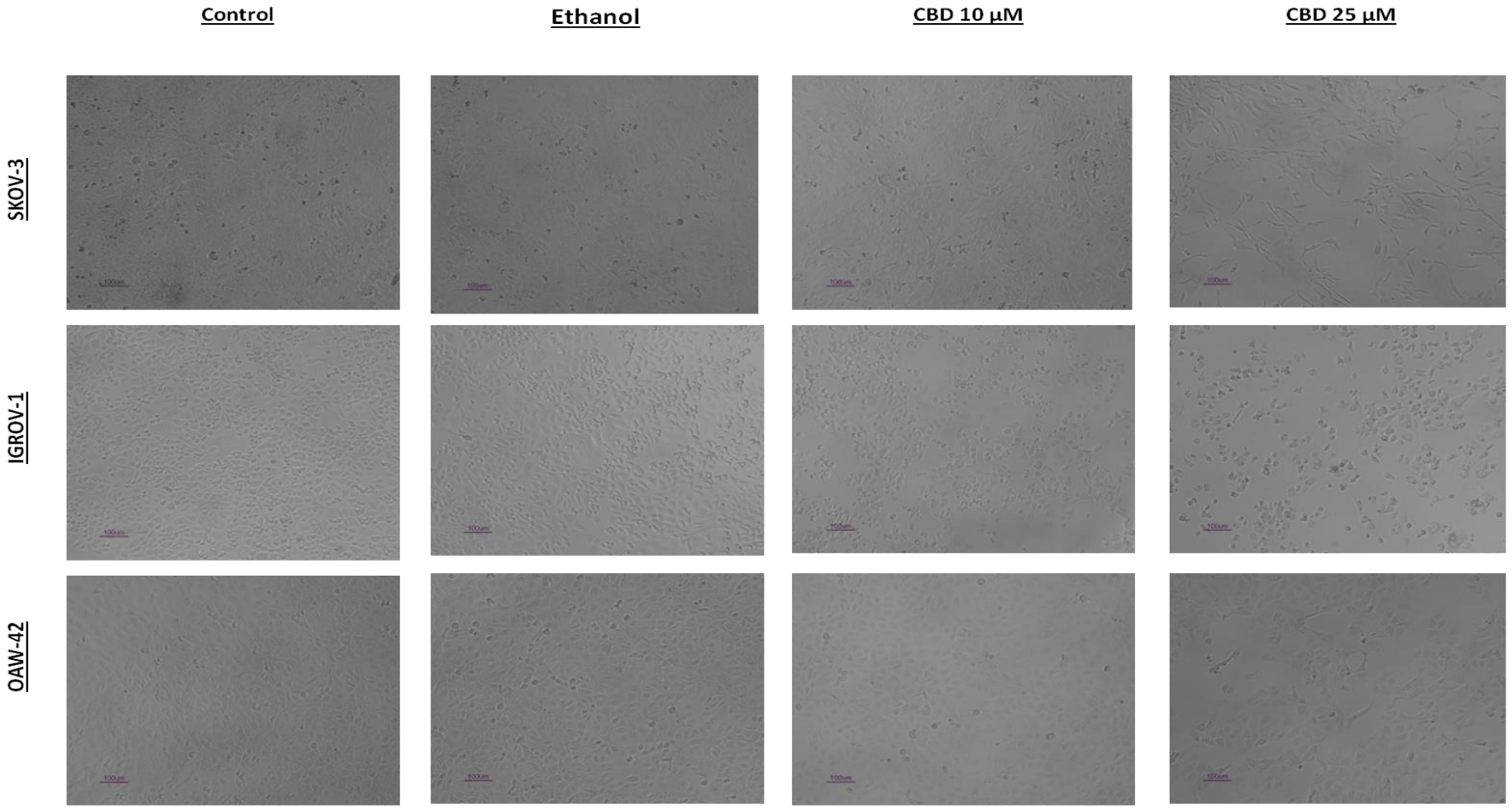


Figure S2: Images of SKOV-3, IGROV-1 and OAW-42 cells treated with CBD in solution for 48 hours. Control cells were treated with cell culture medium and ethanol contains the equivalent amount of CBD in solution (<0.01%). Magnification: x 10.

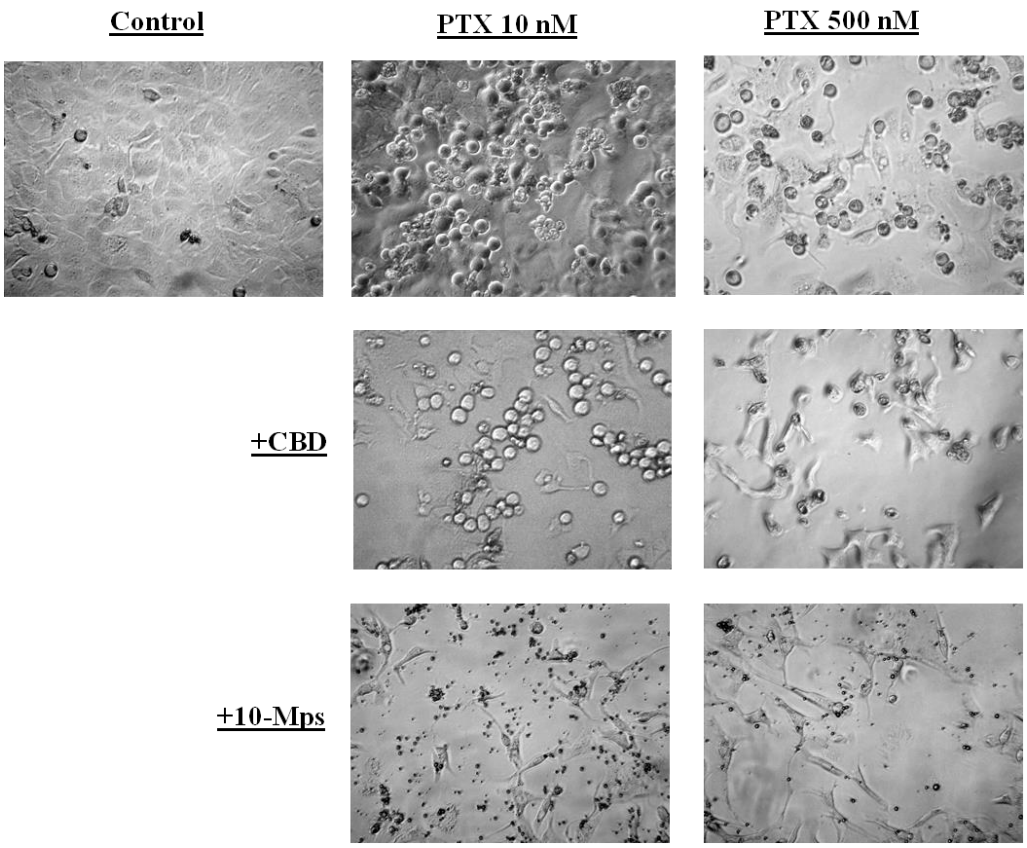


Figure S3: Images of SKOV-3 treated with RPMI medium (control), CBD in solution or encapsulated into microparticles (10-Mps) at a CBD concentration of 10 µM for 72 hours in combination with paclitaxel at a concentration of 10 or 500 nM. Paclitaxel was added after 24 hours of CBD treatments. These images correspond with treatment Schedule 3. Magnification: x20.

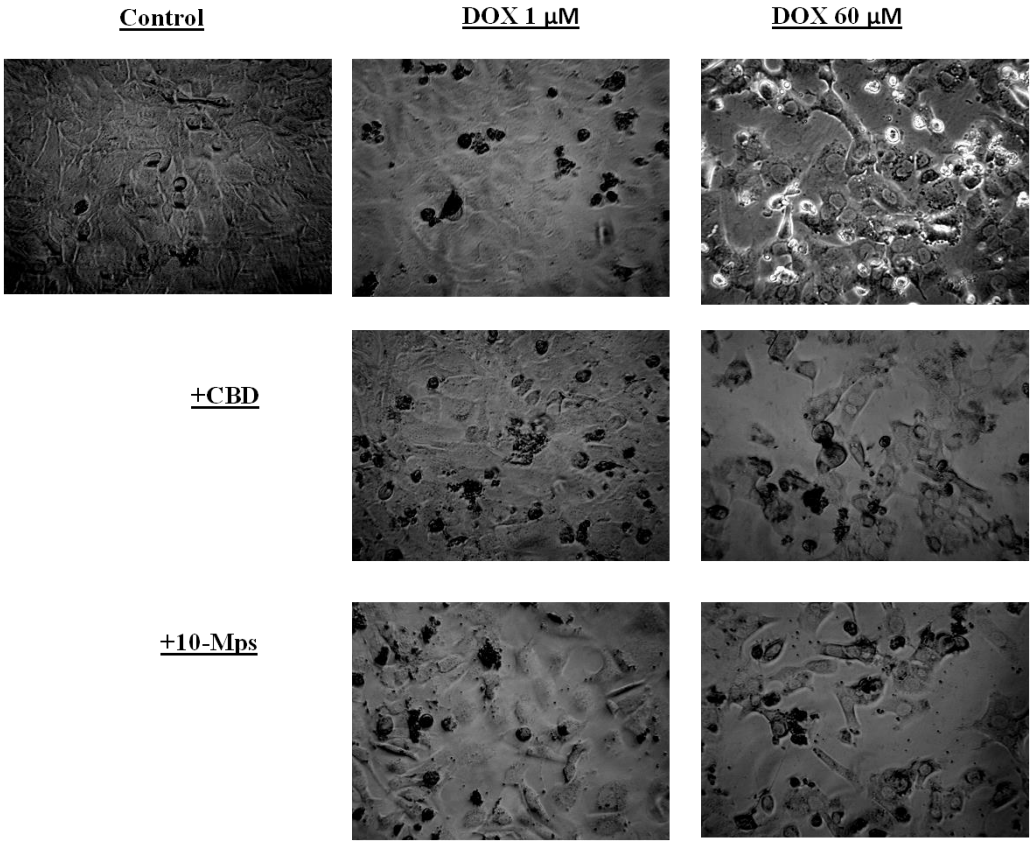


Figure S4: Images of SKOV-3 treated with RPMI medium (control), CBD in solution or encapsulated into microparticles (10-Mps) at a CBD concentration of 10 µM for 72 hours in combination with doxorubicin at a concentration of 1 or 60 µM. Doxorubicin was added after 24 hours of CBD treatments. These images correspond with treatment Schedule 3. Magnification: x20.

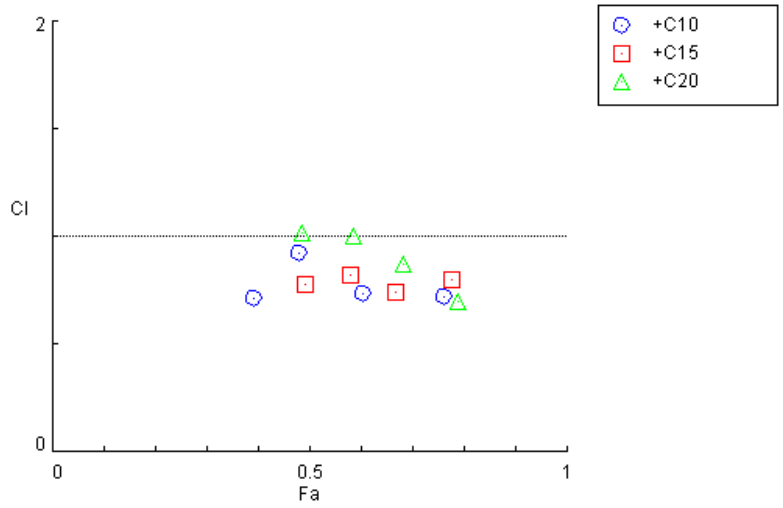


Figure S5: Combination indexes of PTX and CBD 10µM (blue), 15 µM (red) and 20µM (green).

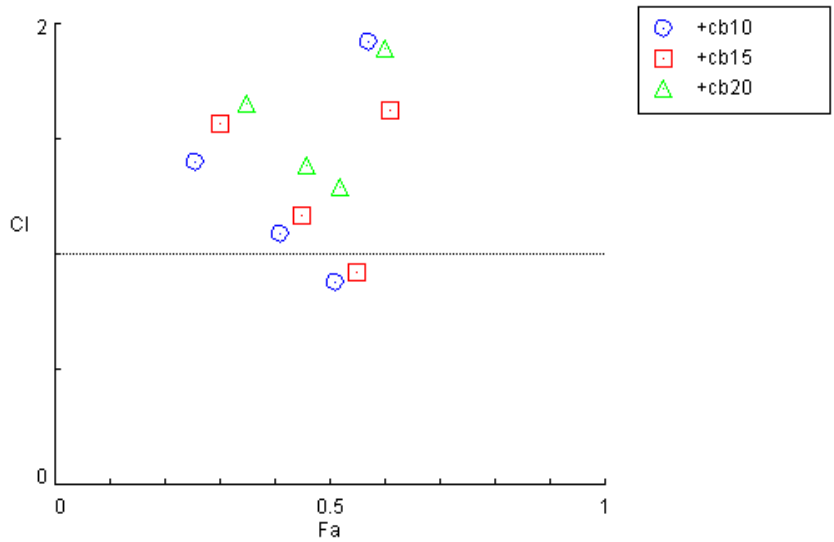


Figure S6: Combination indexes of DOX and CBD 10µM (blue), 15 µM (red) and 20µM (green).

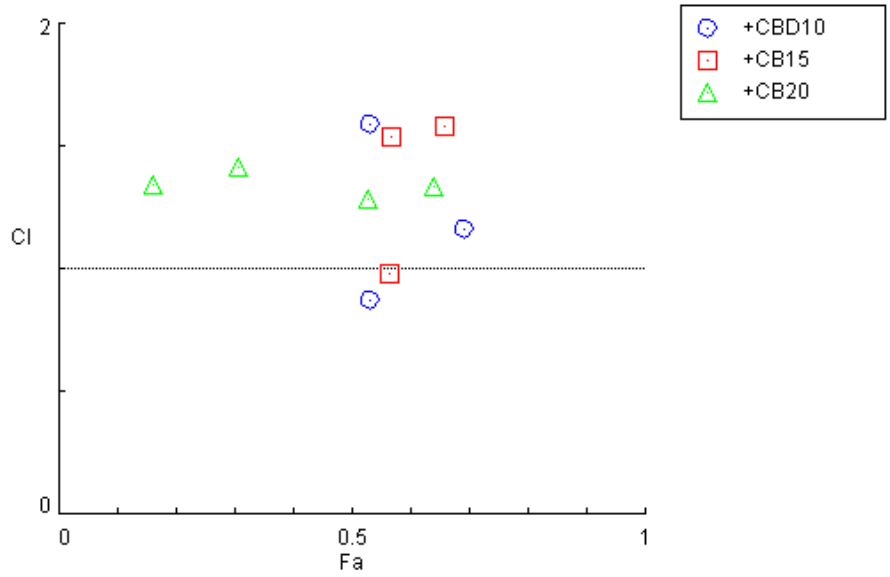


Figure S7: Combination indexes of CIS and CBD 10µM (blue), 15 µM (red) and 20µM (green).

TABLES

PTX (nM)	+ CBD 10 (µM)	+ CBD 15 (µM)	+ CBD 20 (µM)
10	0.70	0.77	1.01
50	0.92	0.82	1.00
100	0.73	0.74	0.87
500	0.72	0.80	0.70

Table S1: Combination indexes values of PTX and CBD combination.

DOX (µM)	+ CBD 10 (µM)	+ CBD 15 (µM)	+ CBD 20 (µM)
0.5	1.32	1.4	1.6
1	1.0	1.1	1.3
5	0.8	0.92	1.2
60	1.8	1.5	1.8

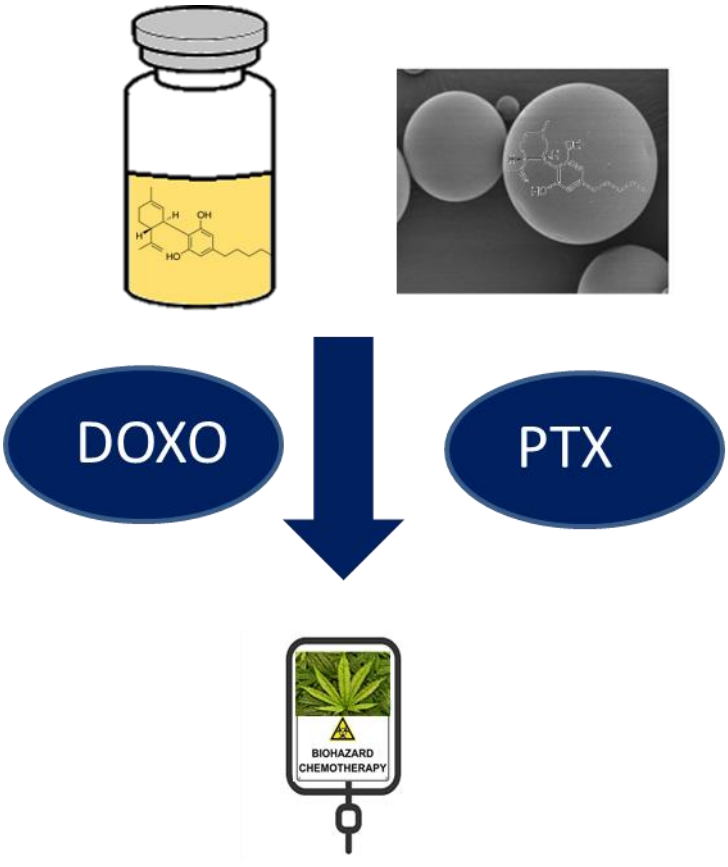
Table S2: Combination indexes values of DOX and CBD combination.

CIS (µM)	+ CBD 10 (µM)	+ CBD 15 (µM)	+ CBD 20 (µM)
1	2.58	2.43	1.3
5	2.70	2.03	1.4
20	0.97	0.98	1.2
50	1.49	1.53	1.3
100	1.16	1.58	2.1

Table S3: Combination indexes values of CIS and CBD combination.

PUBLICACIÓN 7

CBD loaded microparticles as a potential formulation to improve paclitaxel and doxorubicin based chemotherapy in breast cancer



CBD loaded microparticles as a potential formulation to improve paclitaxel and doxorubicin based chemotherapy in breast cancer.

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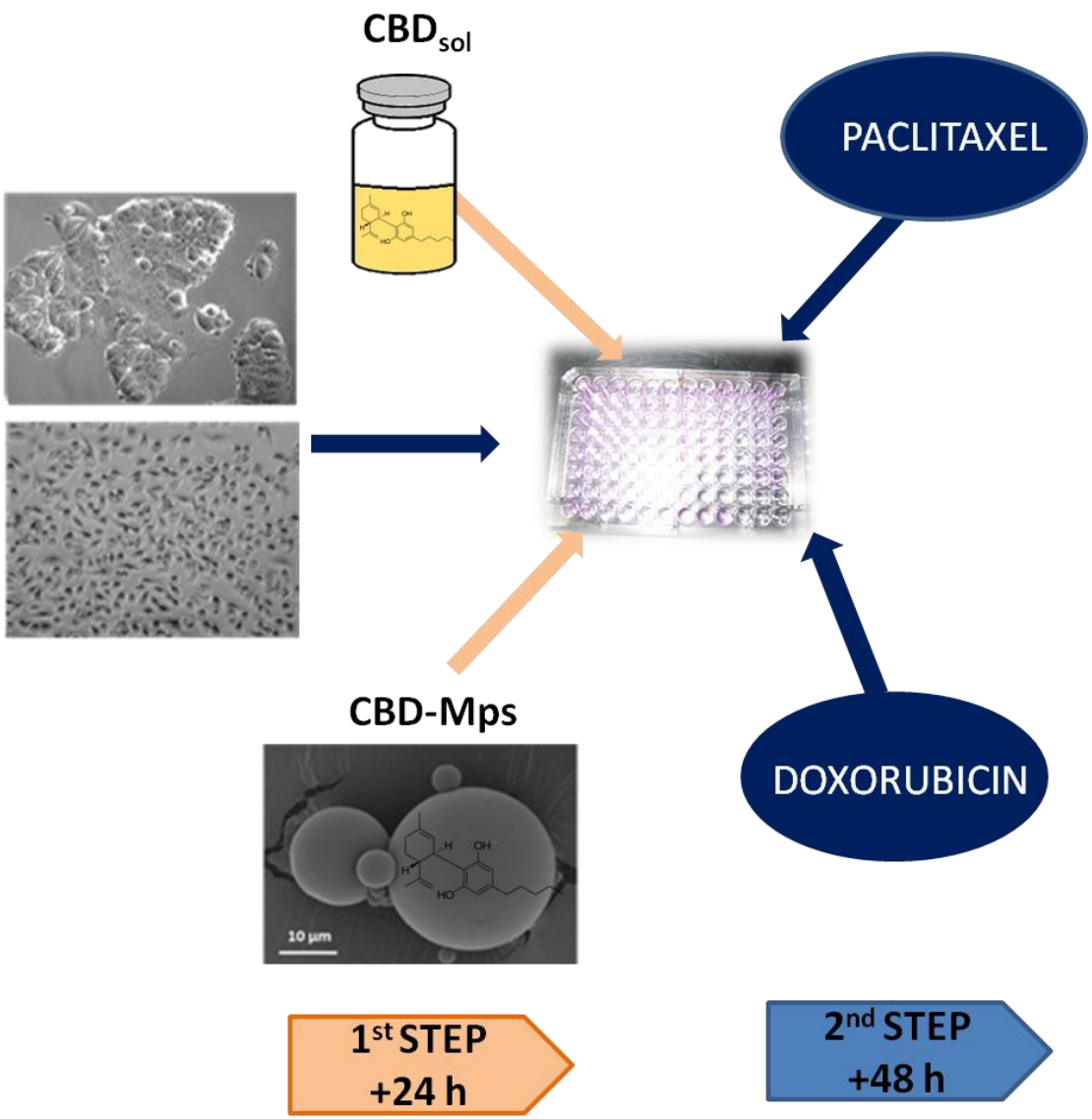
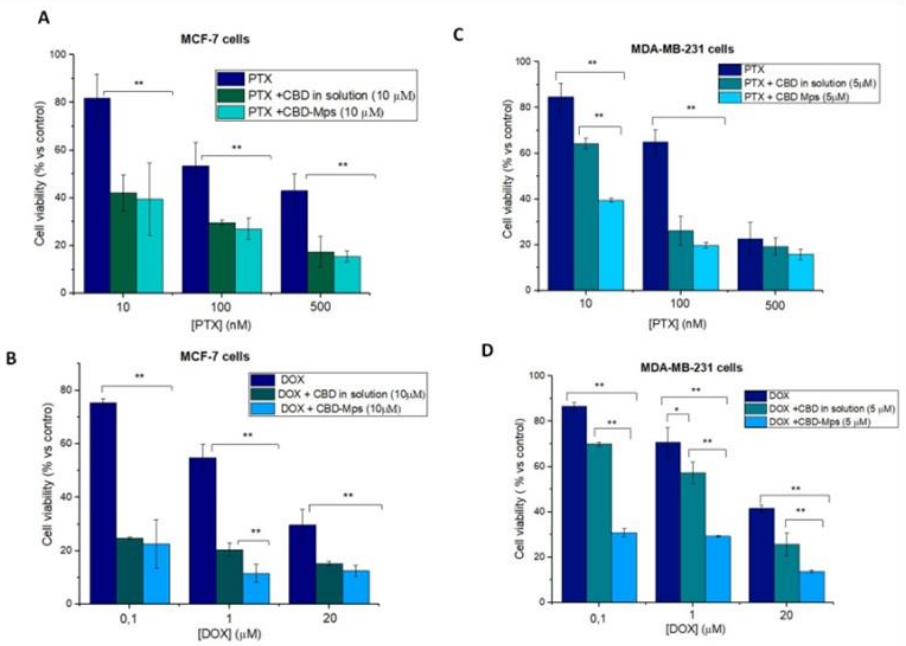
GRAPHICAL ABSTRACT





ABSTRACT

Cannabidiol (CBD) has emerged as a potential agent for the management of breast cancer. In this work, it has been evaluated for the first time the potential use of cannabidiol in solution and encapsulated into polymeric microparticles in combination with paclitaxel and doxorubicin in the treatment of breast cancer. Oestrogen receptor positive (MCF-7) and triple negative (MDA-MB-231) cell lines were used as model of breast cancer. The previous administration of CBD in solution (CBD_{sol}) at suboptimal concentrations (cell death lower than 10%) enhanced the activity of PTX and DOX in both cell lines, with a statistically significant reduction in IC_{50} values of these antineoplastic drugs. The co-administration of CBD_{sol} +PTX or CBD_{sol}+ DOX was also effective. A synergistic effect was detected in MCF-7 for both PTX and DOX. However, in MDA-MB-231 synergism was only observed at the maximum DOX and PTX concentrations, appreciating an additive effect in the rest of combinations. Notwithstanding, this synergistic effect was especially more pronounced in MCF-7 cells. PLGA microparticles loaded with CBD exhibited an extended antiproliferative activity for at least 10 days in both mammary tumour cell lines. The previous administration of CBD-Mps followed by the administration of PTX or DOX showed a high antiproliferative effect. Therefore, PLGA microparticles could be a good strategy to administer CBD and to improve conventional chemotherapy in both oestrogen receptor positive and triple negative mammary tumour cells. These results show the interest of the combination of CBD with PTX or DOX, allowing the reduction of the administered dose of these antineoplastic drugs and, as a consequence, their adverse effects.

KEYWORDS: Breast cancer, cannabinoids, cannabidiol, Doxorubicin, drug delivery, Paclitaxel.

1. Introduction

Cancer disease is one of the major health problems of the 21st century. In women, breast cancer is the most frequent malignancy, with the estimation of more than 23 million of diagnoses till 2030 [1, 2]. The implementation of early diagnostic technologies and the development of novel therapies have considerably increased the rate of survival. Nevertheless, breast tumours continue being one of the leading causes of death in women [3].

Breast cancer is a heterogeneous pathology, grouped into several subtypes according tothe aggressiveness grade (in situ and infiltrating carcinomas), the molecular profile (considering the expression of oestrogen and progesterone receptors and the overexpression of human epidermal growth factor receptors), the histopathology (tubular, ductal or medullary) and the stage of the disease (from stage 1, with a limited localization in the breast, to stage 4, with spreading to distant organs like bone or brain) [4-6].Nowadays, there are available several therapeutic approaches depending on cancer subtype: surgery, hormone therapy, radiotherapy, immunotherapy and chemotherapy[7].

In the case of breast cancer chemotherapy, it is usually recommended before (to shrinkthe tumour facilitating its elimination) or after surgery (to eliminate remnant cancer cells), and in advanced breast carcinomas (where it constitutes the main treatment strategy). Usually includes combinations of several anticancer agents: i) taxanes like paclitaxel or docetaxel, ii) anthraciclines like doxorubicin or epirubicin, iii) 5-fluorouracil, iv)platinum agents like carboplatin or cisplatin and v) gemcitabine, among others [8, 9]. However, conventional chemotherapeutic treatments are highly toxic. In this way, the appearance of novel agents with a lower toxicity are really desirable.

In the last decades cannabinoids have attracted a great deal of interest in cancer disease. They have demonstrated to be useful to improve appetite, pain and nausea and vomiting resulting from chemotherapy [10, 11], and in fact, several formulations based on cannabinoids are already approved as palliative agents in cancer disease. But cannabinoids also have shown interest as antitumor drugs per se; inhibiting the proliferation, neovascularisation, invasion and chemoresistance of tumours [12-14].

Cannabidiol (CBD) is one of the most promising cannabinoids, due to its lack of psychoactive properties and its high anticancer activity. CBD has reported to exert a high antitumor activity in breast carcinomas, decreasing the proliferation of tumour cells [15-17] and reducing breast cancer metastasis, due to its ability to inhibit the expression of Id-1 factor [18-21]. In fact, a recent study undertaken in metastatic tumour patients has demonstrated that pharmaceutical grade synthetic CBD have a potential therapeutic interest in breast carcinomas with a well-tolerated safety profile [22].

In the last years, CBD has emerged as a potential clinical tool to overcome cancer chemoresistances, due to its ability to inhibit the expression of multidrug resistance transporters [23, 24], improving the sensitivity of tumour cells to cytotoxic agents. For example, it has been demonstrated that enhances the sensitivity of anti-leukaemia drugs such as vinblastine and vincristine [25, 26], and of temozolomide and radiotherapy in glioblastoma [27, 28]. In fact, several clinical studies are being undertaken to evaluate the safety and efficacy of CBD in combination with chemo- and radiotherapy in gliomas (NCT03246113, NCT03529448), gastrointestinal malignancies and multiple myeloma (NCT03607643).

Despite the potential interest of CBD to be included in chemotherapeutic regimens, its low aqueous solubility and stability problems (it is sensible against light, temperature and oxidation [29-31], hinder the development of effective formulations. Microencapsulation could resolve these challenges, also allowing an extended antitumor activity after a single administration, which would be desirable in cancer disease where long term treatments are required.

In the present study, poly (lactic-co-glycolic acid) (PLGA) microparticles loaded with CBD were prepared. PLGA, FDA-approved, is a widely used polymer for the design and development of parenteral controlled release systems due to its biocompatible and biodegradable properties.

The aim of this work was to evaluate, for the first time, the activity of CBD in solution and encapsulated into PLGA-microparticles when combined with paclitaxel or doxorubicin in breast cancer cells in order to get a possible synergistic effect.

2. Materials and methods

2.1. Materials

Cannabidiol (CBD) was obtained from THC-Pharma (Frannkfort, Germany). Poly-(lactide-co-glycolic acid-resomer RG® 502(PLGA-502) (i.v. 0.16–0.24 dL/g) was obtained from Evonik® Industries (Essen,Germany).Doxorubicin hydrochloride and Paclitaxel (Acros Organics) were obtained from Fisher Scientific (Madrid, Spain). Polyvinyl alcohol (PVA, Mw= 30,000–70,000), Sigmacote® and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Aldrich (Missouri, USA). Acetonitrile, dichloromethane (DCM), dimethyl-sulfoxide (DMSO) and methanol (all HPLC grade) were obtained from Fisher Scientific (Madrid, Spain). Potassium di-hydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate dehydrate (Na_2HPO_4•2 H_20) and Tween®-80 were supplied by Panreac (Barcelona, Spain). RPMI-1640, DMEM and Gentamicin (10 mg/ml), were obtained from Gibco (Life technologies, California, USA). Fetal bovine serum (FBS) was supplied by Biowest (Nuaillé, France). 24-well Inserts (3µm pore size) were purchased from Falcon (Corning, Nueva York, USA). Demineralized Milli-Q® water (Millipore, Spain) was used. All chemicals and reagents were used as received.

All glass material was pre-treated with Sigmacote® to avoid cannabidiol binding.

2.2. *In vitro* antitumor activity of CBD in solution

2.2.1. Cell culture

MCF-7 (oestrogen receptor positive) and MDA-MB-231 (oestrogen, progesterone and HER-2 receptors negative also called as triple negative) breast cancer cells were obtained from American Type Culture Collection. MCF-7 and MDA-MB-231 cellsweregrown in RPMI-1640 and DMEM medium respectively, supplemented with 10% (v/v) of FBS and 1% (v/v) of gentamicin. Cells were maintained at 37 ±0.5ºC in a humidified atmosphere containing 5% of CO_2.

For cytotoxic studies, cells in exponentially growing phase were used.

2.2.2. Cell viability studies with free CBD

The antiproliferative activity CBD in solution (CBD_{sol}) was tested in both MCF-7 and MDA-MB-231 cell lines. Briefly, cells were seeded in a 96-well-plate at a density of $1.5 \cdot 10^4$ cells/cm^2 and treated, 24 hours after seeding, with CBD_{sol} (1.25-50 µM). Cell viability was determined by MTT 24 and 48 hours after treatments. Briefly, the medium was removed and 100µL of a MTT solution (0.5 mg/ml) were added. Then the cells were incubated for 3.5 hours.After that, the medium was gently discarded and 100 µL of DMSOwere added to dissolve formazan crystals. Plates were stirred for 10 minutes and read at 570 nm (Varioskan Flash, Thermo Scientific). Cell culture medium and Triton X at 1% were used as negative and positive controls of cell death.

The effect of low CBD concentrations (100-1000 nM) was also investigated for 24-96 hours of incubation using the protocol aforementioned.

2.2.3. Combination studies: modulatory effect

The ability of sub-optimal concentrations of CBD_{sol} (with a cell death lower than 10%) to increase the antiproliferative activity of paclitaxel (PTX) and doxorubicin (DOX) was evaluated in both MCF-7 and MDA-MB-231 cells. CBD_{sol} concentrations of 2.5, 5 and 10µM were used in MCF-7 cells, and concentrations of 1.25,2.5 and 5µM in MDA-MB-231 cells. DOX and PTX stocks were prepared in sterile physiological solution and cell grade DMSO respectively. The final well concentration of DMSO was lower than 0.01% (v/v). Nevertheless, the maximum amount of DMSO was tested to evaluate its effect on cell viability. For all these experiments, cells were seeded in a 96-well-plate at a density of $1.5 \cdot 10^4$ cells/cm^2 and treated 24 hours after seeding. Briefly, in a first step cells were treated with CBD_{sol} for 24 hours. Afterwards, the medium was removed and DOX (0.1-20µM) or PTX (10-500nM) treatments were added and incubated for 48 hours. Cell viability was determined by MTT as previously described.

IC_{50} of PTX and DOX were also determined for comparison purposes. A reduction in IC_{50} values would allow to achieve the same antiproliferative activity with a lower concentration of these antineoplastics. For this reason, the results of combination studies was considered as follows:

- Highly positive effect: statistically significant differences in IC_{50} at p value <0.01
- Positive effect: statistically significant differences in IC_{50} at 0.01<p <0.05
- No-effect: no statistically significant differences in IC_{50} at p value >0.05.

2.2.4. Combination studies: synergistic effect

The co-administration of CBD_{sol} with PTX or DOX was also evaluated. For these studies, MCF-7 cells were treated with CBD at 10,15 and 20 µM. However, in MDA-MB-231 cells CBD concentrations of 5,10 and 15µM were used due to the highest sensitivity of these cells to CBD. Briefly, MCF-7 and MDA-MB-231 cells were seeded at a density of $1.5 \cdot 10^4$ cells/cm^2 in 96-well plates and treated, 24 hours after seeding, with different drug pairs (CBD_{sol} + PTX or CBD_{sol} + DOX). 48 hours later cell viability was determined by MTT. From data of dose vs cell death(%) [32], combination index (CI) values were obtained using CompuSyn Software (ComboSyn, Inc. NJ, USA). The CI values were used to define the effect of drug combinations: synergism (CI < 1), additive effect (CI ≈1) and antagonism (CI>1)[33].

2.3. Elaboration and characterization of CBD loaded microparticles.

PLGA-502microparticles with a CBD: polymer ratio of 10:100 (CBD-Mps) (w/w) were prepared by the oil-in-water (O/W) emulsion–solvent evaporation technique. Briefly, PLGA and CBD were dissolved in 5 mL of DCM and dropped onto 250 mL of a 0.5% (w/v) PVA aqueous solution under stirring at 4000 rpm for 6 min. Afterwards, the resulting O/W emulsion was continuously stirred at 200 rpm using a turbine homogenizer for 3-4 hours to allow the evaporation of the organic solvent and the hardening of the microparticles. Finally, microparticle suspension was filtered using a 5 µm PTFE membrane. Collected microparticles were washed with demineralised water to remove residual PVA and lyophilised for 18h at −50 °C and 0.2 mbar.

Unloaded microparticles (PLGA-Mps) were prepared by the same protocol.

Microparticles were characterized in terms of size, drug loading and drug release. The particle size was determined by laser diffraction using a Microtrac® S3500 Series Particle Size Analyzer (Pennsylvania, USA). The mean particle size was calculated as volume diameter.

For the quantification of encapsulated CBD into PLGA microparticles and CBD released from microparticles, a High Performance Liquid Chromatography (HPLC, Agilent 1200 series, California, USA) method was used. The analytical conditions were as follows: i) a mobile phase containing a mixture of methanol: acetonitrile: water at pH-4.5at a ratio of 52:30:18,ii) a flow rate of 1.8ml/min; iii) a reversed-phase Mediterranea® C18 column (15×0.46 cm, 5 μm) (Teknokroma®, Barcelona, Spain) , iv) 20μL of injection volume and v) detection at 228nm.

The amount of CBD encapsulated into PLGA microparticles was determined as follows: 10 mg of microparticles were dissolved in 1 ml of DCM and the CBD was extracted with the addition of 9 ml of mobile phase, which also promoted polymer precipitation. The samples were filtered using PTFE 0.45μm syringe filters and analysed by HPLC.

The encapsulation efficiency (EE) was calculated using the following equation:
EE (%) = [(CBD:PLGA ratio $_{Experimental}$)/ (CBD:PLGA ratio$_{Initial}$)] x100

The CBD released from microparticles was determined indirectly by calculating the remaining CBD into microparticles. Briefly, vials with10 mg of microparticles suspended in 5 ml of PBS (pH 7.4) containing Tween® 80 at 0.5% (w/v) to maintain sink conditions were disposed in a thermostatic shaking water bath at37±0.5ºC and an agitation rate of 100rpm. At specific time points (2h,8h,1,2,4,7,10,14,21,28, 31 and 42 days) the supernatant was removed, CBD was extracted from microparticles as aforementioned and samples were analysed by HPLC.

2.4. Stability studies of CBD microparticles

In order to evaluate the stability of CBD-Mps, long term stability studies over 12 months were undertaken. For this, 15 mg of Mps were placed into sealed vials and stored in refrigerator at 5 ± 3ºC and in stability chamber at 25±2ºC/60 ±5 % of relative humidity (Memmert UN30, Germany). At predetermined time points (1, 3, 6, 9 and 12 months) samples were analysed in relation to particle size and drug content.

Due the fact that CBD is a photo-sensitive molecule, photo-stability of CBD-Mps was also evaluated. Briefly, 15 mg of microparticles were placed into sealed vials an incubated at 5±3ºC in a photo-stability chamber (CCI: Xenoterm 1500-RF, CCI, Barcelona, Spain) equipped with a D65/ID65 xenon lamp for 72 hours in order to guarantee an overall illumination greater than 1.2 million of lux h and an integrated near ultraviolet (above 320 nm) energy greater than 200 W h/m^2 [34]. Samples in dark were used as control. At predetermined time points, CBD content was analysed by HPLC. The same experiment, was also evaluated with N_2flushed samples to evaluate the participation of oxygen in CBD photo-degradation.

2.5. *In vitro* antitumor efficacy of CBD microparticles

MCF-7 and MDA-MB-231 cells were seeded in a 24-well plate at a density of $1.5 \cdot 10^4$ cells/cm^2. 24 hours after seeding, the medium was removed and the treatments were added. For these experiments, release studies of CBD from Mps were undertaken in parallel. At pre-determined time points (2,4,6 and 8 days) release medium was removed after centrifugation, and microparticles were collected, suspended in cell culture medium and positioned in the top of 3μm pore size inserts, which were placed in each well (24 well-plates were used). The amount of microparticles was calculated according to *in vitro* CBD release. The results were compared with those obtained when an equivalent concentration of CBD_{sol} was administered. Unloaded microparticles were also tested.

Cell viability measurement was evaluated by MTT as previously described in 2.2.1 section with some modifications.

2.6. Combination studies with CBD microparticles

The antiproliferative efficacy of CBD-Mps when combined with PTX or DOX was also tested. MCF-7 and MDA-MB-231 cells were seeded as previously described in a 24 well-plate. Briefly, 24 hours after seeding, an amount of CBD-Mps were added in the top of 3μm pore size inserts and placed in each well to achieve a daily administration of CBD of 5 and 10 μM in MDA-MB-231 and MCF-7 respectively.24 hours later, PTX (10-500 nM) or DOX (0.1-20 μM) was added to cells. After 48h of incubation cell viability was determined by MTT as aforementioned. The activity of microparticles was compared to the daily administration of the equivalent amount of $CBD_{sol.}$

2.7. Statistical analysis

The data were reported as a mean ± S.D (standard deviation) of at least three experiments (n=3). Multiple groups were compared using one-way analysis of variance (ANOVA). Significant differences were reported as follows: $*p < 0.05$ and $**p < 0.01$. All the graphs and statistical analysis were performed using Origin 2017 software (Origin lab, Massachusetts, USA).

3. Results and discussion

3.1. Combination studies of CBD in solution

Breast cancer is one of the tumour types where CBD has showed a high anticancer activity. In fact, numerous authors have reported that CBD inhibits the proliferation, migration and invasion of both oestrogen-receptor-positive and triple negative breast tumour cells [16-19, 21].

Nevertheless, other authors have showed a biphasic cannabinoid effect on tumour cells, indicating that at low concentrations (in the nanomolar range) cannabinoids may stimulate cancer cell proliferation. This could challenge the use of microparticles as carriers for cannabinoid administration due to the slow drug release phases from these systems. However, in this work CBD did not increased the proliferation neither MCF-7

nor MDA-MB-231 cells in a range of concentrations of 100-1000nM (supplementary material, figure S1 and S2).

The previous administration of CBD_{sol} in MCF-7 cells treated with PTX showed a positive effect at CBD concentrations of 2.5 and 5 μM and a highly positive effect when it was administered at 10μM, demonstrating that CBD sensitized these breast cancer cells against PTX (Fig. 1 A). In fact, the pre-administration of CBD_{sol} at 10 μM conducted to a ≈7-fold reduction in PTX concentration to get a cell death of 50% (Table 1). The co-administration of CBD_{sol} plus PTX was also effective (highly positive effect) as depicted in Fig. 1B. A considerable and significant decrease in PTX IC_{50} was achieved for all CBD concentrations (Table 1). In fact, as it could be observed in Fig. 1E, combination indexes below 1, in the range of 0.59-0.83, were obtained for all tested combinations, indicating a synergistic effect between CBD and PTX (supplementary material, table S1).

In respect of DOX, it has been reported that WIN55,212-2 (a synthetic cannabinoid) increases the anticancer effect of this drug in triple negative breast tumours (4-T1 cells were used as model) [35].In our work a positive effect was only detected with CBD pre-treatmentat10 μM. In the cells pre-treated with CBD at 2.5 or 5 μM the reduction in IC_{50} were not significant. The co-administration of CBD_{sol} plus doxorubicin reported a positive effect, except when CBD_{sol} was used at 10μM. In addition, combination indexes below 1, except for the combination of DOX 1 μM with CBD 10 μM (supplementary material, table S2), were obtained, indicating a slightly synergistic effect between CBD and DOX in these oestrogen receptor positive cells (Fig.1 F).

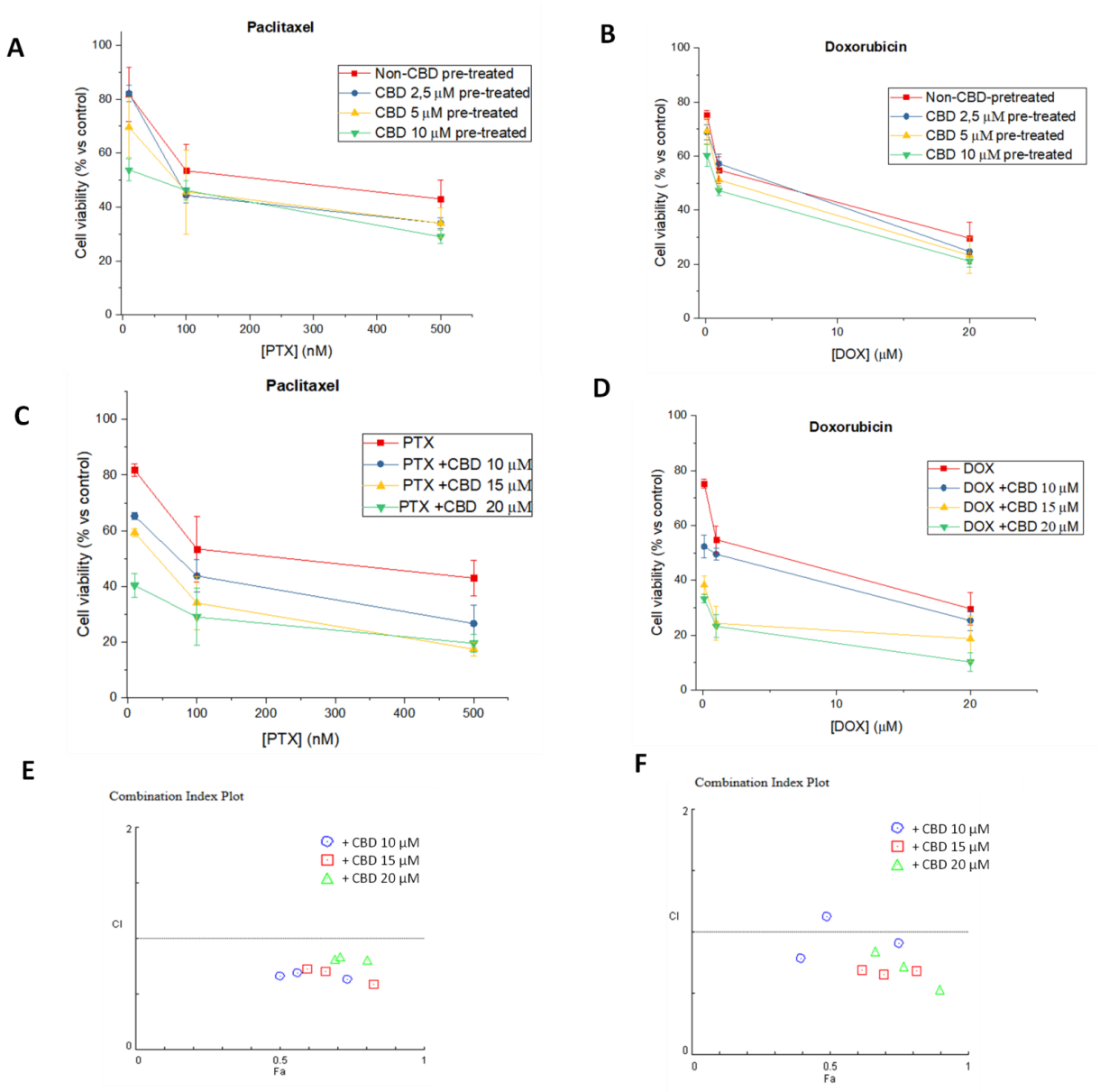


Figure 1: Cell viability of MCF-7 cells pre-treated with $CBD_{sol}$for 24 hours followed by the administration of PTX (A) or DOX (B) for 48 hours. Cell viability of MCF-7 cells treated with CBD_{sol}+PTX (C) or CBD_{sol}+DOX (D) for 48 hours. Combination indexes obtained for CBD_{sol}+PTX (E) and CBD_{sol}+DOX (F) combinations.

	PTX (nM)	DOX (µM)
Singledrug	228.28 ± 55.92	3.09 ± 1.32
+ Pre-treatment CBD 2.5 µM	111.60 ± 22.50 *	2.3± 0.89
+ Pre-treatment CBD 5 µM	72.53 ± 8.76 *	1.22 ± 0.1
+ Pre-treatment CBD 10 µM	33.46 ± 11.74 **	0.88± 0.13*
+ CBD 10 µM	55.19 ± 11.11 **	1.11 ± 0.98
+ CBD 15 µM	24.88 ± 1.43 **	0.30 ± 0.17*
+CBD 20 µM	5.52 ± 4.83 **	0.03 ± 0.025 *

Table 1: IC_{50} values of paclitaxel and doxorubicin in MCF-7 cells when combined with CBD_{sol}.* ($0.01<p\ e<0.05$) and **($p<0.01$) describe significant differences between non-CBD treated and CBD treated cells.

MDA-MB-231 cells (highly invasive breast tumour cells) are more sensitive to CBD action, showing a lower IC_{50} after 48 hours of incubation (11.37± 1.82 µM) than MCF-7 cells (20.64±2.97 µM) (cell viability curves of CBD_{sol} are depicted as supplementary material). The previous administration of suboptimal concentrations of CBD_{sol} also reported the sensitization of these cells to PTX and DOX (Fig. 2 A and B), showing a highly positive and positive effect respectively, but the combination was less effective than in MCF-7 cells, with a ≈3- and ≈1.7-fold decrease in IC_{50} values of PTX and DOX respectively when CBD_{sol} was administered at the highest concentration (5µM) (Table 2). CBD_{sol} co-administered with PTX also significantly improved PTX results, demonstrating a highly positive effect in all tested concentrations (Table 2). A synergistic effect (CI<1) was detected at the maximum PTX concentration (500 nM) when combined with CBD 5, 7.5 and 10 µM. However, at lower PTX concentrations an additive effect was observed with combinations indexes in the range of 0.80-1.1 (Fig. 2C) (supplementary material, table S3).

Regarding DOX combination, the pre-administration of CBD_{sol} showed a positive effect at all CBD concentrations tested (Fig. 2B). In the case of CBD_{sol} and DOX co-administration (Fig. 2 D), while a positive effect was appreciated when DOX was combined with CBD 5 µM, a highly positive effect was observed in the other combinations (CBD 7.5 and 10 µM) (Table 2). However, a moderate synergism was only observed with DOX administered at 20µM. At lower concentrations an additive effect,

with combination indexes in the interval of 0.92-1.1, was appreciated (Fig. 2 F) (supplementary material, table S4).

All these studies carried out on MCF-7 and MDA-MB-231 cell lines indicate, first, that the combination of CBD and both antineoplastic drugs could be really interesting, especially in oestrogen positive breast tumours, where the best results were found.A significant reduction in $IC_{50}$and a synergistic effect was appreciated in both CBD_{sol}+ PTX and CBD_{sol}+ DOX combinations. In triple negative breast tumour cells, the decrease observed in the IC_{50} values was statistically significant, although not synergism was detected in all combinations tested. In this case, an additive effect could also be interesting, especially if the invasiveness, the poor prognosis and the difficulty of the treatment of this tumour type are considered. In fact, the main advantage of the combination is due to the possibility of decreasing the dose of PTX and DOX with the reduction of toxicity related to chemotherapy. Moreover, it has been demonstrated in murine models that CBD palliates the peripheral neuropathy related to PTX [36-38] and the cardiotoxicity associated with doxorubicin (which is the major problem of doxorubicin based chemotherapy)[39, 40], making more promising the combination CBD plus PTX or CBD plus DOX.

	PTX (nM)	DOX (µM)
Single drug	142.77 ±18.24	10.73 ± 2.49
+ Pre-treatment CBD_{sol} 1.25 µM	102.09 ± 7.66**	6.11± 1.01*
+ Pre-treatment CBD_{sol} 2.5 µM	67.08 ± 7.01**	6.29± 0.68*
+ Pre-treatment CBD_{sol}5 µM	49.46 ± 2.47**	4.84± 0.68 *
+ CBD_{sol} 5 µM	79,22 ± 10.35 **	5.49± 1.04*
+ CBD_{sol} 7.5 µM	44.82 ± 4.60 **	3.84± 1.05**
+CBD_{sol} 10 µM	22.86 ± 4.8 **	1.41± 0.51**

Table 2: IC_{50} values of paclitaxel and doxorubicin in MDA-MB-231 cells when combined with CBD in solution.* ($0.01<p<0.05$) and ** ($p<0.01$) describe significant differences between non CBD treated and CBD treated cells.

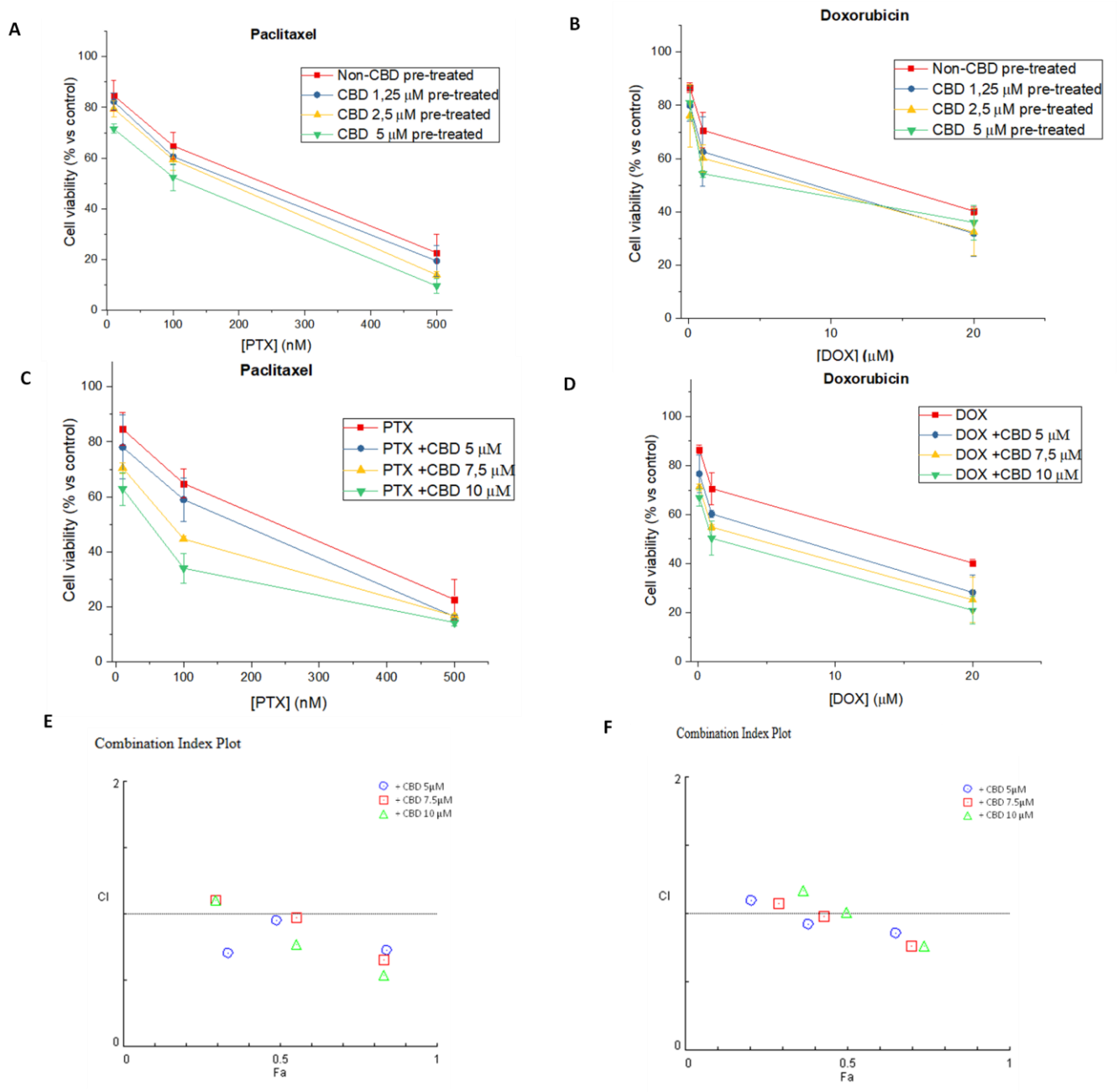


Figure 2: Cell viability of MDA-MB-231 cells pre-treated with $CBD_{sol}$for 24 hours followed by the administration of PTX (A) or DOX (B) for 48 hours. Cell viability of MDA-MB-231 cells treated with CBD_{sol}+PTX (C) or CBD_{sol}+DOX (D) for 48 hours. Combination indexes obtained from CBD_{sol}+PTX (E) and CBD_{sol}+DOX (F) combinations.

3.2. Microparticle preparation and characterization

Despite the potential clinical interest of CBD, the low aqueous solubility of this compound hampers its intravenous administration. The use of microparticles as CBD carriers would resolve this challenge.

When characterizing the elaborated microparticles, a similar mean particle size was obtained in both unloaded and CBD-Mps, with values of 24.03±2.01µm and 24.17±2.32 µm respectively. This mean particle size is suitable for parenteral administration. Unimodal size distribution curves were obtained, although the size distribution was not monodisperse, with span values around 2 in both unloaded and CBD-Mps. The CBD content was 8.601±0.42mg /100 mg Mps, with an encapsulation efficiency above 90% (94.62±4.62 %).

The release profile of CBD from PLGA microparticles is depicted in Fig.3. A controlled drug release for more than one month was detected. Regarding release kinetic, a tri-phasic release profile was observed: a 1st faster phase until day 2 with almost 30% of the CBD released, a 2nd slower phase from day 2 to day 14, with around the 45% of the drug released, and a final and slightly faster 3rd phase from day 14 to day 42, with more than 95% of the CBD released (Fig. 3).

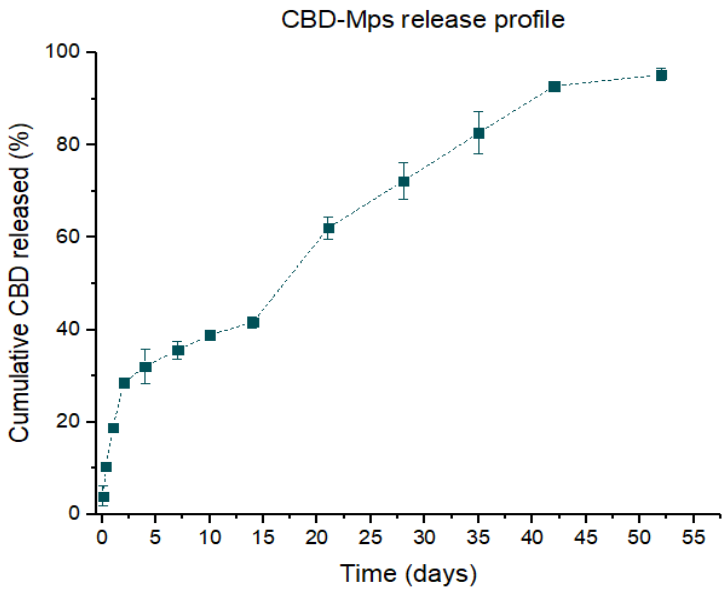


Figure 3: Release profile from CBD-loaded-microparticles.

3.3 Stability studies

It has been reported that cannabinoids show, in general, high instability[29].Thismay compromises their efficacy and has to be considered in cannabinoid formulation development and storage. In order to evaluate the preservation of CBD into the microparticles and recommend storage conditions, long term stability studies were conducted for a period of 12 months, evaluating CBD content and also the mean particle size and polydispersion, since microparticle aggregation is usual during storage [41].

No significant changes in the mean particle size were detected over the 12 months of the study when CBD-Mps were stored in refrigerator. However, in Mps stored at room temperature a significant higher particle size and polydispersion were detected after 6 months, which could be attributed to particle aggregation (Fig. 4 A and B). Regarding drug content, in microparticles stored at 25ºCaround 33%of encapsulated CBD was degraded in the first month. Nevertheless, no significant drug degradation was appreciated after storage at 5ºC during all the study (Fig. 4C). These data indicate that CBD-Mps, remained stable for at least 12 months when stored at 5ºC.

Due to the instability of cannabinoids against light, photo-stability studies with CBD-Mps have also been performed (in supplementary material is illustrated the photo-stability of CBD in solution, figure S3). As it could be observed in Fig.4D, while light protected samples maintained stable during all the experiment, CBD degradation around 25% was appreciated in light exposed samples after 72 hours of storage at 5ºC.Several authors have reported that microencapsulation protects the drug form photo-degradation [42, 43]. In this work microencapsulated CBD was photo-unstable. Moreover, it has also been demonstrated that cannabinoids, including CBD, are sensible to oxidation. In fact, they are potent antioxidants. As depicted in Fig.4Ewhen photo-stability samples where previously flushed with N_2 in order to eliminate the air chamber, no CBD loss during the 72 hours of the study at 5ºC was detected, suggesting that CBD photo-degradation reaction is oxidative.

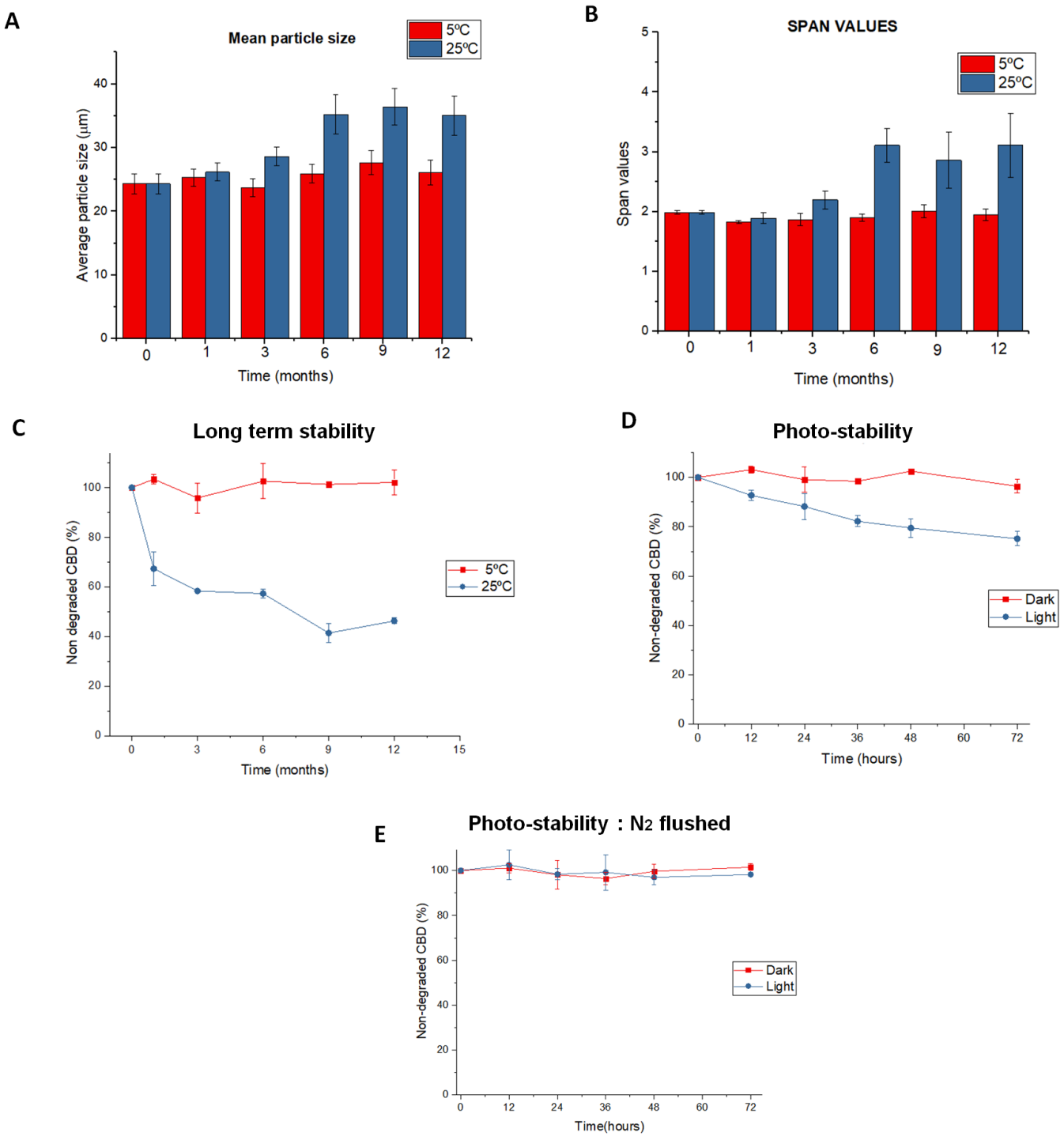


Figure 4: Graphs of long term stability studies of CBD loaded microparticles: mean particle size (expressed as volume diameter) (A), span values (B) and CBD content (C). Graphs of photo-stability studies with samples without (D) and with N_2flushing (E).

To sum up, stability studies showed that CBD-Mps should be stored in refrigerator and protected from light. N_2flushing should be also recommended to improve their stability. Stored under these conditions, CBD-Mps are physically and chemically stable for at least 12 months.

3.4. *In vitro* antitumoral efficacy of CBD-Mps

The use of microparticles as CBD carriers would allow to achieve an extended antitumor activity after a single administration. In fact, polycaprolactone microparticles loaded with CBD have shown a promising extended antitumor efficacy in triple negative breast tumour cells [44].

When unloaded microparticles were administered no cytotoxic effect was appreciated neither in MCF-7 cells nor MDA-MB-231 cells. Nevertheless, CBD-Mps inhibited the cell proliferation throughout the time interval evaluated (10 days) in both breast cancer cells (Fig.5A and B). In MCF-7 cells 3.8mg of CBD-Mps (containing 0.335 mg of CBD) were administered. However, in MDA-MB-231 cells, due to its higher CBD sensitivity, 2.92 mg of CBD-Mps (containing 0.251 mg of CBD) were administered. These values were calculated using CBD *in vitro* release data during the 2nd slower phase and approximately correspond with IC_{70} values after 48 hours of incubation previously calculated (an overall concentration of CBD 25and 18μM in MCF-7 and MDA-MB-231 cells respectively).

The higher antiproliferative activity during the first two days of the experiment is related to CBD release profile. This period of time corresponds to the 1st faster phase, in which a high free CBD concentration, above 100μM, is reached in both MDA-MB-231 and MCF-7 cells. This explains the high cell viability inhibition, above 85%, detected in both cell lines. According to the release study, after day 2, the 2nd slow release phase of CBD from Mps starts (finished at day 14). As a consequence, during the rest of the antitumor activity assay (2-10 days) an overall free CBD concentration around 18 and 25 μM was achieved in MDA-MB-231 and MCF-7 cells respectively, leading to a cell proliferation inhibition of 60% that was similar to that obtained with CBD_{sol} daily administered. Therefore, these results indicated that the CBD encapsulated into polymeric microparticles maintained its antiproliferative activity against both mammary breast

tumour cells. This antiproliferative activity remained constant during at least 10 days, indicating that this formulation could be a good strategy to get an extended CBD antiproliferative activity after a single administration.

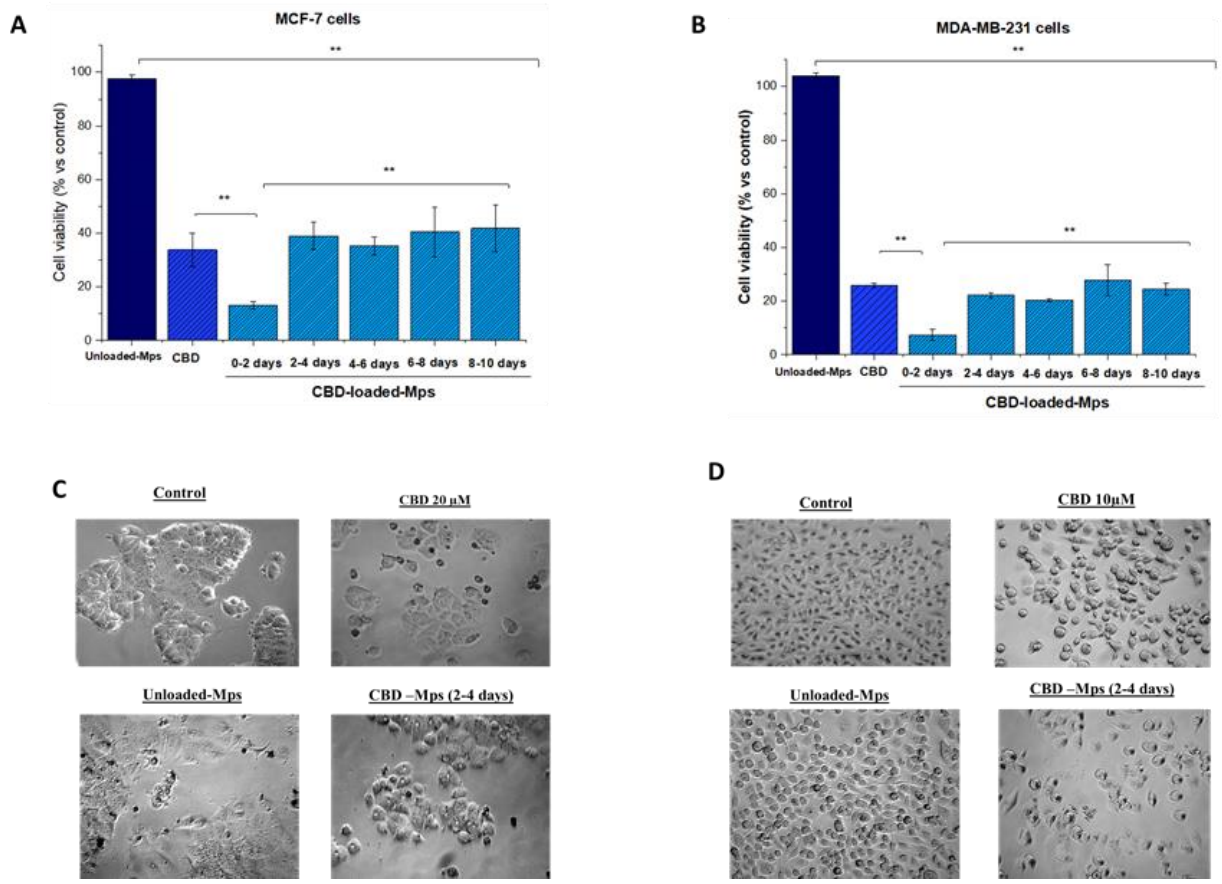


Figure 5: Antiproliferative activity of CBD-loaded microparticles in MCF-7 (A) and MDA-MB-231 cells (B). Images obtained by optical microscopy of MCF-7 (C) and MDA-MB-231 cells (D) after CBD_{sol} and microparticle formulation treatments. Image magnification: x20

3.6. Combination activity: CBD loaded microparticles

As previously mentioned, the results obtained in the combination studies of CBD in solution with PTX and DOX showed that the administration of CBD before or during PTX or DOX treatments could be interesting strategies to enhance the antiproliferative activity of these drugs in breast carcinomas. In fact, the pre-treatment or the co-administration of CBD would allow to reduce the dose of antineoplastic agents. Now, both strategies (CBD pre and co-administration) were combined looking for an optimal effect of CBD on the antiproliferative activity of PTX or DOX. For this, CBD was administered in two different formulations: as solution and in Mps. On the one hand, CBD_{sol} was added to cell culture in a 1st step of pre-treatment and after 24 hours in co-administration with PTX or DOX. On the other hand, CBD-Mps were added in a single administration 24h before the administration of PTX or DOX. In these experiments, the amount of administered microparticles was calculated using in vitro release data during the first 72 hours, the total duration of this study. The administration of CBD in Mps leaded to its continuous release before and after PTX or DOX administration and in addition, to protect CBD from possible degradation due to external agents. In fact, it has been reported that CBD is unstable in aqueous medium at 37ºC.

In MCF-7 cells this schedule (CBD pre and co-administration) was really effective, with statistically significant differences between IC_{50} values for single drugs (PTX or DOX) with respect to their combination with CBD administered in solution or in microparticles. For these experiments0.650 mg of CBD-Mps (containing 55.9µg of CBD) were administered, achieving a daily free CBD concentration of ≈10µM (Fig. 6A and B). Their activity was compared with the daily administration of CBD_{sol} at 10µM. The single administration of CBD-Mps showed a similar or even better efficacy than the daily administration of CBD in solution. Nevertheless, no statistically significant differences were appreciated between both treatments, except in the combination of CBD-Mps plus doxorubicin 1µM. These data suggest that the use of polymeric microparticles could be a good strategy for CBD administration, so that the activity of both PTX and DOX in oestrogen positive breast cancer cells can increase after a single administration of CBD-Mps.

Regarding experiments with MDA-MB-231 cells, 0.325 mg of CBD-Mps (containing 27.95 µg of CBD) were administered, getting a daily free CBD concentration of ≈5µM.Their effect was compared to the daily administration of CBD_{sol} at the same concentration. In respect of CBD and PTX combination(Fig. 6C), a higher and statistically significant (p value<0.01) antiproliferative activity was detected in both CBD_{sol} and CBD-Mps treated cells, compared to single PTX, except for the highest PTX concentration, probably due to the high death detected when PTX was administered at 500nM. Nevertheless, statistically significant differences between both CBD treatments were only detected at the lowest PTX concentration, where CBD-Mps showed more effective.

In the case of DOX, a significant higher antiproliferative activity (p value <0.01) was also detected in CBD treated cells (Fig. 6D). Even more, CBD-Mps reported higher activity than $CBD_{sol,}$ with statistically significant differences (p<0.01).CBD instability could explain, in part, the higher efficacy of CBD-Mps compared to CBD_{sol}. As previously mentioned, CBD is unstable in aqueous medium. While CBD encapsulated into microparticles is protected, CBD in solution is more susceptible to degradation, decreasing its final concentration and as a consequence its activity. It has to be taken into account that MDA-MB-231 cells (where considerably differences between CBD_{sol} and CMD-Mps were detected) are more sensitive to CBD than MCF-7 cells, anda constant CBD release from microparticles could be more effective, explaining the higher efficacy of CBD-Mps compared to the daily administration of CBD in solution.

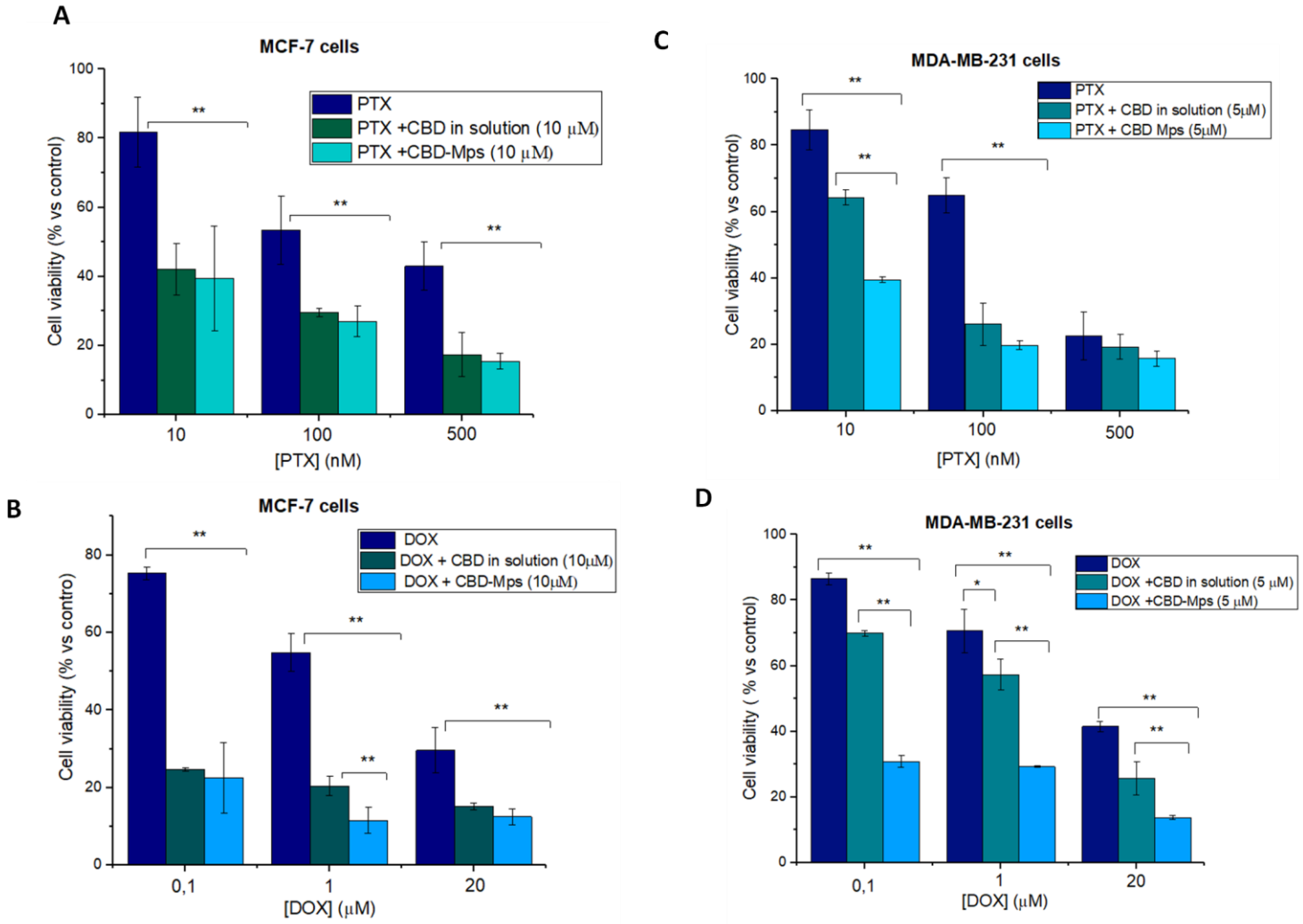


Figure 6: Cell viability of breast cancer cells pre-treated with CBD in solution or encapsulated into polymeric microparticles for 24 hours followed by the administration of these treatments plus PTX (A and C) or doxorubicin (B and D) for 48 hours.

4. CONCLUSION

The combination of cannabidiol with paclitaxel or doxorubicin will allow to reduce the effective concentration of these antineoplastic agents in MDA-MB-231 and MCF-7 cells. On the one hand, the previous administration of CBD in solution reported a significant sensitization of both breast cancer cell lines when they were subsequently treated with these drugs. On the other hand, the co-administration of CBD with paclitaxel or DOX showed synergistic effect in MCF-7 cells and an additive or synergistic effect, depending on DOX and PTX concentrations, in MDA-MB-231 cells.

Polymer microparticles loaded with CBD showed an extended antiproliferative activity for at least 10 days in MDA-MB-231 and MCF-7 cells. These CBD microparticles are shown as an interesting alternative to the pre + co-administration of CBD in solution in combination with paclitaxel or doxorubicin, being achieved similar or even improved antiproliferative effect in both type of breast cancer cells.

Although further studies are required, this work provides by the first time, the potential use of CBD loaded microparticles in combination with paclitaxel or doxorubicin for both oestrogen receptor positive and triple negative breast tumour cells. The combination of CBD-MPs with PTX or DOX in breast cancer could allow the reduction of the administered dose of these drugs and, as a consequence, their adverse effects.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

FIGURES

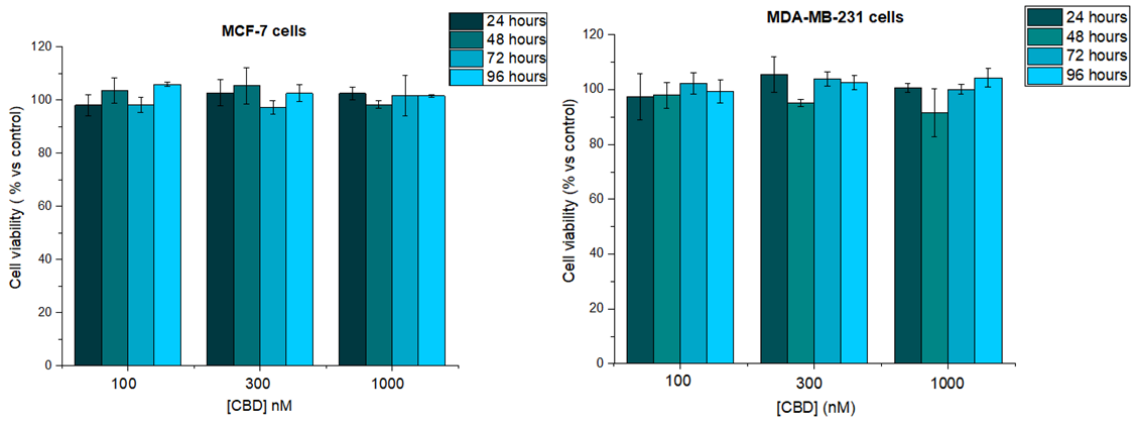


Figure 1: MCF-7 and MDA-MB-231 cells treated with CBD in solution at low concentrations (100-1000 nM) for 24, 48,72 and 96 hours.

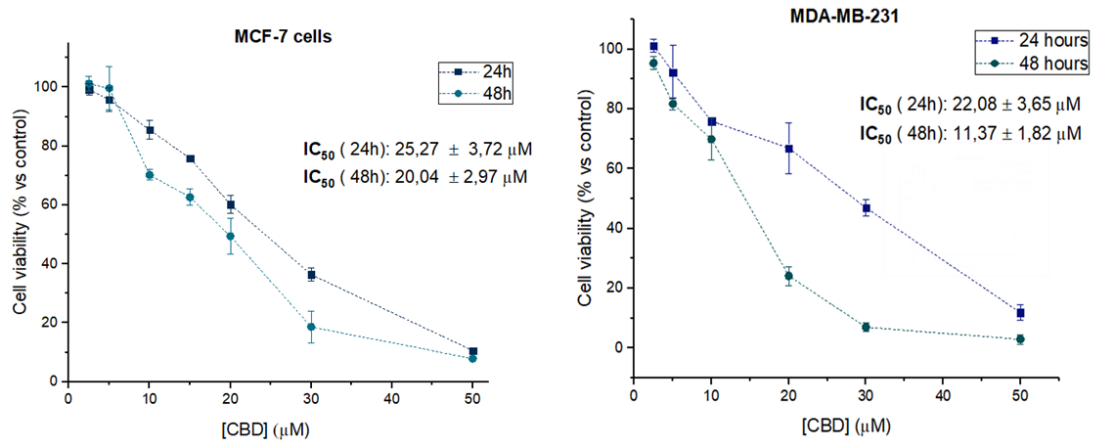


Figure 2: Antiproliferative curves of CBD in solution (2.5-50µM) inMCF-7 and MDA-MB-231 cells after 24 and 48 hours of incubation.

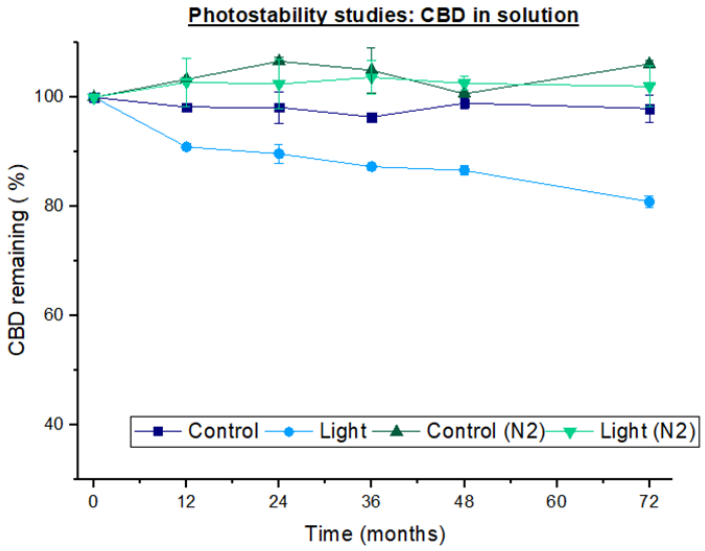


Figure 3: Photo-stability study of CBD in solution (100µM) non bubbled and N_2 bubbled. The study was undertaken at 4ºC.

TABLES

PTX (nM)	+CBD 10 µM	+CBD 15 µM	+CBD 20 µM
10	0.66±0.05	0.72±0.17	0.81± 0.09
100	0.68±0.12	0.70±0.21	0.83± 0.16
500	0.63±0.20	0.59±0.08	0.80± 0.13

Table S1: Combination indexes of CBD and PTX drug pair in MCF-7 cells.

DOX(µM)	+CBD 10 µM	+CBD 15 µM	+CBD 20 µM
0.1	0.78± 0.11	0.68± 0.05	0.84± 0.31
1	1.12± 0.22	0.65± 0.06	0.72± 0.47
20	0.91± 0.04	0.68± 0.12	0.53± 0.10

Table S2: Combination indexes of CBD and DOX drug pair in MCF-7 cells.

PTX(nM)	+CBD 5 µM	+CBD 7.5 µM	+CBD 10 µM
10	0.8 ±0.14	1.1 ±0.02	1.08 ±0.10
100	1.07 ±0.17	0.87 ±0.1	0.88 ±0.12
500	0.55 ±0.007	0.54 ±0.05	0.63 ±0.08

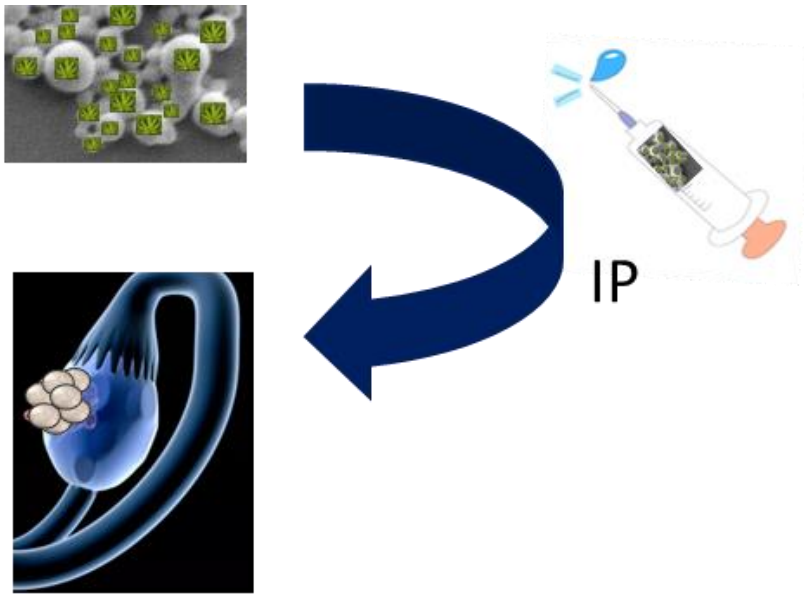
Table S3: Combination indexes of CBD and PTX drug pair in MDA-MB-231 cells.

DOX(µM)	+CBD 5 µM	+CBD 7.5 µM	+CBD 10 µM
0.1	1.09±0.06	1.07±0.04	1.00±0.07
1	0.92±0.04	0.97±0.1	1.1±0.02
20	0.86±0.64	0.75±0.3	0.76±0.16

Table S4: Combination indexes of CBD and DOX drug pair in MDA-MB-231 cells.

PUBLICACIÓN 8

CBD-PLGA nanoparticles as a novel formulation for ovarian cancer therapy: *in vitro* and *in ovo* assessment.



CBD-PLGA nanoparticles as a novel formulation for ovarian cancer therapy: *in vitro* and *in ovo* assessment.

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ABSTRACT

The intraperitoneal administration of chemotherapeutics has emerged as a potential route in ovarian cancer. The use of nanoparticles as carriers of these agents could increase the retention time in the peritoneal cavity and enhance the antitumor efficacy. These systems are also useful to administer drugs with a low aqueous solubility. This is the case of CBD, which also shows several stability problems. In the last decades, CBD has attracted a great deal of interest in therapeutics, especially in the field of cancer due to its ability to inhibit the proliferation, migration, invasion and angiogenesis of several kinds of tumours. Therefore, the aim of this work was to develop polymeric nanoparticles as CBD carriers for ovarian cancer treatment. Developed nanoparticles (CBD-Nps) exhibited a spherical shape, a particle size around 240 nm and a negative zeta potential (-16.6 ±1.22 mV). The encapsulation efficiency was really high, with values above 95%. A controlled CBD release for 96 hours was achieved. A considerably particle internalization in SKOV-3 cells was detected after 2 hours of incubation, and it was increasing up to 6 hours, where all particles seemed to be completely internalized. The encapsulation of CBD maintained its antiproliferative activity in ovarian cancer cells. In fact, CBD-Nps showed a lower IC_{50} values than CBD in solution. Both CBD in solution and CBD-Nps produced the expression of PARP, indicating an induction of apoptosis. Similar antitumor results were found in SKOV-3 derived tumours formed in the chick embryo model, where a slightly higher tumour growth inhibition was appreciated with CBD-Nps. Nevertheless, statistically significant differences between CBD-Nps and CBD in solution were not detected in *in vitro* or *in ovo* models. To sum up, PLGA nanoparticles could be a good strategy to administer CBD for ovarian cancer treatment.

Keywords: Cannabinoids, cannabidiol, gynaecological cancer, nanomedicines.

1. Introduction

Ovarian cancer is one of deadliest carcinomas in women worldwide, with 295,414 new cases and 184,799 deaths in 2018 [1]. The symptoms are not specific and similar to other less dangerous disorders like irritable bowel syndrome, hampering the diagnosis of this pathology. In fact, this neoplasm is usually diagnosed in advanced stages of the disease (in the 85% of the cases), when disease has already spread (usually within the intraperitoneal cavity). This hinders cancer treatment and increases the risk of death. In fact, this neoplasm shows a low 5-year survival rate, it is around 45% [2, 3]. In this way, the appearance of novel therapeutic agents for ovarian cancer treatment is necessary.

In the last decades, cannabidiol (CBD), the main non-psychoactive constituent of the plant from the genus Cannabis, has emerged as a potential therapeutic tool in cancer disease [4-6]. In general, CBD is a well-tolerated compound with no major safety events, especially compared to conventional anticancer drugs. It should be highlighted that cannabinoid receptors (specifically the type I cannabinoid receptor) have been found overexpressed in ovarian cancer and this overexpression has been associated with the invasive grade of the disease, suggesting their participation in ovarian carcinomatosis and also the potential use of cannabinoids as anticancer drugs in this neoplasm [7].

Ovarian cancer trends to spread within peritoneal cavity and the intraperitoneal administration of antineoplastic agents could improve their antitumor efficacy. Administered by this route the drugs are maintained in close proximity to the target tumour tissues, leading to more rapid and prolonged tumour accumulation [8, 9]. However, it should be considered that the drug delivery into the peritoneum is hampered by their clearance. The use of nanoparticles as drugs carriers could resolve this, increasing the retention of chemotherapeutics within the peritoneal cavity compared to the administration of free drugs [10, 11].

It should be considered that CBD shows a low aqueous solubility, which hampers its administration, especially by a parenteral route. In fact, the administration of CBD to animals required the use or organic solvents like ethanol and/or dispersing agents (e.g. Cremphor EL or Pluronic F68), increasing the toxicity and limiting the administered dose [12, 13]. Moreover, nanoparticles could change drug biodistribution and increase their

availability to tumour cells, since lipophilic drugs trend to distribute in fatty tissue. CBD is also an unstable molecule. Therefore, the development of polymeric nanoparticles could be a good strategy to administer CBD intraperitoneally and to improve its stability and modify its biodistribution. Among all the polymers, poly-lactic-co-glycolic acid (PLGA), due to its biodegradability and its FDA and EMA approval for human administration is one of the most used polymers [14].

Therefore, the main objective of this work was to design and to develop PLGA nanoparticles loaded with CBD capable of being internalised by ovarian cancer cells to overcome the administration and stability challenges of this drug and to improve its antiproliferative effect. The physicochemical properties of the formulation, physical state of the drug and short-term stability were evaluated. Antitumor efficacy was tested using *in vitro* and *in ovo* models of ovarian cancer.

2. Materials and methods

2.1 Materials

Cannabidiol (CBD) was obtained from THC-Pharma (Frannkfort, Germany). Poly-(lactide-co-glycolic acid-resomer RG® 502 (PLGA-502) (i.v. 0.16–0.24 dL/g) was purchased from Evonik® Industries (Essen, Germany). Polyvinyl alcohol (PVA, Mw= 30,000–70,000), Sigmacote®, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and Phalloidin Atto -488 were purchased from Sigma-Aldrich (Missouri, USA). DiO and DAPI were obtained from Invitrogen Molecular probes (Thermofisher Scientific, Massachusetts, USA). Ibidi-8 well plates was obtained from Ibidi labware (Gräfelfing, Germany). Methanol, acetonitrile, dichloromethane (DCM) and dimethyl-sulfoxide (DMSO) HPLC grade were obtained from Fisher Scientific (Massachusetts, USA). Potassium di-hydrogen phosphate (KH2PO4), disodium hydrogen phosphate dehydrate (Na2HPO4•2 H20) and Tween®-80 were provided by Panreac (Barcelona Spain). RPMI Medium 1640- GlutaMAX™-I, Gentamicin (10 mg/ml) Hank's Balanced Salt Solution (HBSS) and Geltrex®were supplied by Gibco (Life technologies, California, USA). Fetal bovine serum (FBS) was obtained from Biowest (Nuaillé, France). Cleaved PARP (Asp 214) rabbit monoclonal antibody and anti-rabbit HRP-linked antibody were purchased from cell signalling (Thermofisher Scientific, Massachusetts, USA). GAPDH rabbit antibody (6C5) was obtained from Santa Cruz (Santa Cruz Biotechnology, Texas USA). Demineralized Milli-Q® water

(Millipore, Spain) was used. All chemicals and reagents were used as received.

2.2. Development of PLGA nanoparticles

Nanoparticles loaded with CBD at 1.5% (w/w) were elaborated by emulsion solvent evaporation technique, using PLGA RG® 502 as polymer. Briefly, PLGA (100 mg) and CBD were dissolved in 1 ml of dichloromethane and emulsified in 13 ml of PVA at 1% (w/v) by ultrasonication (Fisher Scientific sonicator, Massachusetts , USA) in an ice bath for 2 minutes with an amplitude of 90% and a sonication: pause cycle of 40:20 seconds. Then, particle suspension was stirred for 3 hours at 500 rpm at room temperature to allow solvent evaporation. Nanoparticles were collected by centrifugation (15000 rpm for 35 min) (Beckman coulter Avanti, California, USA) and washed three times with demineralised water in order to eliminate remnant PVA. Finally, 1 ml of sucrose at 3% (w/v) was added as cryoprotectant. Then samples were freeze dried for 24 hours at −50 °C and 0.2 mbar.

For internalization experiments DiO was used as fluorescent agent and DiO-labelled nanoparticles, at a DiO: PLGA ratio of 0.015:100 mg, were prepared using the aforementioned protocol.

2.3. Characterization of developed formulations

2.3.1. Measurement of particle size and zeta-potential

The mean particle size, expressed as volume diameter, polydispersity index (PDI) and zeta potential of developed nanoformulations (suspended in demineralised water) were determined by dynamic light scattering using a Microtrac®-Zetatrac™ Particle Analyzer (Microtrac Inc., USA).

2.3.2. Morphological examination

The morphology of nanoformulations was evaluated by scanning electron microscopy (SEM), using a SEM Jeol, JSM-6335F microscope (Jeol, Japan). With the aim of eliminate the cryoprotectant, nanoparticles were suspended in ultrapure water and centrifuged (15000 rpm 15 min) three times. Samples were prepared by dropping

nanoparticle aqueous suspension (≈100 μg/ml) in a coverslip adhered to a stub and allowing water to evaporate (at room temperature for 24 hours). Then dried samples were coated with a 10-nm gold palladium thickness and examined by SEM.

2.3.3. DSC studies

Thermal analysis of nanoformulations were carried out using a Mettler differential scanning calorimeter (DSC820, Toledo Tech., Switzerland) equipped with a aTAC 7/DX instrument controller and aSTARe SW9.10 system software for the data acquisition. The temperature was calibrated using indium standards. Pure CBD, raw PLGA, and unloaded and CBD loaded nanoparticles were analysed. Samples were weighted (5 mg) directly into perforated aluminium pans, heated (under a nitrogen flow of 70ml/min) at a rate of 10 ºC/min from 20 to 100ºC, cooled from 100 to 20ºC and heated again up to 100ºC. An empty pan was used as reference. All samples were measured by triplicate. The value of polymer glass transition was calculated in the second heating cycle [15].

2.3.4. Determination of drug content and encapsulation efficiency

The amount of CBD encapsulated into formulations was determined by HPLC (HPLC, Agilent 1200 series, Agilent Technologies, California, USA) as follows: i) a mobile phase constituted by a mixture of methanol: acetonitrile: water at pH4.5 (52:30:18 v/v), ii) a flow rate of 1.8ml/min; iii) a reversed-phase Mediterranea® C18 (15×0.46 cm i.d. pore size 5 μm) (Teknokroma®) column, iv) an injection volume of 20μL and v) a detection wavelength of 228nm.

To determine the content of CBD, lyophilized nanoparticles were dissolved in DCM. CBD was extracted by the addition of mobile phase by vortexing. Then the samples was filtered and analysed by HPLC. The encapsulation efficiency (EE) was determined by the following equation:

$$EE\ (\%) = \frac{\text{Amount of CBD loaded (mg)}}{\text{Amount of CBD added (mg)}} * 100$$

2.3.5. *In vitro* drug release studies

In vitro drug release studies were performed by suspending nanoparticles in phosphate buffered saline (PBS) (pH 7.4) containing 0.5% (w/v) of Tween® 80 to keep the sink conditions during all the experiment. Samples were incubated in a thermostatic shaking bath at 37±0.5 ºC and under a mechanical stirring of 100 rpm for 96 hours. At predetermined time points aliquots were withdrawal, centrifuged at 15000 rpm for 20 min, filtered using a 0.22µm syringe filter and measured by HPLC.

2.3.6. Stability studies

The stability of CBD-Nps stored at 5 ± 3ºC was evaluated during 3 months. At predetermined time points (2, 4, 6, 8 and 12 weeks) nanoparticles were dispersed in demineralised water and characterized by determining their particle size, PDI and zeta potential as aforementioned. The content of CBD was also evaluated.

2.4. Cell culture experiments

2.4.1. Cell line

The highly invasive human ovarian cancer cell line SKOV-3 was used as a model of ovarian cancer. This cell line that belongs to epithelial serous group, the most frequent ovarian tumours, was purchased from American Type Culture Collection (ATCC). Cells were grown in RPMI-1640-Glutamax medium supplemented with 10% (v/v) of FBS and 1% (v/v) of gentamicin, and incubated at 37ºC in a humidified atmosphere containing 5% of CO_2.

2.4.2. *In-vitro* cytotoxicity

The cytotoxic activity of CBD in solution (CBD_{sol}), unloaded and CBD-loaded nanoparticles was tested in SKOV-3 cells. Cells were seeded into 96-well plate at a density of 1.5 10^4 cells/cm^2. 24 hours after seeding, the cells were treated with CBD_{sol} and nanoparticle formulations for 24 and 48 hours. CBD stock solution was prepared in absolute ethanol and then diluted in complete cell culture medium to reach each concentration. Both unloaded and CBD-loaded nanoparticles were suspended in complete cell culture medium. Then, the medium of each well was gently discarded, the cells were gently washed with fresh medium and 100 µL of an MTT solution in complete RPMI

medium (at a concentration of 0.5 mg/ml) were added to each well. Then, the plates were placed into the incubator for 3.5 hours. Afterwards, the medium was gently removed and 100µL of DMSO were added to each well to dissolve formazan crystals. The plates were stirred for 10 minutes and the absorbance was measured at 570nm with a microplate reader (Varioskan Flash, Thermo Scientific). CBD treatments were added at a concentration in the range of 10-50 µM. Cells treated with complete medium and with Triton-X at 1% served as negative and positive control of cell death.

The half maximum inhibitory 50 (IC_{50}) was calculated for each treatment for comparison purposes using Compusyn Software (ComboSyn, Inc. NJ, USA.) All the experiments were performed at least in triplicate.

2.4.3. *In-vitro* cellular uptake

The uptake of PLGA nanoparticles was determined qualitatively by fluorescence microscopy. For this purposes, DiO-labelled nanoparticles was used. SKOV-3 cells were seeded into Ibidi-8 well plates at a density of 30.000 cells/ well. Cells were treated with nanoparticle suspension (at a concentration of 1mg/ml) in complete RPMI-1640 medium 24 hours after seeding and incubated at 37ºC in a humidified atmosphere containing 5% of CO_2 for 0.5, 1, 2, 4, 6 and 8 hours. At each time points, the medium was removed and cells were rinsed three times with PBS, fixed with 4% paraformaldehyde and stained with DAPI and Phalloidin. Finally, cells were washed and observed by fluorescence microscopy (Invitrogen™, EVOS™ , Fisher Scientific, USA).

2.4.4 Western blot analysis

SKOV-3 cells were seeded at a density of 250000 cells/ well in a 6-well plate. 24 hour after seeding, the medium was removed and the cells were treated with CBD_{sol} and CBD-Nps at a CBD concentration of 40µM for 6 and 12 hours. Unloaded nanoparticles were also tested. After the treatments, cells were lysed using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % NP-40, 0.1 % SDS, 2 mM EDTA). Protein content was measured by Bradford assay and equal amounts were electrophoresed in SDS polyacrylamide gel and then transferred onto nitrocellulose membranes. Membranes were subsequently immunoblotted with monoclonal cleaved PARP antibody. A goat HRP-

linked antibody was used as secondary antibody. Specific signal was detected using Amershan ECL Prime Western Blotting Detection Reagent (Ge Healthcare, Buckinghamshire, UK). The detection of GAPDH was used as loading control.

2.5. *In ovo* antitumor activity

The cytotoxicity of CBD in solution and encapsulated into polymeric nanoparticles was also tested *in ovo* in SKOV-3 derived tumours formed on the choriallantoic membrane (CAM) of fertilized chicken eggs. For this experiments, fertilized chicken eggs were incubated in an automated incubator at 37°C and 47% humidity in a rotating mode. On incubation day (ID) 4, a small window was drilled in the eggshell and sealed with tape to prevent desiccation. Then, eggs were placed again into the incubator in a stationary mode. On ID 8 the window was enlarged and CAM membrane was gently scratch to inoculate SKOV-3 cells ($2*10^6$ cells suspended in Geltrex® matrix were inoculated per egg). On ID 11, SKOV-3 derived tumours were formed and surrounded with a silicone o-ring. At this point, tumour area of each egg was measured (tumour area initial) and treated topically with CBD_{sol} (100 μM), unloaded or CBD loaded nanoparticles (with an equivalent concentration of CBD 100μM). After the treatments, eggs were placed again into the incubator. On ID 13.5, tumour area of each egg was evaluated (tumour area final). Eggs treated with complete RPMI-1640 medium served as control. All the treatments were added daily from ID 11 to ID 13.5. At least 7 eggs per condition were analysed. The tumour growth was determined as follows:

$$Tumour\ growth\ (\%) = \frac{Tumour\ area\ final}{Tumour\ area\ initial} * 100$$

After the experiments, SKOV-3 derived tumours were collected and paraffin-embedded for histopathological examination. Samples were cut and stained using haematoxylin and eosin.

3 RESULTS

3.2 Nanoparticle characterization

3.2.1 Physical characterization

Table 1 illustrates the processes yield, particle size, polydispersion index and zeta potential of developed CBD nanoparticles. Unloaded and DiO labelled nanoparticles were also prepared. The elaboration protocol reported processes yield above 50% in all formulations. Nevertheless, it was slightly higher in unloaded and DiO-loaded nanoparticles. Dynamic light scattering analyses reported a mono-modal size distribution for all nanoformulations with a mean particle size, expressed as volume diameter in the range of 228 to 236 nm. No differences in particle size were detected with CBD and DiO incorporation. Regarding polydispersity index values higher than 0.1 were obtained in all cases, indicating a polydisperse particle size distribution [16]. Albeit, all the values were lower than 0.2. In general, in the field of polymer based nanoparticles values lower than 0.2 are commonly deemed acceptable [17].

Formulation	Size (nm)	PDI	Zeta Potential (water)	Process yield
PLGA-Nps	228.47 ± 8.36	0.141 ± 0.019	-24.7 ± 1.48	61.8 ± 4.27
CBD-Nps	236.08 ± 12.02	0.165± 0.009	-16.6 ±1.22	51.2 ± 6.22
DiO-Nps	230.27 ±7 .58	0.175 ± 0.021	-28.2 ± 1.52	62.5 ± 2.33

Table 1: Size, PDI, zeta potential and process yield of nanoparticles.

Zeta potential was evaluated after the freeze-drying process. As depicted in table 1, all nanoparticles exhibited a negative zeta potential, which are related to the uncapped carboxylic groups of the PLGA of the polymeric matrix [18]. Unloaded nanoparticles exhibited a mean zeta potential around -24.7 ±1.48 mV, similar to the values obtained by other authors for blank PLGA nanoparticles [19, 20]. Nevertheless, CBD-Nps exhibited a less negative zeta potential (-16.6 ±1.22), which is related to drug incorporation. This probably means that although drug is entrapped into the polymeric matrix, CBD is also exposed to particle surface [21]. Changes in zeta potential were also detected in DiO-labelled nanoparticles. However, in this case higher negative values were found.

Regarding the morphology of nanoparticles, it was evaluated by SEM. As depicted in figure 1, spherical particles with a smooth and slick surface were obtained in unloaded and CBD loaded formulations. SEM images revealed that more than one size population were appreciated, which is in accordance with obtained PDI values. DiO-labelled nanoparticles exhibited and analogous morphology to CBD-Nps.

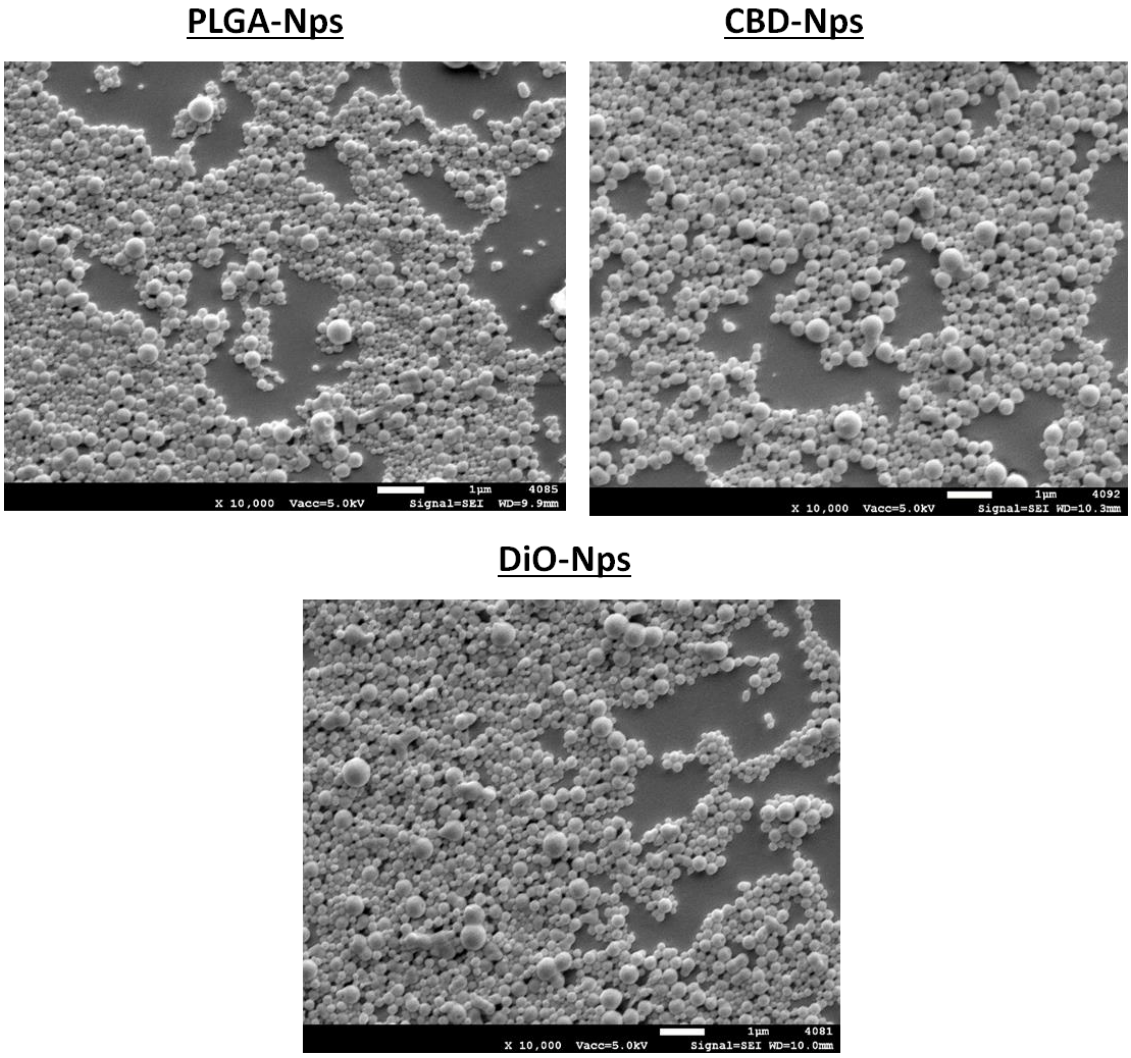

Figure 1: SEM images of unloaded, CBD-loaded and DiO loaded nanoparticles.

It is known that the cellular uptake of nanoparticles is influenced by several morphological and physicochemical properties of nanoparticles, including their particle size and zeta potential [22]. For this reason, DiO-Nps should be showed similar properties that CBD-Nps to assumed a similar internalization process. In this work, CBD-Nps and DiO-Nps exhibited a similar morphology and an analogous particle size. Zeta potential was significantly lower in DiO-Nps, which could interfere in the uptake of both formulations. Nevertheless, other authors have reported that higher surface charge values implies a higher uptake, and as a consequence, in this way, a similar or even better internalization could be expected with CBD-Nps (-16.6 ±1.22) compared to DiO-Nps (-28.2 ± 1.52) [23]. As a consequence, it could be supposed that DiO-Nps could be a good strategy evaluate the possible internalization of CBD-Nps.

The DSC thermogram of pure CBD denoted a sharp endothermic peak at 69.68ºC that corresponds to the melting point of CBD and shows an enthalpy value of 75.60 ±0.95 J/g. This value is pretty similar to the reported in literature [24]. Nevertheless, in CBD-Nps this peak was not appreciated. This indicates that CBD was dissolved into the polymeric matrix. The representation of DSC results is depicted in supplementary material.

In respect of glass transition temperature (Tg), a value of 41.97±1.01 ºC was obtained. A similar value was also detected in unloaded nanoparticles (42.38±0.80 ºC). Nevertheless, a slightly lower value was found in CBD-Nps (40.44 ± 0.27). This reduction in polymer Tg could indicate a lightly plasticizer effect of CBD. The DSC representation is illustrated in supplementary material (Figure S1)

3.2 Drug loading, entrapment efficiency and *in vitro* drug release

Developed CBD loaded nanoformulation showed a drug loading of 140.2 ± 6.25 µg CBD/10mg Nps and a high encapsulation efficiency with values close to 100% (95.23 ±3.3%). Regarding *in vitro* drug release, as depicted in figure 2, a controlled CBD release over a period of 96 hours was detected, with the 100% of the CBD released by this time. A high burst effect was observed within the first hour of the experiment with ≈35% of the CBD released in this time. The CBD release profile was fitted to a zero order kinetics (r=0.952) with a CBD release rate of 21.6µg CBD day $^{-1}$ /10 mg nanoparticles. As a

consequence, the single administration of this formulation would let a constant CBD release for 96 hours.

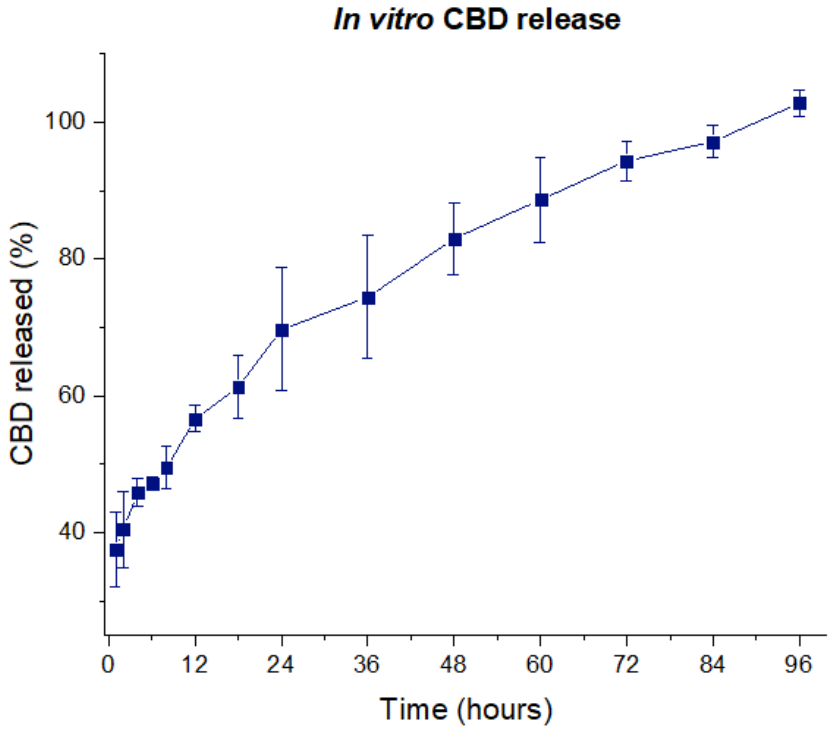

Figure 2: CBD release profile.

As illustrated in figure 3, neither particle deformation nor polymer degradation could be appreciated in CBD-Nps during release experiments, indicating that CBD release probably follows a diffusion mechanism. In fact, the fitting to Korsmeyer -Peppas modelling (r=0.971) confirmed these results. An n value of 0.228 was obtained. This value was lower than 0.43, indicating that CBD release follow a diffusion based mechanism.

As we mentioned previously, CBD shows a low aqueous solubility, hampering its administration. The developed formulations showed a suitable strategy to vehiculize CBD, making possible the administration of this drug without organic solvents. Moreover, our research group reported in other work that CBD stability is affected by multiple factors (temperature, oxygen and light). In this way, the encapsulation of CBD into polymeric nanoparticles could also protect it.

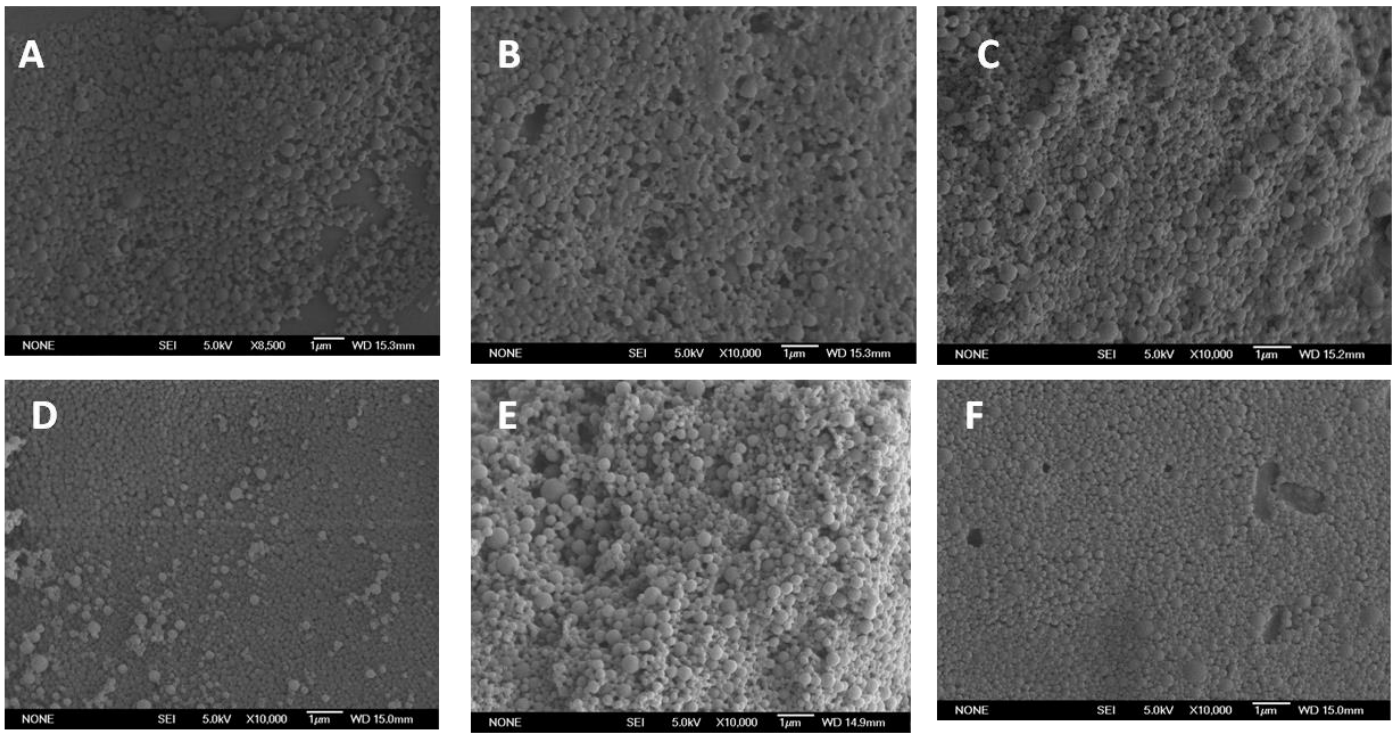

Figure 3: SEM images of CBD-Nps during release experiments after 2 (A), 4 (B), 6 (C) , 24 (D) 48 (E) and 96 (F) hours.

3.5. Stability studies

It should be considered that PLGA must be stored refrigerated. For this reason, and also due to the fact that CBD is unstable against temperature, this formulation is intended to be stored refrigerated. Short term stability studies at 5ºC over a period of 3 months were undertaken; evaluating the physical and chemical stability of CBD-Nps. As depicted in table 2, CBD-Nps remained physically stable for at least 3 months. Particle were easily dispersed, with no significant changes neither mean particle size nor PDI during the evaluated period (supplementary material, figure S2). These data suggest that no particle aggregation was produced. This is an important point, thus the formation of aggregates avoid the uptake of nanoparticles by cancer cells. No-significant changes in zeta potential were also appreciated. Regarding CBD content, no drug degradation was also detected, indicating that CBD encapsulated into the polymeric matrix remained stable for at least 3 months (supplementary material, figure S3).

Time (weeks)	Particle size (nm)	PDI	Zeta potential (mW)	CBD remaining (%)
0	239,93 ± 5.2	0,147 ± 0.02	-15.9 ± 0.89	100
2	249,17 ± 2.27	0,171 ±0.007	-17.2 ± 2.1	101,13 ± 1.27
4	243,23 ± 8.5	0,182 ± 0.03	-15.4 ± 1.13	93,66 ± 3.22
6	247,25 ± 9.27	0,187 ± 0.02	-15.9 ± 1.21	97,62 ± 2.15
8	250,53 ± 3.56	0,190 ± 0.06	-16.65 ± 3.1	99,09 ± 1.2
12	247,48 ± 2.21	0,166 ± 0.08	-15.25 ± 2.25	96,09 ± 4.21

Table 2: Particle size, PDI, zeta potential and percentage CBD remaining (%) of CBD-Nps stored at 5ºC during stability study.

3.6. Nanoparticles uptake

The internalization process of this formulation was evaluated qualitatively using DiO labelled nanoparticles. This dye has been used previously to evaluate the uptake of polymeric particles by other authors [25,26]. As depicted in figure 4, no nanoparticle internalization was detected during the first 30 min. After one hour of incubation, a slightly particle uptake was observed. However, the internalization of this formulation started to be significant after 2 hours of incubation. Since that moment, the number of internalized nanoparticles was increasing up to 8 hours. This study indicated that DiO-Nps were adequate for its internalization at least after 2 hours.

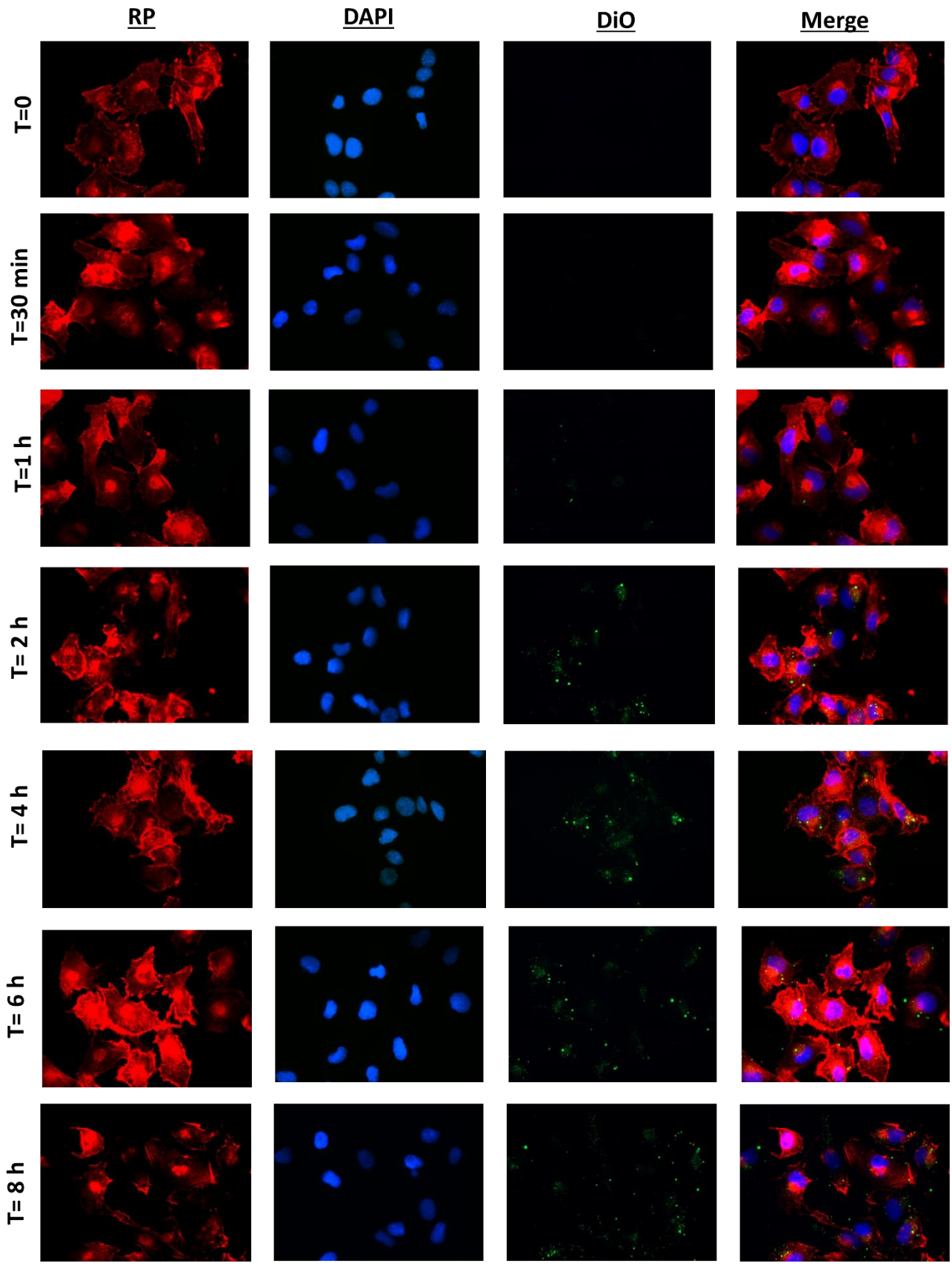

Figure 4: Images obtained by fluorescence microscopy of SKOV-3 cells incubated with DiO-Nps (at a concentration of 1mg/ml) for 30 min to 8 hours. Red images indicate

cell cytoskeleton staining (Red phaolloidin, RP). Blue images indicate nucleus staining (DAPI). Green images showed DiO-Nps.

3.7. *In vitro* cytotoxicity

The anticancer activity of CBD has been extensively demonstrated in a broad range of tumours, including cervix cancer, other gynaecological neoplasms [27]. Nevertheless, the effect of CBD in ovarian cancer has not been published by other authors. In fact, our research group has been the first to evaluate and to report the antiproliferative activity of CBD_{sol} in ovarian carcinoma in a previously. In this work, it has been evaluated the antiproliferative activity of both CBD_{sol} and encapsulated into PLGA nanoparticles.

As depicted in figure 5A and 5B, unloaded nanoparticles were no cytotoxic even at the highest concentration. Nevertheless, CBD_{sol} as well as CBD encapsulated into PLGA nanoparticles inhibited the proliferation of SKOV-3 cells. This inhibition was dose- and time-dependent. The IC_{50} values of CBD_{sol} and CBD-Nps are illustrated in table 3.

	24 hours	**48 hours**
CBD_{SOL}	33.19 ± 2.57 µM	23.47 ± 4.10 µM
CBD-Nps	29.64 ± 2.94 µM	20.88 ± 1.25 µM

Table 3: IC_{50} of CBD_{sol} and CBD-Nps in SKOV-3 cells.

After 24 hours of incubation, statistically significant differences between the cell death induced by CBD_{sol} and CBD-Nps were only detected at low and intermediate concentrations (5-20 µM). In fact, it was observed that at low CBD concentrations (5-10 µM) CBD-Nps tended to produce a higher antiproliferative effect than CBD_{sol}. This could be attributed to the internalization of these nanoparticles. Nevertheless, at high CBD concentrations no significant differences were found between both CBD formulations, probably due to the high cell death that was appreciated. After 48 hours of treatment, the

same tendency was observed at low concentrations, however significant differences were not found except for CBD administered at 5µM.

Apoptosis plays a critical role in determining cell survival. It has been reported that CBD induces programmed cell death in numerous types of tumours such as breast, prostate or cervix cancer [27-30]. In this work, the induction of apoptosis have been investigated by western blotting with the evaluation of PARP cleavage. As illustrated in figure 5C and 5D, the treatment of CBD_{sol} for 6 and 12 hours at a concentration of 40 µM induced PARP cleavage in SKOV-3 cells (supplementary material, figure S4). A higher expression of cleaved PARP was not detected in unloaded nanoparticles treated cells compared to the control, thus this nanoparticles did not show a cytotoxic effect in these cells even after 48 hours of incubation. Nevertheless, CBD-Nps treated cells also exhibited PARP cleavage. In fact, CBD-Nps exhibited a higher PARP cleavage compared to CBD_{sol}. After 6 hours of incubation, while CBD_{sol} reported a 3.7 fold induction of cleaved PARP expression, CBD-Nps showed a 4.9 fold-induction. This was also evident after 12 hours of treatment, with a 9.01 and 12.03 fold induction for CBD_{sol} and CBD-Nps respectively. However, significant differences between CBD_{sol} and CBD-Nps were not detected. After 6 hours of incubation, CBD-Nps showed a considerable internalization, with could explain this higher PARP cleavage. Cleaved PARP is a key arbitrator in apoptosis and, as a consequence, this study indicates that this mechanism is also responsible for the cell death induced by CBD in these ovarian cancer cells.

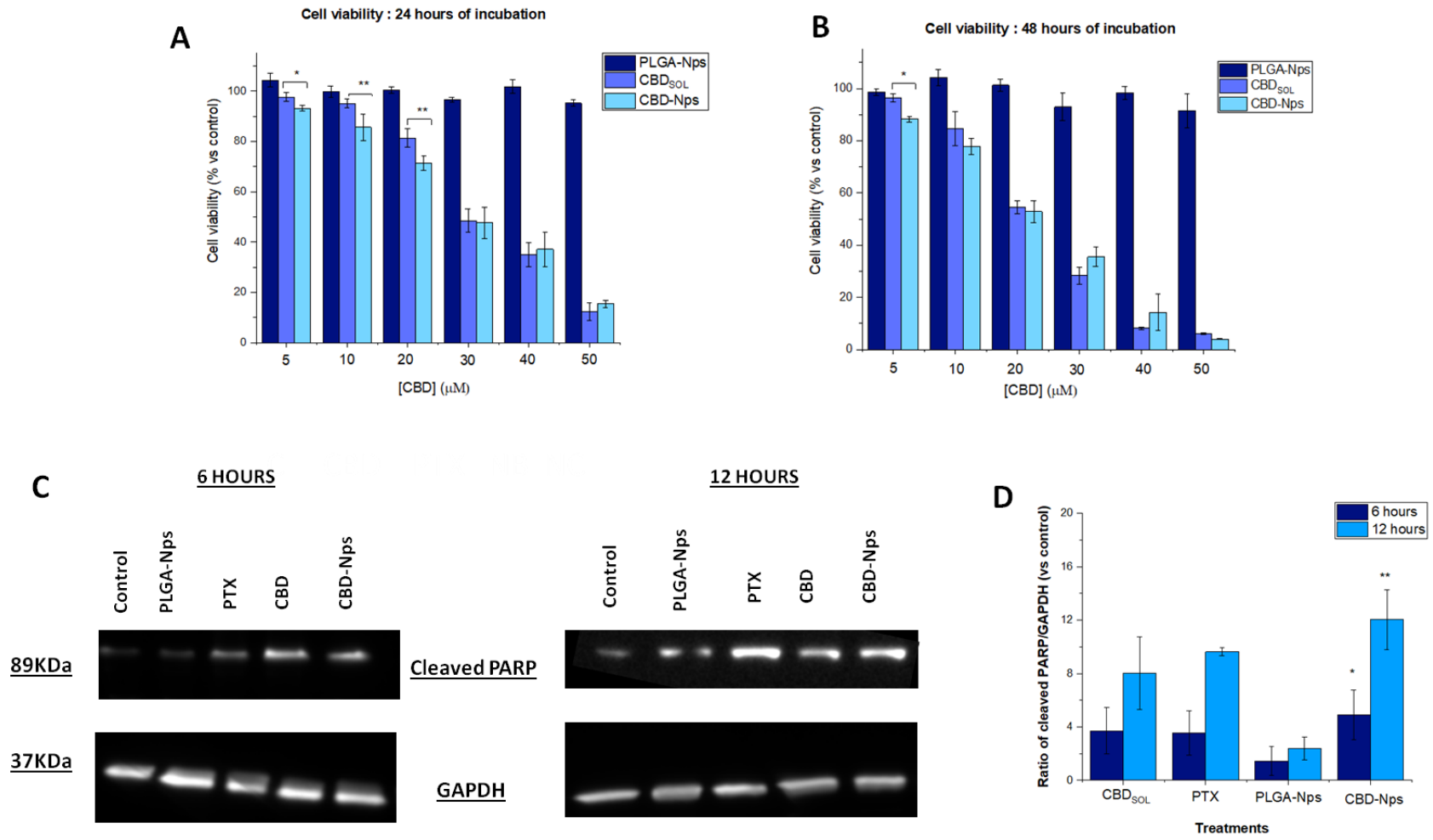

Figure 5: Antiproliferative activity of CBD_{sol}, PLGA-Nps and CBD-Nps in SKOV-3 cells after 24 (A) and 48 (B) hours of incubation. Western blott analysis of SKOV-3 cells after 6 and 12(C) hours of incubation with CBD_{sol} and CBD-Nps at a CBD concentration of 40µM,

PLGA-Nps and PTX 100 nM. Cells treated with cell culture medium served as control. Ratio of cleaved-PARP/GAPDH (D). * (p value <0.05) and ** (p value <0.01) mean statistically significant differences between PLGA-Nps and CBD-Nps.

3.8. *In-ovo* antitumor activity

CAM model has been proposed as an alternative *in vivo* preclinical technique in cancer research due to the feasibility to induce tumour formation. The chick embryo is not immunocompetent and cells and tissues from different species could be successfully inoculated [31, 32]. In fact, SKOV-3 derived tumours have been formed successfully in this model [33, 34]. Interestingly, these tumours closely resembles the ovarian cancer patient tumours since a histological point of view [35], reinforcing the use of this model to evaluate the activity of novel formulations designed for ovarian cancer treatment. In the present work, nanoparticles were designed for intraperitoneal administration. For this reason, the tumours formed on CAM membrane were treated topically, since this administration could resemble better the intraperitoneal route.

In this work, SKOV-3 derived tumours were successfully formed as reveal figure 6A. Its histophatological examination exhibited that tumour cells invaded CAM membrane (Figure 6B).

As depicted in figure 6C, while unloaded nanoparticles were no toxic, both CBD_{sol} and CBD encapsulated into polymeric nanoparticles significantly reduced tumour growth (p value <0.01). Although CBD-Nps exhibited a slightly higher growth inhibition compared to CBD_{sol} (1.5- vs 1.38- fold reduction respectively), no significant differences were appreciated (Figure 6D).

Like in in vitro experiment, the studies undertaken in this model also suggested that the encapsulation of CBD maintains its antitumor activity in ovarian carcinoma. In fact, CBD-Nps trend to be more effective in producing an antitumor effect in ovarian cancer in both in vitro and in ovo models of ovarian cancer.

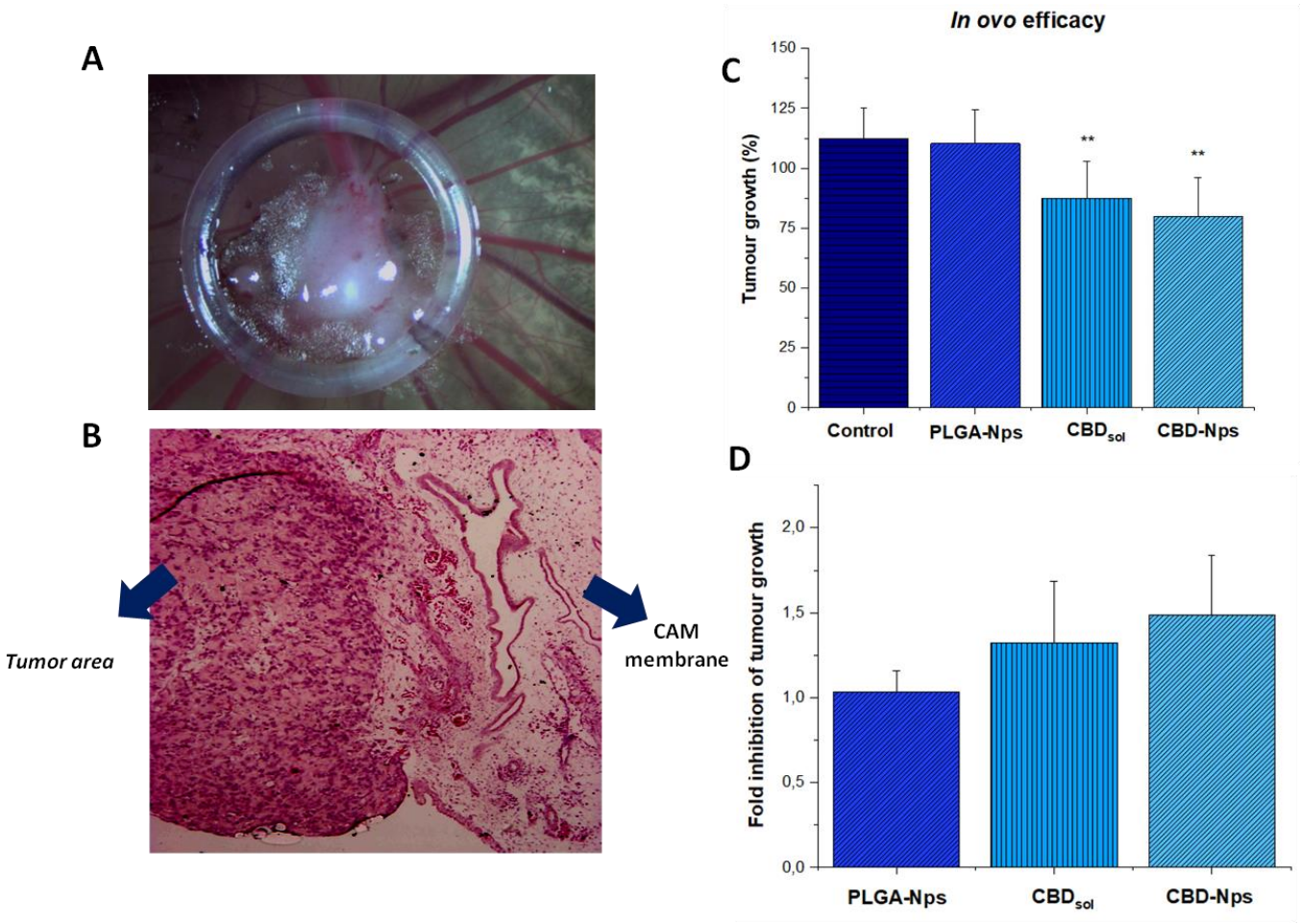

Figure 6: SKOV-3 derived tumour formed on CAM membrane corresponding with incubation day 11 (A), haematoxylin and eosin staining (B). Tumour growth of SKOV-3 derived tumour after several treatments (C). Fold inhibition of tumour growth of the treatments (D). ** Statistically significant differences (p value<0.01) compared to the control (cell culture medium).

Finally, it should be highlighted that CBD shows a low aqueous solubility, which hinders its intraperitoneal administration. In this sense, nanoencapsulation resolve this problem and provide a suitable form to administer this drug without the necessary of using organic solvents and/ or dispersing agents. Moreover, the use of this strategy could modify the biodistribution of the drug, increasing its availability to cancer cells. In fact, developed nanoparticles started to be considerably internalized after 2 hours. During this time around the 40% of the CBD was released. However, the remaining 60% would be release inside the cells.

CONCLUSIONS

CBD was successfully encapsulated into PLGA nanoparticles, showing a suitable particle size to be internalised. The use of CBD-Nps shows several advantages. This formulation avoids the use of organic solvents and/ or dispersing agents for CBD administration and also improves its stability. The CBD-Nps were efficaciously internalised by SKOV-3 ovarian cancer cells, which allow the internal CBD release. This formulation shows an antiproliferative activity in both *in vitro* and *in ovo* models of ovarian cancer.

Although further studies are necessary, this work evidenced that PLGA nanoparticles could be a good strategy to administer CBD intraperitoneally in ovarian cancer treatment.

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SUPPLEMENTARY MATERIAL

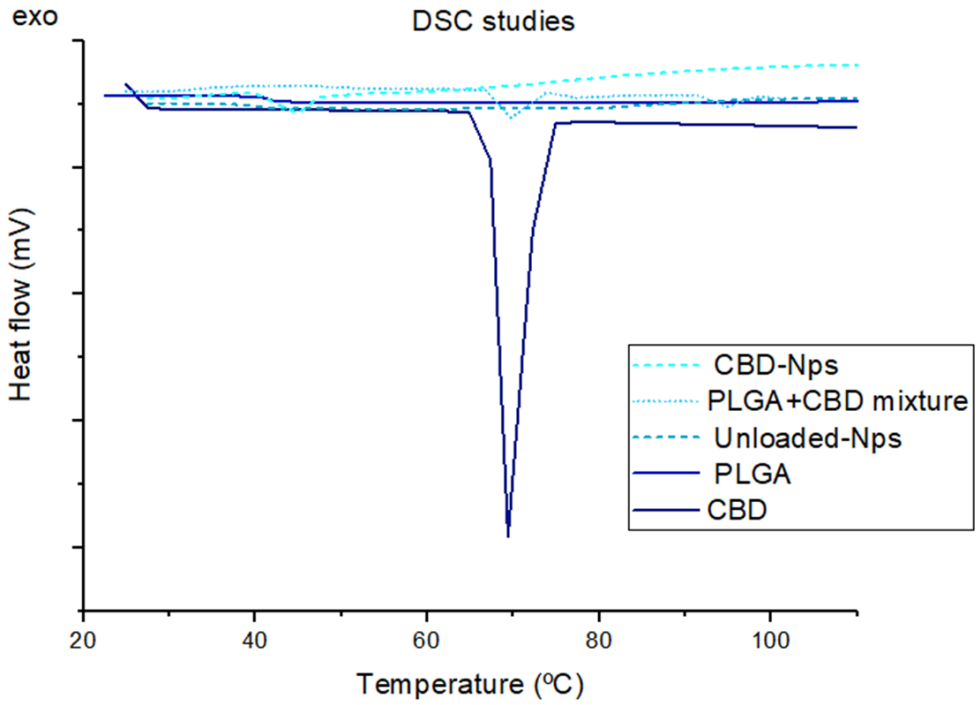

Figure S1: DSC thermograms of pure CBD, raw PLGA, their physical mixture, unloaded and CBD-loaded nanoparticles.

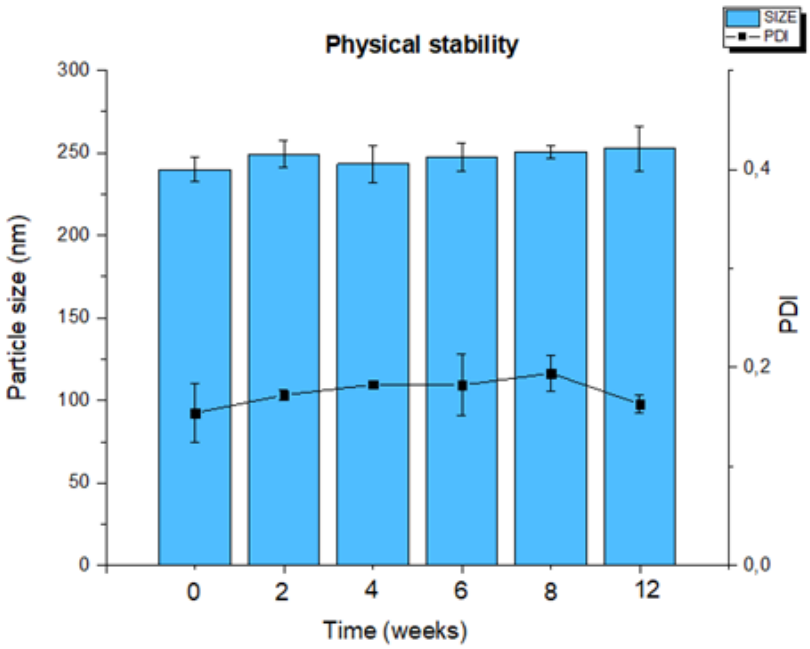

Figure S2: Particle size and PDI values of CBD-Nps during short-term stability studies.

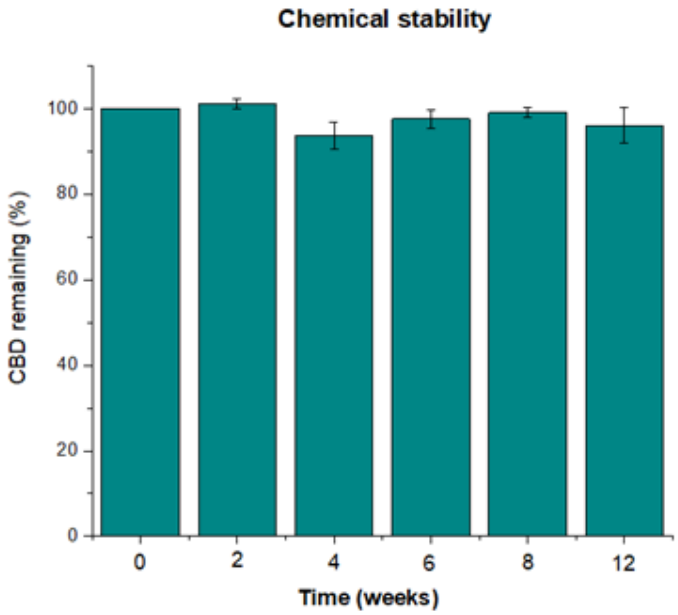

Figure S3: CBD remaining of CBD-Nps during short-term stability studies.

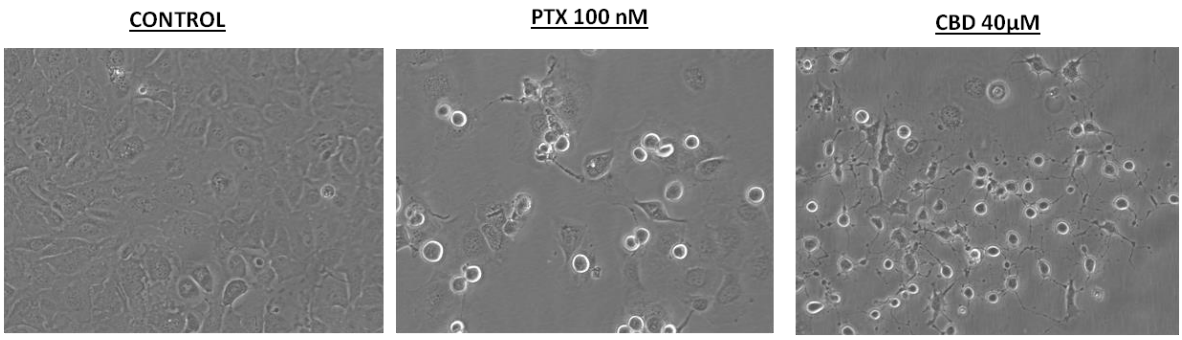

Figure S4: Images of SKOV-3 cells treated with cell culture medium (control), PTX (100 nM) or CBD_{sol} (40 µM) for 12 hours.

DISCUSIÓN FINAL

A pesar de los avances llevados a cabo en el tratamiento de cáncer con el desarrollo de nuevos sistemas de diagnóstico precoz y la aparición de nuevas terapias, esta patología continúa siendo uno de los principales problemas de salud en todo el mundo, con una estimación global de 23 millones de casos hasta el año 2030 [1, 2]. En la población femenina, el cáncer de mama y ovario son dos de las neoplasias más importantes. Por un lado, el cáncer de mama es el tumor más frecuente en mujeres de todo el mundo, y constituye una de las de las principales causas de mortalidad por cáncer en este colectivo [3]. Por otro, aunque menos frecuente, el cáncer de ovario presenta una mortalidad muy elevada, con una tasa de supervivencia a cinco años en torno al 45%. Debido a su sintomatología inespecífica y a la falta de técnicas de detección precoz es habitualmente diagnosticado en estados avanzados de la enfermedad (85% de los casos), lo que dificulta el tratamiento y aumenta la mortalidad [4, 5].

Ambas neoplasias son altamente variables, de manera que la estrategia de tratamiento depende del tipo de tumor. Una de las más importantes es la quimioterapia, especialmente en estados avanzados de la enfermedad. Sin embargo, los tratamientos quimioterápicos disponibles son altamente tóxicos, presentando numerosos efectos adversos importantes que limitan la dosis utilizada. En este sentido, la aparición de nuevos activos con una baja toxicidad y que potencien el efecto de la quimioterapia convencionales altamente deseable, ya que permitirían disminuir la dosis de los agentes antineoplásicos y por consiguiente sus efectos adversos.

En las últimas décadas, los cannabinoides (compuestos terpeno fenólicos presentes en las plantas del género cannabis) han surgido como activos de interés en el tratamiento del cáncer. Por un lado, son útiles como agentes paliativos para combatir las náuseas y vómitos relacionados con la quimioterapia y la pérdida de apetito y dolor asociados al cáncer. De hecho, existen diversas formulaciones comercializadas con este propósito. Pero además, se presentan como potenciales activos antitumorales, ya que han demostrado tener un efecto antitumoral per se al inhibir la proliferación, migración e invasión de las células tumorales, y también una posible acción adyuvante al potenciar el efecto de diversos antineoplásicos [6, 7].

Muchas de las investigaciones llevadas a con los cannabinoides empleaban extractos de la planta del cannabis enriquecidos en diversos cannabidiol (CBD), cannabigerol, cannabinol y/o Δ^9-Tetrahidrocannabinol. Debido a la gran diferencia en el contenido y proporción de estos compuestos se obtenían resultados muy variables. Por ello, se empezaron a utilizar los cannabinoides puros (de síntesis).

Las investigaciones llevadas a cabo por Munson y cols. en modelos *in vitro* e *in vivo* de adenocarcinoma pulmonar de Lewis pusieron de manifiesto por primera vez la actividad antitumoral de los cannabinoides en 1975 [8] . Desde entonces, numerosos autores han demostrado la capacidad de estos compuestos de inhibir la proliferación, migración e invasión de las células tumorales. Estos efectos anticancerígenos se han demostrado en numerosos tipos de tumores, tales como mama, próstata, glioma, neoplasias gastrointestinales y leucemia entre otros [9-12].Precisamente, una de las neoplasias donde los cannabinoides presentan un importante potencial antitumoral es el cáncer de mama. De hecho, investigaciones llevadas a cabo en modelos *in vivo* de cáncer mama agresivo han establecido que diversos cannabinoides reducen significativamente el crecimiento tumoral y la aparición de metástasis. Además, son capaces de bloquear la generación de nuevos tumores.

De entre todos los cannabinoides, el cannabidiol (CBD) es uno de los más prometedores en terapéutica. Este compuesto fue aislado por primera vez en 1940 por Adam y col. [13], pero su estructura no fue elucidada hasta 1960 [14]. Es el segundo cannabinoide más abundante presente en la planta del cannabis, cuya principal ventaja frente a otros cannabinodes es la ausencia de efectos psicoactivos [15]. De hecho, diversas investigaciones han demostrado que el CBD disminuye los efectos psicótropos de otros cannabinoides, sobre todo delΔ^9-Tetrahidrocannabinol; que es el principal responsable de los mismos [12]. En terapia antitumoral, es además uno de los más eficaces. Por ello actualmente se están llevando a cabo diversos ensayos clínicos para evaluar la eficacia y seguridad de CBD en combinación con quimioterapia y radioterapia en glioma, mieloma múltiple y neoplasias gastrointestinales (NCT03246113, NCT0360764and NCT03529448).

En la presente tesis doctoral se ha evaluado por primera vez la actividad antitumoral del CBD en cáncer de ovario (descrito en la **publicación 6**). Este cannabinoide ha demostrado inhibir *in vitro* la proliferación de todas las líneas celulares de ovario evaluadas (IGROV-1, OAW-42 y SKOV-3). Tal y como se puede observar en la tabla D1, que muestra los valores de concentración inhibitoria 50 (IC_{50}) tras 24 y 48 horas de incubación, las células OAW-42 fueron las menos sensibles a la acción del CBD. Las diferencias pueden atribuirse al distinto perfil histológico de estas líneas celulares. Todas ellas, proceden de tumores de ovario epiteliales. No obstante, las células OAW-42 son de tipo seroso, las SKOV-3 una mezcla de tipo seroso y de células claras, y las IGROV-1 de tipo mixto (seroso, endometroide, células claras y mucinoso). De hecho, también se han encontrado diferencias en la sensibilidad a los tratamientos antineoplásicos antitumorales convencionales: mientras las células OAW-42 son resistentes al paclitaxel y sensibles al cisplatino, las células SKOV-3 y IGROV-1 presentan una sensibilidad opuesta [16].

	IGROV-1	**SKOV-3**	**OAW-42**
IC_{50} (24 hours)	24.01±1.21 μM	29.71±1.89 μM	34.55±0.90 μM
IC_{50} (48 hours)	18.57±2.72 μM	22.73±1.18 μM	31.80±1.01 μM

Tabla D1: Valores de IC_{50} de las líneas de cáncer de ovario evaluadas tras 24 y 48 horas de incubación con CBD en solución.

De entre todas las líneas celulares de cáncer de ovario, la SKOV-3 es una de las líneas más utilizadas como modelo. Debido al alto grado de invasividad y a la dificultad de tratamiento que presenta este tipo de tumores, las células SKOV-3 se eligieron como modelo de cáncer de ovario para el resto de los estudios en cultivos celulares de la presente tesis doctoral.

Uno de los mecanismos propuestos implicados en la muerte celular debida al CBD es la inducción de apoptosis. Esto ha sido demostrado en numerosos tipos de cáncer, incluyendo mama, próstata y cérvix [17-21]. En la presente tesis doctoral (estudios descritos en la **publicación 8**) se ha demostrado que el tratamiento de células SKOV-3

con CBD 40µM durante 6 y 12 horas induce la fragmentación de la enzima poli ADP ribosa polimerasa (PARP), indicando que la inducción de apoptosis es uno de los mecanismos responsables de la muerte celular en cáncer de ovario.

Tal y como mencionamos previamente, una de las principales potenciales aplicaciones de los cannabinoides en general y del CBD en particular en terapia antitumoral es su combinación con agentes antineoplásicos comunes. En la presente tesis doctoral se ha evaluado por primera vez en cáncer de ovario el potencial uso del CBD en combinación con paclitaxel (PTX), doxorrubicina (DOX) y cisplatino (CIS), fármacos habituales en el tratamiento de este tipo de neoplasias (resultados descritos en la **publicación 6**). Así mismo, se ha estudiado la combinación de este cannabinoide con paclitaxel y doxorrubina en cáncer de mama receptor de estrógenos positivo (células MCF-7) y en cáncer de mama triple negativo (células MDA-MB-231) (resultados descritos en la **publicación 7**).

En cáncer de ovario, la combinación de CBD y PTX fue altamente eficaz. Por un lado la administración previa de concentraciones sub-eficaces de CBD (muerte celular inferior al 10%) (10 µM) demostró sensibilizar a las células SKOV-3 frente a este agente antineoplásico, disminuyendo de manera significativa la IC_{50} del PTX. Por otro lado, la co-administración de ambos activos, CBD (10-20µM) y PTX (10-500 nM) demostró un efecto sinérgico, ya que se obtuvieron, en todos los casos, índices de combinación comprendidos en el intervalo de 0.71-1.01. Sin embargo, las combinaciones de CBD+DOX y CBD+CIS no fueron efectivas. En el caso de la doxorrubicina, se observó una disminución no significativa (p valor <0.05) en sus valores de IC_{50}, tanto en las células tratadas previamente con CBD (1-10µM), como en la co-administración del CBD (10-20 µM) y la DOX (1-60 µM). En el caso del cisplatino (5-100µM), un ligera disminución (no significativa) sólo se observó en la co-administración con CBD 15 y 20 µM. Valores de índice de combinación superiores a 1 fueron obtenidos en ambas combinaciones, CBD +DOX y CBD+CIS, indicando que se produjo antagonismo [22]. Este efecto fue especialmente evidente en la combinación de CBD con cisplatino, donde índices de combinación de 0.98 a 2.57 fueron obtenidos.

En el caso de cáncer de mama, la combinación de CBD con PTX también resultó eficaz, tanto en células MCF-7 como en células MDA-MB-231. Por un lado, la

administración previa de concentraciones sub-eficaces de CBD (concentraciones de CBD de 1.25-5 µM y 2.5-10 µM se emplearon en células MDA-MB-231 y MCF-7 respectivamente) moduló el efecto del PTX (10-500 nM) en ambas líneas celulares, disminuyendo significativamente los valores de IC_{50}de este fármaco. Por otro lado, la co-administración del CBD+PTX fue también efectiva, observándose un efecto sinérgico. Este sinergismo fue más marcado en células MCF-7, con valores de índices de combinación de 0.59-0.83, mientras que en células MDA-MB-231 únicamente se detectó sinergismo a la máxima concentración de PTX, detectándose un efecto aditivo a concentraciones más bajas del antitumoral. En cuanto a la combinación de CBD con DOX (0.1-20µM), ésta resultó algo menos eficaz. La administración previa de CBD en solución disminuyo los valores de IC_{50} de DOX en ambas líneas celulares. Sin embargo, mientras que en células MDA-MB-231 disminuciones estadísticamente significativas se observaron en todas las combinaciones (CBD 1.25-5 µM) en células MCF-7 sólo se apreció cuando el CBD se administró a 10 µM. La co-administración de CBD+DOX fue también efectiva, especialmente el células MCF-7 donde se observó un efecto sinérgico, ya que índices de combinación inferiores a 1 se observaron en la mayoría de las combinaciones evaluadas (salvo en la combinación DOX 10µM + CBD 10µM, con un valor de índice de combinación de 1.12 ±0.22). En las células MDA-MB-231 únicamente se detectó un efecto sinérgico cuando la DOX se administró a la concentración más alta (20µM), observándose un efecto aditivo en el resto de combinaciones (con índices de combinación de 0.92-1.1) [22].

Estos resultados indican que el CBD presenta un importante potencial para ser combinado con paclitaxel en el tratamiento de cáncer de ovario y de mama, tanto en cáncer de mama con receptores hormonales positivos como en cáncer de mama triple negativo. Con la combinación de ambos fármacos se obtuvo un efecto sinérgico (en el caso de las células de cáncer de mama triple negativo únicamente detectado a altas concentraciones del antitumoral mientras que a bajas concentraciones el efecto fue aditivo), lo que permitiría reducir considerablemente la dosis de paclitaxel y como consecuencia sus efectos adversos. Además estudios recientes en modelos *in vivo* han indicado que el CBD disminuye la neuropatía periférica relacionada con el paclitaxel, lo que hace aún más interesante esta combinación [23-25].

Sin embargo, la combinación de CBD y DOX únicamente resultó eficaz en células de cáncer de mama, especialmente en cáncer de mama con receptores hormonales positivos donde se observó un mayor efecto sinérgico. No obstante, dada la toxicidad de este agente antitumoral, principalmente a nivel cardíaco, el efecto aditivo detectado en células de cáncer de mama triple negativo también sería interesante, especialmente teniendo en cuenta que el CBD ha demostrado disminuir en modelos *in vivo* en ratones la cardiotoxicidad asociada a este fármaco[26, 27]. Por lo tanto, la combinación de CBD y DOX podría resultar también útil en cáncer de mama triple negativo, que es especialmente agresivo y difícil de tratar.

A pesar del potencial antitumoral que presenta el CBD tanto en cáncer de mama como en cáncer de ovario, los cannabinoides en general y el CBD en particular, presentan diversas características que dificultan su manipulación y comprometen el diseño y desarrollo de formulaciones eficaces, como son su baja hidrosolubilidad y elevada inestabilidad.

Los cannabinoides son compuestos altamente inestables frente a diversos factores como la luz, la temperatura y el oxígeno [28-30]. Aunque diversos autores han publicado datos de estabilidad del CBD frente a la temperatura en extractos acuosos y oleosos de la planta del cannabis, no se han encontrado datos de estabilidad sobre el CBD en estado puro. Por ello, en la presente tesis doctoral se ha evaluado la estabilidad del CBD en solución frente a diversos factores (resultados descritos en la **publicación 5)**.

Previamente, para cuantificar el CBD en los estudios de estabilidad y en los estudios de caracterización de las formulaciones desarrolladas se ha validado un procedimiento analítico por HPLC en fase reversa. El método desarrollado resultó ser lineal y proporcional en un intervalo de concentraciones de 1-150 µg CBD/ml, así como preciso. Este procedimiento analítico se validó empleando soluciones de CBD en etanol como patrones. Sin embargo, muchos de los ensayos se realizarán en soluciones acuosas. Para comprobar la posibilidad de emplear rectas elaboradas con patrones en etanol en la cuantificación del CBD en soluciones acuosas se evaluó la exactitud según los criterios de USP. El método descrito resultó ser exacto a este nivel, lo que indica que se pueden emplear patrones en etanol para cuantificar el CBD en medios acuosos.

Hay que destacar, que debido a la elevada liposolubilidad del CBD, con valores de log P(CBD)=6.33 y de solubilidad en agua ≈10µg/ml recogidos en la bibliografía, tanto para la preparación de soluciones acuosas como para mantener condiciones sink en los estudios de cesión in vitro se requiere el uso de agentes tensioactivos. En la presente tesis doctoral se empleó Tween 80 como solubilizante, eligiendo la concentración necesaria en base a un estudio de solubilidad llevado a cabo tanto a 25 como 37 ºC (Tabla 2). En todos los ensayos descritos en este trabajo, la adición de Tween 80 al 0.5% fue suficiente para solubilizar el CBD y mantener las condiciones sink (20% del coeficiente de solubilidad) en los ensayos de cesión in vitro utilizando un volumen de medio de cesión que diera lugar a concentraciones por encima del límite de cuantificación del método analítico.

	Coeficiente de solubilidad 25ºC (mg/ml)	**Coeficiente de solubilidad 37ºC (mg/ml)**
Sin Tween 80	$<7.4\ 10^{-4}$	0,018±0.001
+Tween 80 0.1%	0,513±0.027	1,070±0.052
+Tween 80 0.5%	2,492±0.009	3,211±0.0007
+Tween 80 1%	4,460±0.032	4,644±0.698

Tabla D2: Estudio de solubilidad del CBD en agua a 25 y 37ºC.

En cuanto a la estabilidad del CBD en solución (descrita de manera detallada en la **publicación 5**), en la presente tesis se ha demostrado que se trata de un compuesto altamente inestable frente a diversos factores como la temperatura, la luz y el oxígeno. La temperatura y la presencia de oxígeno son dos de los factores críticos que comprometen la estabilidad del CBD. A temperatura ambiente, el CBD fue altamente inestable, con un periodo de validez (t_{95}) inferior a 4 meses. No obstante, almacenado en refrigerador el CBD en solución fue bastante estable, estimándose un periodo de validez superior a 4 años. Este compuesto es también muy sensible a la oxidación. De hecho, en un ambiente oxidante su período de validez fue realmente bajo, mostrando un t_{95} inferior a 2días, lo que indica que la presencia de oxígeno es un factor crítico que compomete la estabilidad del CBD. Además, es una molécula fotolábil, pero únicamente en presencia de

oxígeno.Por lo tanto, la eliminación del oxígeno y/o el uso de antioxidantes es altamente recomendable para incrementar la estabilidad de este cannabinoide.

Un punto a destacar en cuanto a la estabilidad del CBD en solución, es la influencia del solvente. Las soluciones acuosas de este principio activo son mucho más inestables que las soluciones etanólicas, probablemente debido a la presencia del oxígeno disuelto, ya que el CBD es altamente sensible a la oxidación. Se trata de un punto importante a tener en cuenta ya que muchos ensayos se deben lleva a cabo en medios acuosos y a temperaturas fisiológicas (37ºC), lo que incrementaría la inestabilidad del CBD en estas condiciones. Por ello se evaluó la estabilidad del CBD en condiciones fisiológicas simuladas (PBS pH7.4 + Tween 80 0.5% a 37ºC). En estas condiciones, en torno un 10% de CBD se degradó en 24 horas.

Como ya se ha comentado anteriormente, su elevada inestabilidad en disolución junto con su elevada lipofilia son los principales problemas que plantea el CBD para el desarrollo de formulaciones de administración parenteral con actividad antitumoral. Al igual que ocurre con otros antitumorales convencionales, el caso más típico es el del paclitaxel, se requiere el uso de solventes orgánicos (como el etanol) y /o agentes dispersantes, como el Cremophor, para su administración parenteral [31-34]. El uso de micropartículas como transportadores de CBD permitiría su administración en medios acuosos sin necesidad de emplear estos solventes orgánicos y/o agentes dispersantes. Además, estos sistemas permitirían obtener un efecto prolongado con una única administración, lo que sería muy interesante en terapia antitumoral donde se requieren tratamientos de larga duración. .

El co-polímero del ácido poli-láctico-co-glicólico (PLGA) es uno de los polímeros más utilizados en el desarrollo de sistemas de liberación controlada de administración parenteral, debido a su alta biocompatibilidad y su biodegradabilidad. De hecho, está aprobado por la FDA, existiendo comercializadas diversas formulaciones que lo contienen. Además, desde el punto de vista tecnológico es muy versátil. Hay disponibles numerosos co-polímeros con distinto peso molecular e hidrofilia que permiten modificar las características de los sistemas desarrollados (como tamaño, carga y liberación del activo) y obtener los sistemas que mejor se adapten a nuestros objetivos [35-37].

Por ello, en la presente tesis doctoral se eligió el PLGA como matriz polimérica, concretamente los resómeros PLGA-RG®-502 (Mw: 7000-17000, i.v.: 0.2 dl/g) y PLGA-RG®-504 (Mw: 38000-64000, i.v.: 0.5 dl/g).Las micropartículas se elaboraron mediante la técnica de emulsificación-evaporación del solvente, manteniendo constante la viscosidad de la fase interna de la emulsión (i.v.: 0.2 dl/g).

Con el objetivo de optimizar las características de las micropartículas obtenidas, se hicieron, en primer lugar, estudios preliminares con PLGA-502 a fin de evaluar la influencia de la velocidad de emulsificación (1000-8000 rpm) en el tamaño de partícula (Tabla D3).

Velocidad de agitación	Tamaño de partículas (μm)	SPAN
1000	77.99 ±1.32	2.12 ± 0.2
4000	22.81± 3.27	1.85 ±0.15
8000	12.78±5.32	3.47±0.31

Tabla D3: Tamaño medio de partícula expresado como diámetro volumen y valores de Span de las micropartículas blancas elaboradas a distintas velocidades de agitación.

A pesar de que la micropartículas elaboradas con una velocidad de agitación de 8000 rpm presentaron un tamaño de partícula medio, expresado como diámetro volumen, considerablemente inferior; mostraron una elevada polidispersión. De hecho se obtuvieron curvas distributivas multimodales. Por este motivo se seleccionó la velocidad de agitación de 4000 rpm como óptima para el desarrollo de las formulaciones cargadas. Cabe destacar que no se produjeron cambios significativos en el tamaño y polidispersión de las micropartículas durante el proceso de liofilización.

Una vez definida la velocidad de agitación, se prepararon micropartículas cargadas de CBD utilizando los dos resómeros de PLGA y partiendo de un ratio fármaco: polímero de 10:100 (10-CBD). No se observaron diferencias en cuanto al tamaño de partícula y valor de span en las formulaciones elaboradas con ambos resómeros de PLGA mostrando, en los dos casos tamaños inferiores a 25 μm y valores SPAN en torno a 2

(tabla D4). Sin embargo, si se apreciaron diferencias considerables en cuanto a la carga en principio activo. Mientras que las micropartículas elaboradas con PLGA-502 mostraron una mayor capacidad de carga, con una eficacia de encapsulación cercana al 95%, en las micropartículas desarrolladas con PLGA-504 la carga en activo fue significativamente menor. El uso de polímeros de elevado peso molecular se traduce en un aumento de la viscosidad de la fase interna de la emulsión, lo que dificultaría la difusión del principio activo a la fase externa acuosa y aumentaría la tasa de encapsulación [38, 39]. No obstante, en este trabajo la viscosidad de la fase interna de la emulsión se mantuvo constante. La menor eficacia de encapsulación obtenida con el polímero de mayor peso molecular puede atribuirse a una menor interacción entre el principio activo y el polímero. Otros autores también han encontrado resultados similares en micropartículas de PLGA cargadas con atorvastatina [40].

Debido a la elevada capacidad de carga que presentó el PLGA 502 se elaboraron formulaciones utilizando un ratio fármaco:polímero 20:100 (20-CBD), con el fin de aumentar en contenido en CBD en las micropartículas y así poder reducir la cantidad de micropartículas a administrar. En este caso, se aumentó la velocidad de emulsificación de la fase interna de la emulsión (una velocidad de 6000 rpm fue seleccionada como óptima) para mantener constante el tamaño de partícula. Las micropartículas elaboradas con ratio fármaco:polímero 20:100 mostraron una elevada capacidad de encapsulación de este activo, con un valor de eficacia de encapsulación cercano al 90% y una carga de 14.97±0.20mg CBD/ 100 mg Mps. La elevada eficacia de encapsulación se debe a la baja solubilidad del CBD en medios acuosos. Este activo es altamente lipófilo y tiende a quedar atrapado en la matriz polimérica en vez de difundir a la fase externa de la emulsión.

El estado físico del CBD en las formulaciones de micropartículas se evaluó mediante DSC. En los termogramas del CBD puro se apreció un pico de un pico de fusión en torno a 70ºC con una entalpía de 75.60 ±0.95 J/g, valor similar al encontrado en la literatura [41]. Sin embargo, en las micropartículas cargadas, este pico desapareció indicando que el CBD se encuentra disuelto o molecularmente disperso en la matriz polimérica. Además, en las formulaciones cargadas se detectó una disminución en la temperatura de transición vítrea del polímero con respecto al valor obtenido al analizar el polímero puro o las micropartículas blancas, indicando un efecto plastificante del CBD

sobre la matriz polimérica. Esta disminución de la Tg fue más acusada en las micropartículas con un ratio CBD: polímero 20: 100 debido a un mayor contenido en CBD.

	Tamaño de partícula (µm)	SPAN	Rendimiento del proceso (%)	Carga (mg CBD/ 100 mg Mps)	Eficacia de encapsulación (%)
PLGA 502	24.03±2.01	1.98± 0.05	89.25±1.012	--------	--------
10-CBD-502	24.17±2.32	2.02± 0.08	87.36 ±2.77	8.600± 0.425	94.62±4.62
20-CBD-502	25.14 ±1.27	2.36 ± 0.15	81.05 ±5.33	14.97±0.20	89.86±10.33
PLGA 504	23.02 ± 2.25	2.12± 0.25	79.27±5.21	--------	--------
10-CBD-504	24.12 ±1.25	2.28± 0.17	77.95 ±3.24	5.212± 0.576	57.34±6.34

Tabla D4: Tamaño de partícula, valor de SPAN, rendimiento del proceso de microencapsulación, carga de CBD y eficacia de encapsulación de las micropartículas blancas (PLGA-502 y PLGA-504) y cargadas con CBD con un ratio fármaco: polímero 10:100 (CBD-502 y CBD-504) y 20:100 (20-CBD).

Todas las formulaciones desarrolladas presentaron una liberación controlada del activo durante más de un mes, siendo mucho más rápida en las micropartículas 10-CBD (Figura D1). Esto puede deberse al mayor contenido en activo que presentaron las formulaciones 20-CBD. Como ya mencionamos previamente, el CBD es un compuesto altamente lipófilo lo que dificulta la entrada de agua al interior de la matriz polimérica, retrasando la degradación hidrolítica del polímero y por consiguiente la liberación del CBD [42]. De hecho las imágenes obtenidas por SEM para evaluar la degradación de la matriz polimérica durante los estudios de cesión demostraron que la formulación de 20-CBD-502 presentó una degradación más lenta de la matriz polimérica, justificando los resultados obtenidos en el estudio de cesión. Si comparamos las dos formulaciones de micropartículas elaboradas con un ratio fármaco: polímero 10:100 se apreció una liberación más rápida en las elaboradas con PLGA 502 (Figura D2). Esto se puede atribuir al peso molecular del polímero, ya que polímeros con un peso molecular más elevado muestran una degradación más lenta de la matriz polimérica, y por consiguiente una liberación más lenta del activo [42]. De hecho, esta menor degradación del polímero también se puede apreciar en las imágenes obtenidas por SEM durante los

estudios de cesión. Debido a la mayor carga en CBD que presentaron las formulaciones elaboradas con PLGA 502, se seleccionaron estas para los siguientes estudios.

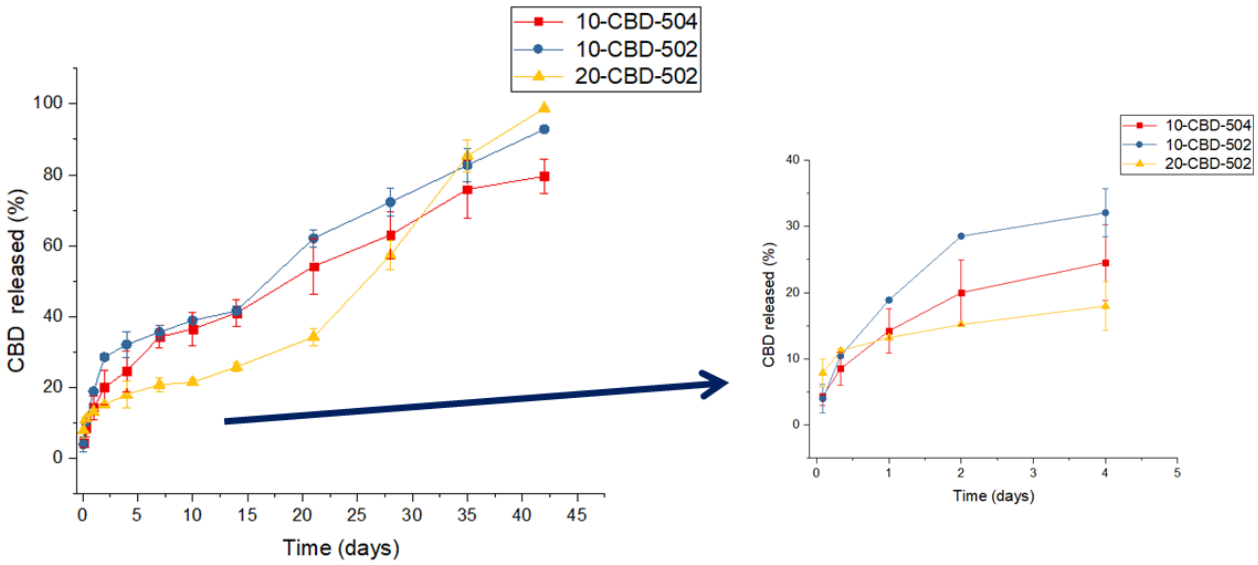

Figura D1: Perfil de liberación de las micropartículas de CBD desarrolladas.

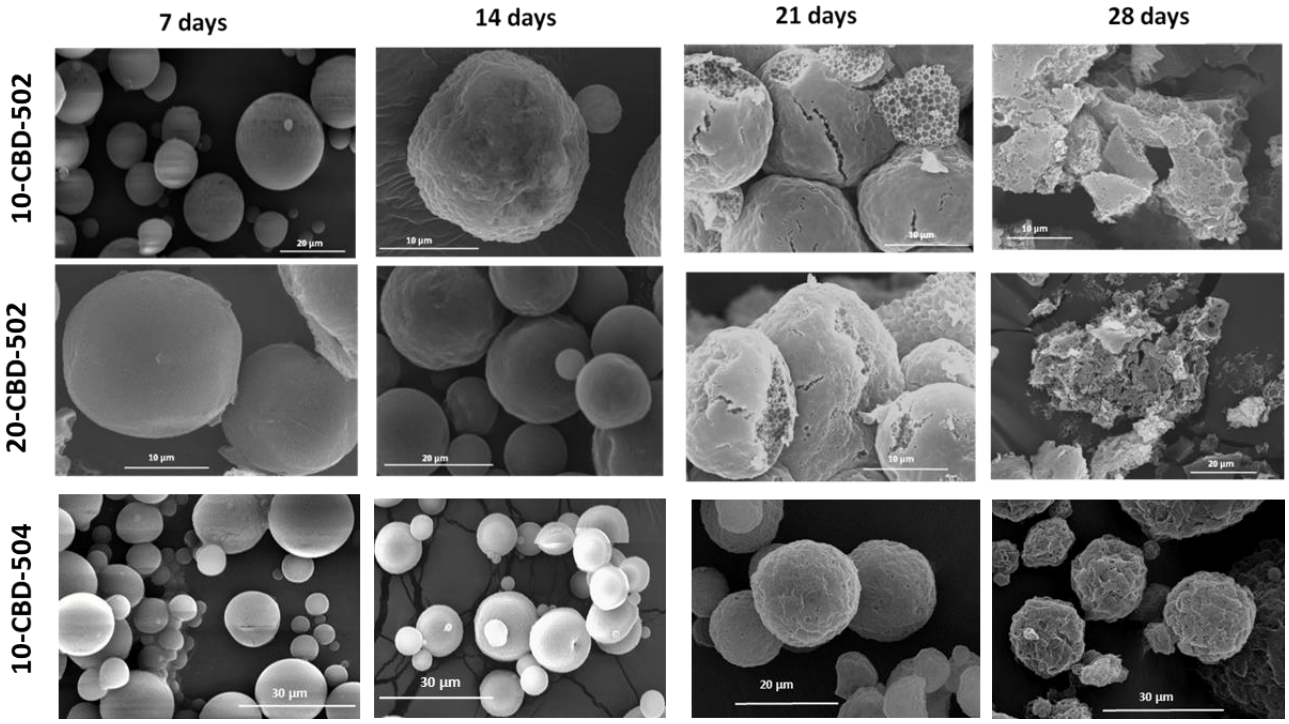

Figura D2: Imágenes obtenidas por SEM para evaluar la degradación de la matriz polimérica de las micropartículas cargadas con CBD durante los estudios de cesión

Las micropartículas cargadas con CBD desarrolladas están destinadas a ser administradas por vía parenteral, donde la esterilidad es un requisito imprescindible. Por ello, en la presente tesis se evaluó el efecto de la esterilización por radiación gamma (se empleó una dosis de 25KGy) en las características de las micropartículas previamente seleccionadas. Cabe destacar, que existen otras técnicas disponibles para esterilizar las micropartículas poliméricas, como es el uso de óxido de etileno. No obstante, el riesgo de residuos tóxicos y la tendencia a aglomerarse de las micropartículas esterilizadas por este método hizo que se seleccionará la esterilización por radiación gamma [43]. Debido a que durante la esterilización por radiaciones se suele producir un aumento de la temperatura que comprometería la estabilidad del CBD, este proceso se llevó a cabo manteniendo las muestras en hielo seco. Tal y como se describe detalladamente en la **publicación 6**, no se observaron cambios significativos en cuanto a las características morfológicas (forma y tamaño) ni en el comportamiento térmico de las micropartículas estériles. Sin embargo, se detectó una disminución en el contenido de CBD. Esta degradación puede atribuirse al aumento de la temperatura durante el proceso de esterilización a pesar del uso de hielo seco. En las formulaciones estériles también se observó un aumento de la velocidad de liberación de este compuesto, probablemente debido a una disminución del peso molecular de polímero. De hecho, la degradación de la matriz polimérica es mucho más rápida. Mientras que en las formulaciones no estériles (tanto las elaboradas con un ratio fármaco:polímero 10: 100 como 20:100) tras 28 días de incubación en las condiciones de los estudios de cesión (PBS pH 7.4 con Tween 80 al 0.5 % y 37ºC)las micropartículas pierden completamente su forma observándose, además, una intensa degradación de la matriz polimérica, en las formulaciones estériles esta intensa degradación se aprecia ya tras 21 días. Precisamente este punto coincide con el final de la fase de cesión del CBD, con más del 90% del fármaco liberado. A pesar de los cambios observados en la liberación, y una cierta degradación del CBD, esta técnica podría suponer una buena herramienta para esterilizar estas formulaciones.

Ya que en los estudios de estabilidad realizados pusieron de manifiesto la inestabilidad de la molécula frente a la temperatura, oxígeno y luz se realizaron estudios de estabilidad con las micropartículas de CBD con el fin de determinar si aumentaban la estabilidad del fármaco. Por un lado se realizaron estudios de estabilidad a largo plazo conforme a las directrices ICH (formulaciones almacenadas a 5ºC y a 25±2ºC/HR 60 ±5%) (Figura 2). Las formulaciones almacenadas en condiciones medioambientales

fueron inestables tanto física como químicamente. Sin embargo, las micropartículas conservadas en refrigerador presentaron una mayor estabilidad, mostrándose químicamente estables durante los 12 meses de estudio, sin apreciarse una degradación significativa del principio activo; aunque , desde el punto de vista físico, mientras las micropartículas elaboradas con un ratio fármaco: polímero 10:100fueron estables durante todo el ensayo, en las formulaciones con un ratio fármaco: polímero 20:100 se detectó un aumento en el tamaño de partícula y en la polidispersión a los 6 meses de almacenamiento, que se achacó a la formación de agregados. Está referido en la bibliografía que las micropartículas de PLGA almacenadas a largo plazo tienden a agregare [44]. La mayor inestabilidad de las micropartículas con un ratio fármaco: polímero 20:100 podría atribuirse al mayor efecto plastificante del CBD, lo que favorecería la formación de agregados.

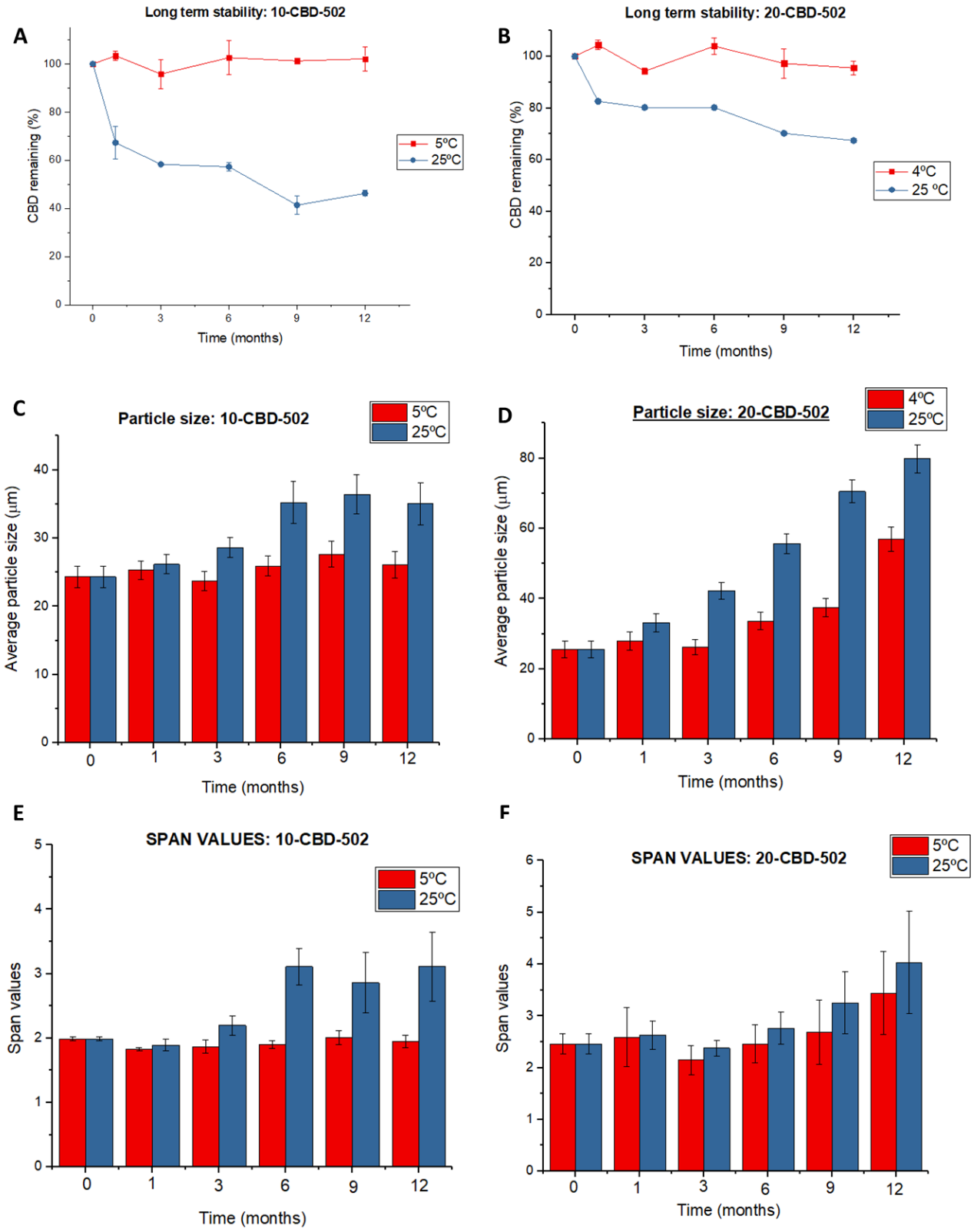

Figure D2: Estabilidad química (A y B) y física (C-F) de las micropartículas de CBD almacenadas a 5 y 25ºC durante 12 meses.

Por otro lado, para evaluar si las micropartículas protegen de la fotodegradación al CBD, se hicieron estudios de fotoestabilidad a 5ºC con las formulaciones 10-CBD-502 y 20-CBD-502. En ambos casos se observó una degradación del CBD en las muestras expuestas a la luz, mientras que las muestra protegidas (en oscuridad) se mantuvieron estables (Figura D3). Tal y como mencionamos al comentar los resultados del estudio por DSC de las micropartículas, este cannabinoide se encuentra disuelto en la matriz polimérica, y probablemente las moléculas localizadas en las capas más superficiales de las micropartículas son las que se degradan en este proceso. Al igual que en los estudios realizados sobre el CBD en solución, la eliminación del O_2 mediante el borboteo de gas nitrógeno evitó el proceso fotodegradativo del CBD encapsulado. Estos resultados corroboran la sensibilidad del CBD a la oxidación.

En conclusión, todos los estudios de estabilidad llevados a cabo con las micropartículas de CBD indican que las formulaciones deben ser conservadas en refrigerador y protegidas de la luz. Debido a la sensibilidad del CBD a la oxidación, el desplazamiento del oxígeno con gas nitrógeno sería, además, altamente recomendable para aumentar la estabilidad de las formulaciones. Conservadas en estas condiciones las micropartículas elaboradas con y ratio: fármaco polímero 10:100 serían estables tanto desde el punto de vista físico como químico durante al menos 12 meses.

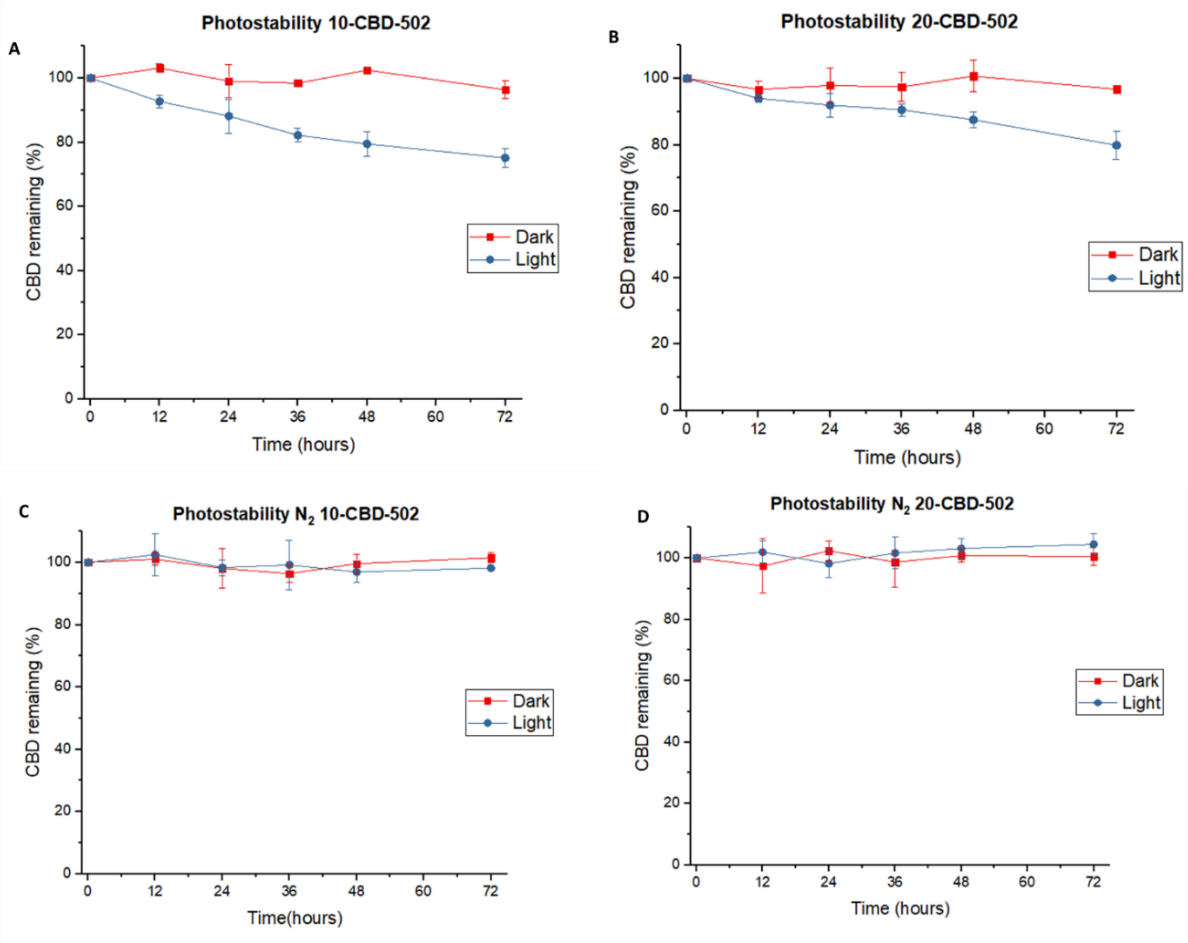

Figura D3: Estudios de fotoestabilidad de las micropartículas de PLGA 502 elaboradas con un ratio fármaco: polímero 10:100 y 20:100.

De las dos formulaciones de PLGA-502 desarrolladas se seleccionaron las elaboradas con un ratio fármaco polímero 10:100 para los estudios de eficacia antitumoral *in vitro* e *in ovo*. Teniendo en cuenta la carga, el uso de las formulaciones 20-CBD-Mps permitiría administrar una menor cantidad de micropartículas. Sin embargo, en esta formulación la liberación del CBD fue mucho más lenta. De hecho, en el perfil de liberación se diferencian claramente dos fases que se ajustan, ambas a cinéticas de orden cero: desde el día 1 al 21 (r^2=0.981, K_0= 13.74 µg/day/10 mg Mps) y del día 21 al 42 (r^2=0.983, K_0= 44.41 µg/day10 mg Mps). La diferencia en la velocidad de de liberación (la constante de velocidad de liberació de la segunda fase es más de tres veces mayor que en la primera fase) dificultaría la dosificación. Por el contrario, la liberación del CBD a partir de las micropartículas 10-CBD-502 se ajusta del día 2 al 42 a una cinética de orden

0 (r^2=0.988, K_0= 14.48 µg/day/10 mg Mps) lo que facilitaría su dosificación. Además, las micropartículas de 10-CBD-502 fueron mucho más estables desde el punto de vista físico. Por ello, esta formulación fue seleccionada para los siguientes ensayos.

La eficacia antitumoral *in vitro* de las micropartículas seleccionadas en cáncer de mama y ovario está recogida en las **publicaciones 6 y 7** respectivamente. Estos estudios, que se realizaron en las líneas tumorales de cáncer de mama MCF-7 (células positivas a los receptores de estrógenos) y MDA-MB-231 (células trile negativas) y en la línea tumoral SKOV-3 de cáncer de ovario, indicaron que mientras las micropartículas blancas no mostraron citotoxicidad, las micropartículas elaboradas con un ratio fármaco: polímero 10:100 inhibieron la proliferación de las células tumorales de mama y ovario. De hecho, se consiguió un efecto antiproliferativo prolongado, durante al menos 10 días, con una única administración. De acuerdo a estos resultados las micropartículas de PLGA podrían suponer una buena estrategia para vehiculizar el CBD y conseguir una actividad antiproliferativa prolongada con una única administración, lo que sería altamente beneficioso en terapia antitumoral donde se requieren tratamientos a largo plazo.

En el caso de cáncer de ovario, la actividad antitumoral de las micropartículas también se evaluó en modelos *in ovo* en los que el tumor se desarrolla sobre la membrana corioalantoidea de embriones de pollo. Precisamente, este modelo ha surgido como una alternativa *in vivo* para el estudio de esta neoplasia, convirtiéndose en un paso entre los ensayos celulares *in vitro* y los estudios en animales de experimentación. De hecho se ha demostrado que los tumores de ovario desarrollados en este modelo son comprables desde el punto histológico a tumores extraídos de pacientes, presentando características similares, lo que refuerza aún más su uso en la evaluación de la eficacia antitumoral de nuevas formulaciones [45]. Para el tratamiento del cáncer de ovario, las micropartículas desarrolladas se pretenden administrar por vía intraperitoneal. Por ello, en este modelo las formulaciones se administran de manera tópica sobre el tumor localizado en la membrana corioalantoidea del embrión, ya que simularía mejor una administración intraperitoneal. Al igual que en los estudios *in vitro*, mientras las micropartículas no cargadas no mostraron toxicidad, las micropartículas de CBD inhibieron el crecimiento de los tumores de ovario. De hecho, la administración de una única dosis de estas formulaciones consiguió un efecto inhibidor del crecimiento tumoral, fue similar a la administración diaria de CBD en solución a la misma concentración que la liberada

diariamente de las micropartículas (100 µM). En este modelo los tumores de ovario fueron tratados durante 60 horas, desde el día de incubación 11 al día 13.5. Cabe destacar, que el ensayo no pudo prolongar más por las leyes de protección de los animales de experimentación de Suiza (estos experimentos se llevaron a cabo en la universidad de Ginebra), que marcan que los embriones de pollo deben ser sacrificados antes del día de incubación 14.

Debido al potencial que presentó la combinación del CBD en solución con paclitaxel, se planteó la evaluación de la eficacia de la combinación de las micropartículas de CBD con este antitumoral, en cáncer de mama y de ovario. Tal y como mencionamos previamente, tanto la pre- como la co-administración de CBD en solución resultaron estrategias eficaces de combinación con paclitaxel en ambos tipos de cánceres. Por ello se planea como el mejor esquema quimoterápico la administración previa del tratamiento de CBD (durante 24 horas) seguida de su co-administración con paclitaxel(durante 48 horas).Precisamente, para este esquema de pre+co-administración de CBD, el empleo de micropartículas sería muy útil ya que debido a la liberación prolongada del CBD se podría llegar a conseguir el efecto potenciador de la actividad del antitumoral con una sola administración, mientras que el CBD en solución requeriría múltiples administraciones. A pesar de que la combinación de CBD en solución con doxorrubicina sólo fue realmente eficaz en cáncer de mama, especialmente en células MCF-7, la combinación de este antineoplásico y las micropartículas de CBD también se evaluó tanto en cáncer de mama como de ovario siguiendo el esquema descrito para el paclitaxel. Los resultados de estas combinaciones están detallados en las **publicaciones 6 y 7**.

En el caso del cáncer de ovario, aunque los estudios descritos con anterioridad demostraron que la pre-o o co-administración del CBD sólo fue eficaz en el caso del paclitaxel, la combinación de ambos esquemas (pre +co-administración de CBD) resultó realmente efectivo en combinación con el paclitaxel y la doxorrubicina. El uso de CBD (a concentraciones de 10 µM) disminuyó significativamente los valores de IC_{50}de ambos agentes antineoplásicos. La administración de una sola dosis de micropartículas (calculada para conseguir una liberación diaria de CBD de 10 µM demostró una eficacia

antitumoral similar o incluso ligeramente superior a la administración diaria de dosis equivalentes de CBD en solución.

Este esquema de combinación de pre+co-administración de CBD fue especialmente eficaz en el caso del paclitaxel. De hecho, las células tratadas con las micropartículas de CBD mostraron un valor de IC_{50} unas 9 veces inferior al paclitaxel en monoterapia. Por este motivo, la combinación del CBD y el PTX siguiendo este esquema también se evaluó *in ovo*. En este modelo, tanto el CBD en solución (administrado diariamente) como las micropartículas cargadas con CBD (administración única) mejoraron el efecto antitumoral del paclitaxel.

En cáncer de mama este esquema de pre+co-administración de CBD fue eficaz tanto en células tumorales triple negativas como en células tumorales positivas a los receptores de estrógenos, donde la administración de concentraciones diarias de CBD 5y 10µM respectivamente, aumentó significativamente la acción antiproliferativa de paclitaxel y doxorrubina, disminuyendo considerablemente sus valores de IC_{50}. Al igual que ocurría en cáncer de ovario, las micropartículas mostraron una eficacia similar e incluso superior en algunos casos a la mostrada por el CBD en solución.

Todos estos resultados confirman la hipótesis de que las micropartículas poliméricas puede ser una buena estrategia para vehiculizar el CBD y mejorar los tratamientos quimioterápicos convencionales de cáncer de mama y ovario.

En los últimos años, la administración intraperitoneal de fármacos antitumorales ha surgido como una vía alternativa en el tratamiento de cáncer de ovario, especialmente en estados avanzados de la enfermedad. El uso de micropartículas como transportadores de los agentes antineoplásicos resulta una buena estrategia para aumentar el tiempo de retención de estos fármacos en la cavidad intraperitoneal, y por consiguiente, su eficacia antitumoral. Sin embargo, hay que tener en cuenta que debido a su tamaño las micropartículas presentan una baja capacidad de penetración en los tumores, así como un riesgo de desarrollar adhesiones tisulares [46]. El empleo de nanopartículas como transportadores de fármacos anticancerígenos también permitiría aumentar, aunque en menor grado, el tiempo de retención del fármaco a nivel de la cavidad intraperitoneal y, además presentarían una mayor penetración en el tumor [47], lo que resulta una ventaja

con respecto a las micropartículas. Por este motivo, en la presente tesis doctoral se han desarrollado nanopartículas poliméricas de PLGA cargadas con CBD que puedan ser internalizadas por las células tumorales de ovario (estudios descritos en la **publicación 8**).

Los objetivo perseguidos con las nanopartículas diseñadas en este trabajo son, facilitar la administración del CBD evitando el uso de disolventes orgánicos, incrementar el tiempo de permanencia de este activo en la cavidad peritoneal respecto a su administración en solución, y favorecer la localización preferente del CBD a nivel de las células tumorales que puedan estar diseminadas en la cavidad peritoneal. Estudios *in vivo* llevados a cabo en ratones con nanopartículas poliméricas de PLGA de un tamaño de partícula en torno a los 250 nm, demostraron, tras una administración intraperitoneal, la permanencia de estas formulaciones en el interior de la cavidad durante al menos 48 horas [48]. De esta manera sería recomendable, al menos, una liberación controlada del CBD a partir de las micropartículas durante al menos ese tiempo (48 h).

Para el desarrollo de las nanopartículas, en una primera etapa, se emplearon diferentes técnicas de nanoencapsulación con el fin de seleccionar el método más adecuado. Los criterios para la elección de dicho método fueron, como ya se ha indicado, el tamaño de partícula (inferior a 250nm), una tasa de encapsulación elevada y una liberación prolongada del CBD durante al menos 48h.

Inicialmente, basándonos en trabajos descritos por otros autores para el desarrollo de nanosistemas poliméricos con otros cannabinoides [49-51], las nanopartículas se elaboraron por el método de nanoprecipitación, empleando acetona como disolvente orgánico. Tal y como se puede observar en la tabla D4, todas las formulaciones desarrolladas por este método presentaron un tamaño de partícula inferior a 200 nm, lo que sería altamente recomendable para favorecer su internalización por parte de las células tumorales. Así mismo, las nanopartículas presentaron una elevada eficacia de encapsulación, con valores superiores al 90%. Sin embargo, estas formulaciones no controlaron la liberación del CBD, de manera que más del 80% de este compuesto se liberó durante los primeros 90 minutos. Esta rápida cesión indica que el CBD no se encuentra completamente encapsulado en la matriz polimérica, lo cual podría atribuirse a la rápida difusión de la acetona hacia la fase externa, lo que dificultaría la encapsulación

del principio activo. Por lo tanto, debido a la rápida liberación del CBD en la primera hora a partir de las nanopartículas elaboradas por nanoprecipitación, este método fue complemente descartado. Como alternativa a este método, se empleó el de evaporación-extracción del solvente, usando diclorometano como disolvente orgánico. Cabe destacar, que con este método se puede llevar a cabo la fase de evaporación a temperatura ambiente, lo que sería recomendable en el caso del CBD debido a sus problemas de estabilidad.

	PLGA (mg)	**CBD (%) (w/w)**	**PVA (%)**	**EE (%)**	**Tamaño medio (nm)**	**CBD liberado 90 minutos (%)**
NP-1	50	1.5	1	91.76±5.31	177.63±3.46	85.75 ± 1.36
NP-2	100	1.5	1	95.16±1.97	180.90±6.22	80.27 ± 4.63
NP-3	100	1.5	3	90.27 ±2.12	170.4 ±5.22	85.32 ±4.08

Tabla D4: Formulaciones elaboradas por el método de nanoprecipitación.

Las formulaciones de nanopartículas desarrolladas por el método de evaporación y extracción del solvente quedan recogidas en la tabla D5. Con este método, se obtuvieron tamaños de partículas superiores a las formulaciones elaboradas por nanoprecipitación, con valores comprendidos en el rango de 220-260 nm. En la optimización del protocolo de encapsulación por este método, se emplearon diferentes tiempos de sonicación, de manera que al aumentar el tiempo se obtuvo una reducción estadísticamente significativa en el tamaño de partícula. Sin embargo, también se produjo una disminución de la carga y un aumento de la velocidad de liberación del CBD. De hecho, otros autores han encontrado resultados similares en el desarrollo de nanopartículas cuando emplean este método [52-53]. En todos los casos, con este método se consigue reducir la liberación del CBD a partir de las nanopartículas en los primeros 90 minutos. Por tanto lo seleccionamos como el más adecuado para la nanoencapsulación del CBD. Además, en base a los criterios previamente establecidos, las nanopartículas elaboradas con CBD al 1.5%, PVA 1% y un tiempo de sonicación de 2 minutos presentaron una tamaño de partícula inferior a 250nm, una elevada eficacia de encapsulación y una liberación más lenta del principio activo, por lo que esta formulación se seleccionó como la más adecuada para nuestros objetivos.

CBD (%) (w/w)	PVA (%)	Tiempo de sonicación	Tamaño medio (nm)	EE (%)	CBD liberado 90 minutos (%)
1.5	1	1	258.19 ± 4.22	92.37± 1.25	37.61
1.5	3	1	247.27± 3.62	95.22 ± 3.12	49.80
1.5	1	5	220.84 ± 5.27	78.13 ± 4.01	59.72
1.5	1	2	231.25 ± 6.27	93.27 ±3.10	41.32
3	1	2	250.01 ± 10.4	80.69 ± 6.22	60.22
3	3	2	240.36 ± 8.31	86.71±2.78	68.27
3	1	5	229.64 ± 2.16	78.08 ± 1.98	72.15

Tabla D5: Características de las nanopartículas preparadas por el método de evaporación-extracción del solvente.

Hay que destacar, que la incorporación a la superficie de las nanopartículas de ligandos reconocidos selectivamente por las células tumorales permitiría su acumulación preferencial en el tumor y por consiguiente un aumento de la eficacia antitumoral [54-55]. En cáncer de ovario, uno de los ligandos más utilizados es el ácido fólico, cuyos receptores se encuentran sobreexpresados en el 90% de los tumores ováricos [56-57]. Por este motivo, en la presente tesis doctoral también se elaboraron nanopartículas cargadas con CBD y funcionalizadas con ácido fólico.

Existen diferentes métodos para la funcionalización de estos sistemas. Algunos autores unen previamente el ligando al PLGA y utilizan el PLGA-ligando, junto con el agente activo, para la formación de las nanopartículas. Otros autores, sin embargo, utilizan un método post-inserción, elaborando previamente las formulaciones cargadas con el agente activo y posteriormente incorporando el ligando a la superficie de las mismas [58-61]. Este último método es más sencillo y permite la funcionalización sobre las nanopartículas ya desarrolladas, lo que en algunos casos podría resultar una ventaja.

En la presente tesis doctoral la incorporación del ácido fólico se realizó sobre la superficie de las nanopartículas previamente formadas. En primer lugar, se activaron los grupos carboxilo libres de la superficie polimérica de las nanopartículas. A continuación, estos carboxilo activados se hicieron reaccionar con el grupo amino libre de la molécula de ácido fólico (Figura D5).

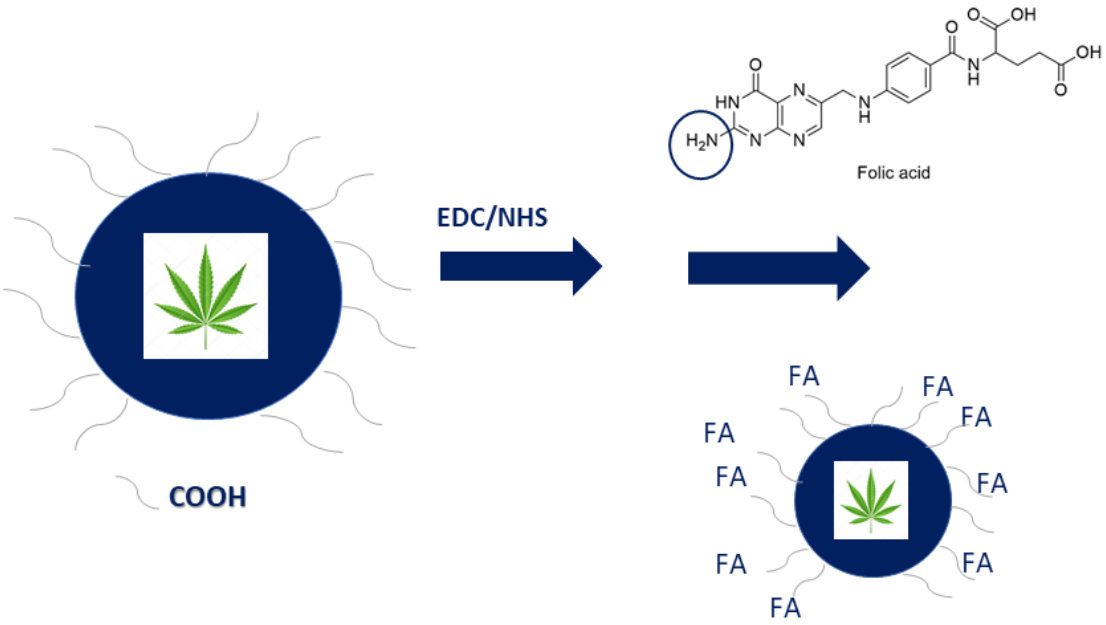

Figura D5: Esquema de funcionalización de las nanopartículas desarrolladas

Tal y como podemos observar en la tabla D6, la incorporación de ácido fólico a la superficie de las nanopartículas produjo un aumento del tamaño de partícula con respecto a las formulaciones no funcionalizadas. No obstante, sólo se apreciaron diferencias estadísticamente significativas en las nanopartículas blancas. Como cabía esperar, se produjo una modificación en el valor del potencial zeta aunque, en todos los casos, esta disminución fue muy pequeña. La unión de los grupos carboxilo activados del PLGA con el grupo amino libre del ácido fólico debería producir un aumento de la carga superficial de las nanopartículas, sin embargo, el ácido fólico presenta dos grupos carboxilos libres; siendo, probablemente, los responsables de esta ligera disminución en el potencial zeta de las nanopartículas funcionalizadas. Por otro lado, desde el punto de vista morfológico, no se apreció ningún cambio. Tal y como se puede apreciar en las imágenes obtenidas por SEM de la figura D6, todas las nanopartículas presentaron una forma esférica y una

superficie lisa y sin poros. En cuanto al contenido en CBD se produjo una ligera disminución en las nanopartículas funcionalizadas probablemente debido a la pérdida de este compuesto durante el proceso de funcionalización. También se observó un ligero aumento en la velocidad de liberación del mismo durante las primeras 48 horas de ensayo (Figura D6).

Formulación	Tamaño (nm)	PDI	Potencial zeta (agua)	Rendimiento del proceso (%)	Carga (µg CBD/10 mg Nps)	EE (%)
PLGA- Nps	228.47 ± 8.36	0.141 ± 0.019	-24.7±1.48	61.8 ± 4.27	------	------
CBD-Nps	236.08± 12.02	0.165± 0.009	-16.6 ±1.22	51.2 ± 6.22	140.2± 6.25	95.23 ± 3.3
FA-PLGA-Nps	259.1± 8.47	0.271±0.031	-29.5 ±0.20	48.62 ± 12.22	------	------
FA-CBD-Nps	241.5± 10.21	0.210±0.033	-18.4 ±0.66	50.94±5.27	120.5±15.57	81.97 ±14.97
DiO-Nps	230.27±7.58	0.175±0.021	-28.2 ± 1.52	62.5 ± 2.33	------	------
FA-DiO-Nps	248.3±9.72	0.227±0.085	-31.27 ± 1.70	50.94±10.22	------	------

Tabla D6: Características de las formulaciones optimizadas elaboradas por el método de evaporación-extracción del solvente.

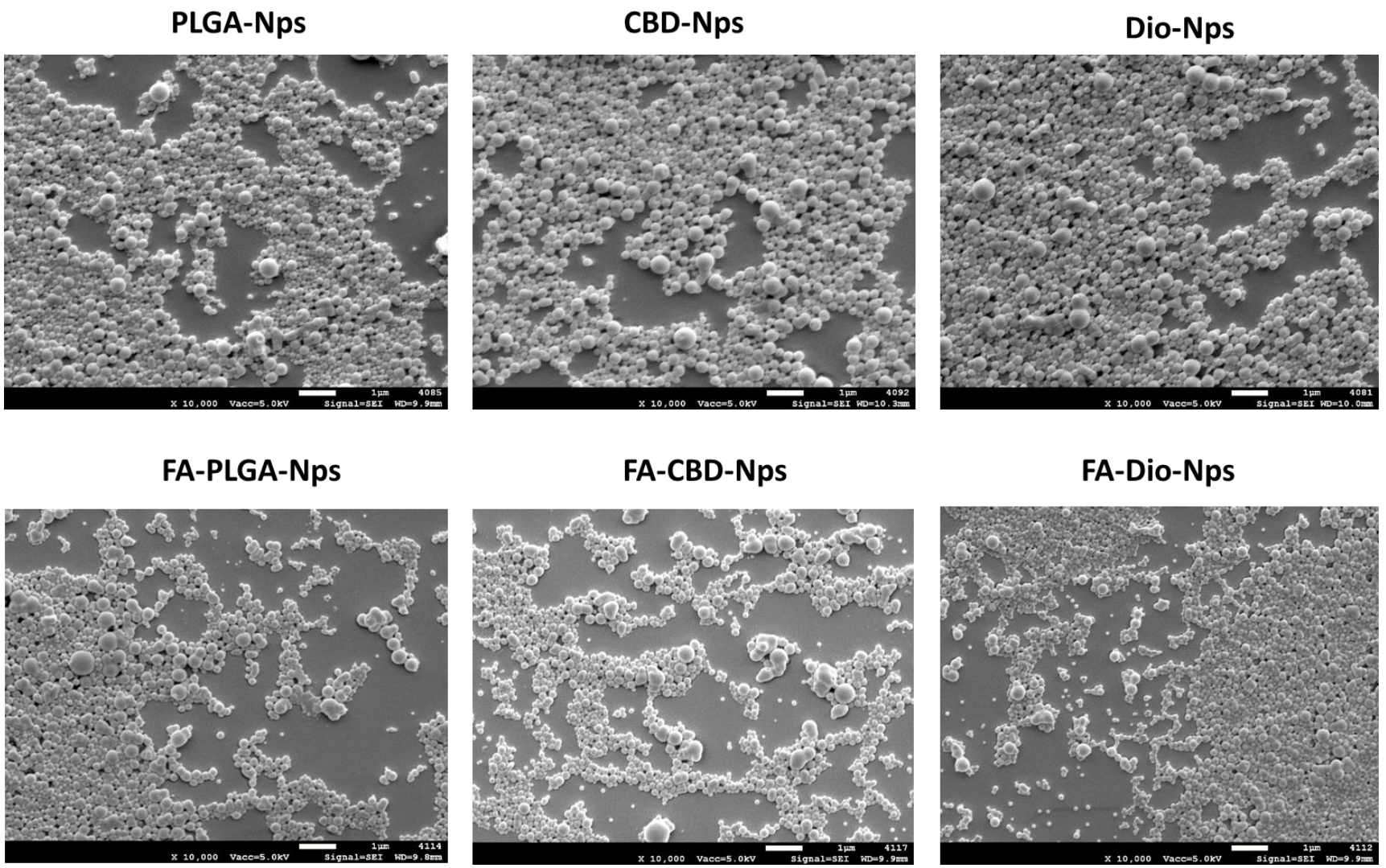

Figura D6: Imágenes obtenidas por SEM de todas la nanopartículas desarrolladas.

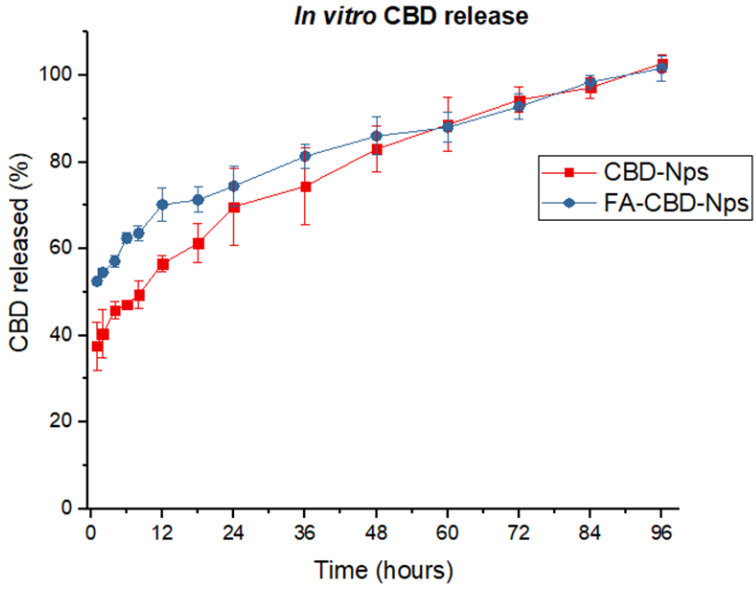

Figura D7: Perfil de liberación in vitro de las nanopartículas de CBD funcionalizadas y no funcionalizadas con ácido fólico.

La incorporación de ácido fólico a la superficie de las nanopartículas debería favorecer su internalización por parte de las células SKOV-3, células tumorales de ovario que sobre-expresan los receptores de folato. En la presente tesis doctoral, la captación de las formulaciones funcionalizadas y no funcionalizadas con ácido fólico se evaluó de manera cualitativa por microscopía de fluorescencia, para lo cual se prepararon nanopartículas cargadas con DiO como agente fluorescente. Tal y como se puede apreciar en la figura D8, se produjo unainternalización más rápida en las formulaciones funcionalizadas con folato. Así mismo, se puede apreciar una mayor captación en estas formulaciones, aunque esta la internalización no ha sido cuantificada. En las formulaciones sin ácido fólico no se apreció una captación considerable hasta las 2 horas de incubación. Sin embargo, en las formulaciones funcionalizadas se observó incluso a los 30 minutos. Precisamente, la internalización a tiempos cortos obtenida con las nanopartículas de folato resulta muy interesante para nuestros objetivos debido a la rápida liberación del CBD a partir de las nanopartículas en los primeros 90 minutos. Sin embargo, tras 8 horas de incubación ambas formulaciones presentaron una captación similar.

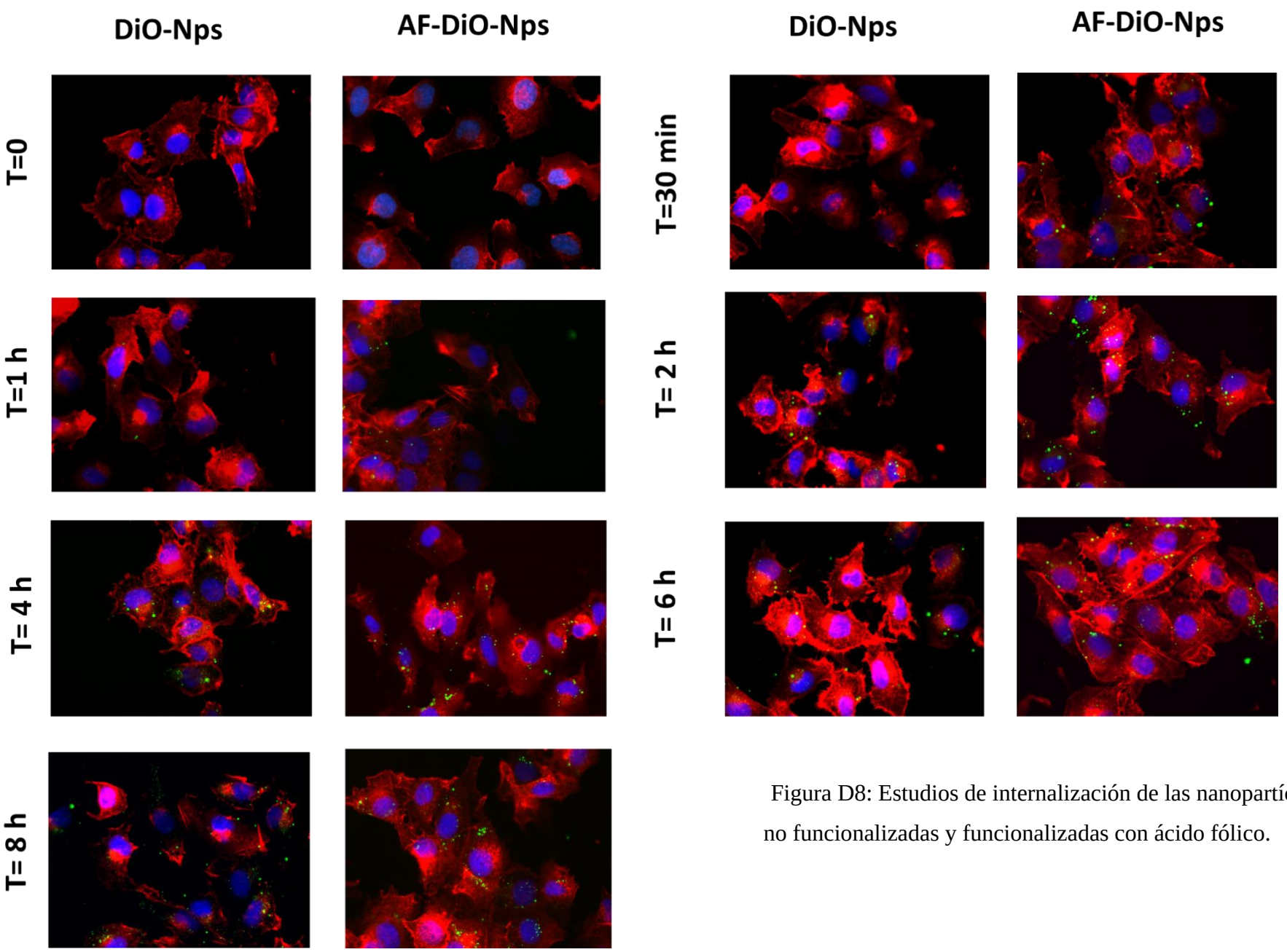

Figura D8: Estudios de internalización de las nanopartículas no funcionalizadas y funcionalizadas con ácido fólico.

Debido a esta más rápida captación, se podría esperar un aumento en la eficacia antitumoral de las nanopartículas funcionalizadas con folato. Sin embargo, ambas formulaciones de nanopartículas presentaron una actividad antiproliferativa similar en las células SKOV-3. Este hecho podría atribuirse a los tiempos a los que se ha realizado el ensayo, ya que a las 24 horas de incubación probablemente ambas formulaciones estén completamente internalizadas.

Sólo se detectaron diferencias en la actividad antiproliferativa de las nanopartículas funcionalizadas y no funcionalizadas con ácido fólico y el CBD_{sol} a concentraciones bajas e intermedias de CBD (5-20µM) tras 24 horas de incubación y a la concentración más baja (5µM) tras 48 horas (Figura D9). No obstante, se obtuvieron valores de CI_{50} ligeramente inferiores al CBD en solución con ambas formulaciones de nanopartículas. Esta disminución fue además más acusada en las formulaciones funcionalizadas, aunque estas diferencias no fueron, en ningún caso, estadísticamente significativas (Tabla D7). Cabe destacar que en estos ensayos el CBD en solución está completamente disponible para las células lo que podría dificultar encontrar diferencias significativas entre la solución y las nanopartículas, en las que el CBD se libra de manera prolongada durante 96 horas. Sin embargo, en modelos *in vivo* el CBD, debido a su elevada lipofilia, tendería a distribuirse a tejidos grasos, mientras que las nanopartículas se podrían internalizar en las células diana. Por este motivo cabe esperar una mayor eficacia de las nanopartículas, en modelos in vivo al permitir la localización del CBD en el lugar de acción.

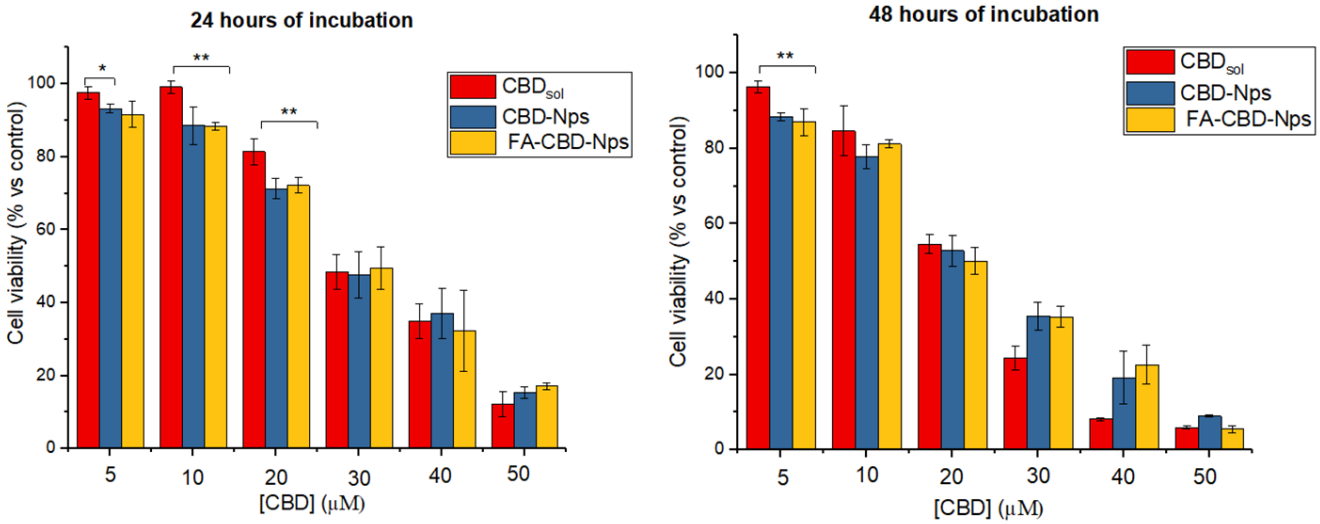

Figura D9: Estudios de viabilidad celular de las nanopartículas cargadas con CBD no funcionalizadas y funcionalizadas con ácido fólico en células SKOV-3.

	CI 50	
	24 horas	**48 horas**
CBD sol	31.19±2.57µM	23.47±4.10µM
CBD-Nps	29.64±2.94µM	20.88±1.25µM
AF-CBD-Nps	26.12±4.32µM	17.63±3.27µM

Tabla D7: Valores de CI_{50} en las células SKOV-3 tratadas con CBD_{sol} y las nanopartículas no funcionalizadas y funcionalizadas de CBD durante 24 y 48 horas.

La eficacia antitumoral de las nanopartículas de CBD desarrolladas también se evaluó en modelos *in ovo*. Para estos estudios, los tumores fueron tratados de manera tópica, ya que como mencionamos previamente sería la administración que mejor simularía la vía intraperitoneal, ruta para la que están diseñadas las nanopartículas. Se comparó la administración diaria de CBD en solución, CBD-Nps y AF-CBD-Nps a una concentración de CBD de 100µM durante 60 horas. Los resultados en estos modelos demostraron una eficacia antitumoral significativamente superior en las nanopartículas funcionalizadas con ácido fólico, comparada con el resto de los tratamientos (Figura D10). Las CBD-Nps mostraron también una mayor inhibición del crecimiento tumoral

que el CBD en solución, aunque en este caso no se apreciaron diferencias estadísticamente significativas. Cabe destacar, que la mayor actividad de las nanopartículas de CBD podría atribuirse, en parte, a una mayor captación en el tumor de este compuesto y a un aumento de su estabilidad.

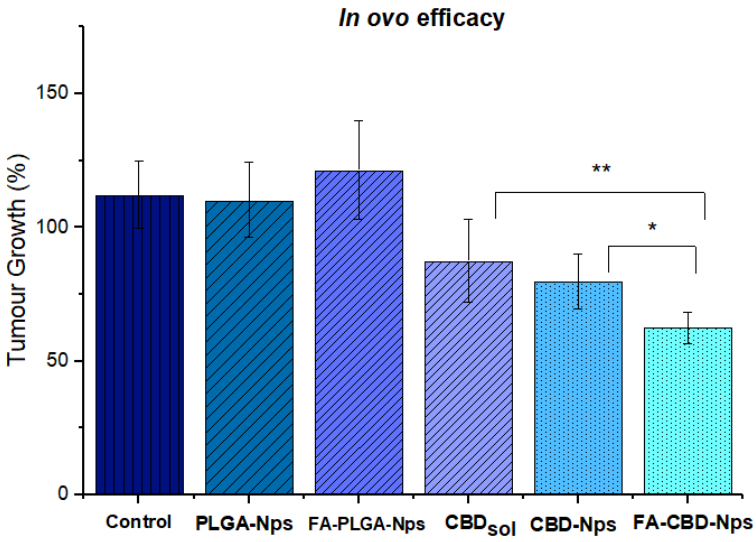

Figura D10: Eficacia antitumoral in ovo de las nanopartículas de CBD no funcionalizadas y funcionalizadas con ácido fólico

Con el objetivo de verificar si la mayor internalización en cultivos celulares de las nanopartículas funcionalizadas, y por consiguiente, el mayor efecto antitumoral *in ovo*, se pueden atribuir a la presencia de ácido fólico se realizaron estudios de internalización con la saturación previa de los receptores de folato. Tal y como se puede observar en la figura D11, la saturación previa estos receptores con ácido fólico libre no produjo diferencias en la internalización de las nanopartículas funcionalizadas. Esto indica que la mayor captación de estas formulaciones no se puede atribuir a una unión de las nanopartículas funcionalizadas con los receptores de folato. De hecho, Chen y cols. demostraron que en la unión del ácido fólico a los receptores de folato tipo αlos grupos aminos de esta molécula interaccionan con aminoácidos negativos del anillo interno del receptor, y los grupos carboxilo con aminoácidos positivos del anillo externo [62]. De esta manera, las nanopartículas pueden generar un cierto impedimento estérico que dificultaría la unión de estas formulaciones a los receptores de folato, ya que el ácido fólico se une a la superficie de estos nanosistemas por el grupo amino.

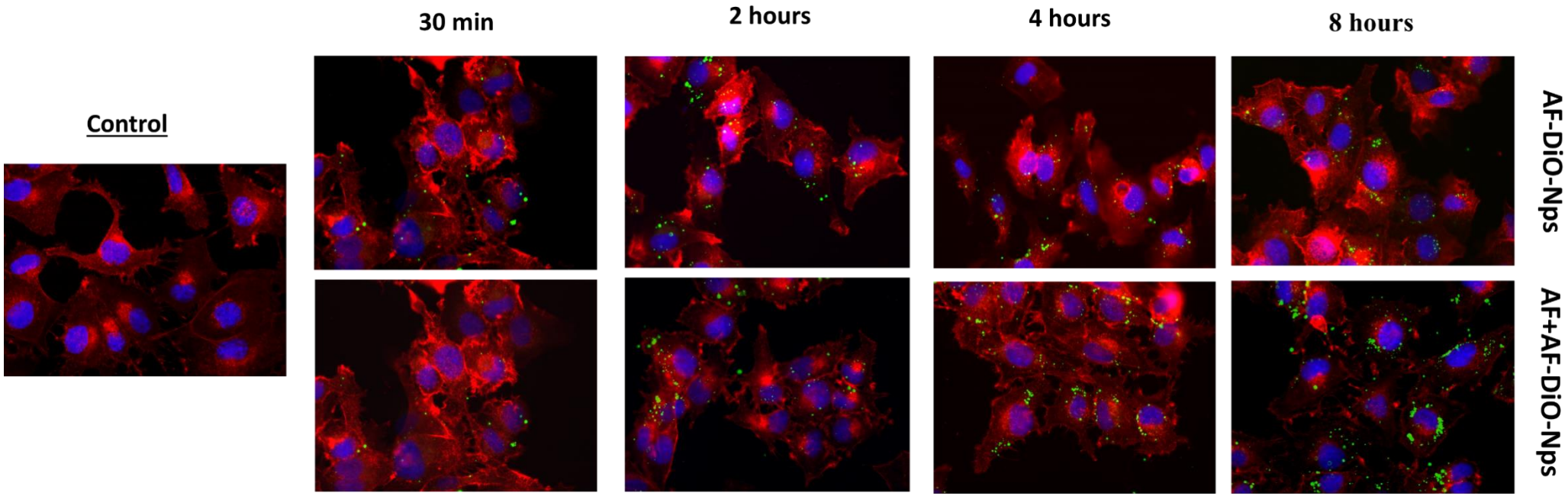

Figura D11: Estudios de internalización de las nanopartículas funcionalizadas con ácido fólico sin saturación (imágenes superiores) y con saturación previa de los receptores de folato con ácido fólico libre.

Por lo tanto, aunque los resultados de eficacia antitumoral obtenidos en los modelos *in ovo* con las nanopartículas funcionalizadas con ácido fólico desarrolladas en la presente tesis doctoral fueron buenos, no podemos determinar cual es su mecanismo de internalización en las celulas tumorales. En este sentido sería interesante modificar el protocolo de funcionalización de manera que se reduzca el posible impedimento estérico del ácido fólico unido directamente a la superficie de las nanopartículas. Esto podría conseguirse uniendo al PLGA ligandos con un grupo amino (como por ejemplo polietilenglicol di-amina). De manera que el complejo PLGA-PEG-NH_2 se uniera posteriormente al grupo carboxilo del ácido fólico, disminuyendo así el impedimento estérico. A pesar de no poder determinar el mecanismo de internalización en las células tumorales, los resultados obtenidos muestran que la nanoencapsulación resulta una buena estrategia para administrar el CBD por vía intraperitoneal, consiguiendo modificar su biodistribución al ser internalizadas en las células tumorales y, probablemente, mejorar su la eficacia antitumoral.

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CONCLUSIONES/CONCLUSIONS

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1. La expresión del sistema cannabinoide endógeno se encuentra alterada en numerosas patologías, convirtiéndose en una prometedora diana terapéutica, y por consiguiente, los cannabinoides en potenciales nuevos fármacos. Una de las patologías donde estos compuestos presentan un gran potencial es el cáncer. El Cannabidiol es uno de los cannabinoides más prometedores a este nivel, especialmente en cáncer de mama, próstata y glioma.

2. El Cannabidiol en solución presenta un efecto antiproliferativo sobre las líneas celulares MCF-7 y MDA-MB-231 de cáncer de mama y sobre la línea celular SKOV-3 de cáncer de ovario, en el rango de concentraciones estudiadas. La administración diaria de CBD a una concentración de 100µM consigue inhibir el crecimiento de tumores de ovario altamente invasivos en modelos *in vivo,* en los que el tumor se ha desarrollado sobre la membrana corioalantoidea de embriones de pollo.

3. En cáncer de ovario la combinación de cannabidiol en solución y paclitaxel resultó muy eficaz. La administración previa de este cannabinoide sensibilizó las células SKOV-3 a la acción de paclitaxel. La co-administración de ambos agentes también fue efectiva, observándose un efecto sinérgico entre ellos. En cáncer de mama, la administración previa de cannabidiol en solución sensibilizó las células MDA-MB-231 (células tumorales de mama triple negativas) y MCF-7 (células tumorales de mama positivas a los receptores de estrógenos) a la acción de doxorrubina y paclitaxel. La co-administración del cannabidiol y paclitaxel o doxorrubicina también resultó eficaz sobre estas células. Mientras que en células MCF-7 el CBD demostró un efecto sinérgico con ambos agentes antineoplásicos, en las células MDA-MB-231 se observó un efecto aditivo o sinérgico dependiendo de la concentración de paclitaxel y doxorrubicina empleadas. El cannabidiol en solución consigue reducir la concentración de estos antineoplásicos necesaria para conseguir el mismo efecto antiproliferativo.

4. La estabilidad del cannabidiol en solución se encuentra comprometida por la temperatura, la luz y la presencia de oxígeno. El disolvente utilizado influye significativamente en la estabilidad de este compuesto, siendo las soluciones acuosas altamente inestables. La estabilidad del cannabidiol en solución presenta una alta

dependencia de la temperatura de almacenamiento, habiéndose encontrado una alta correlación entre las variables de la Ecuación de Arrhenius, lo que permite hacer estimaciones de estabilidad a diferentes temperaturas con bastante fiabilidad. El cannabidiol se oxida con facilidad, y bajo la incidencia de radiaciones se desencadenan reacciones foto-oxidativas. Por todo ello, las soluciones de cannabidiol deben ser almacenadas en refrigerador y protegidas de la luz. La eliminación del oxígeno y/o el uso de antioxidantes serían recomendable para mejorar la estabilidad de este compuesto.

5. Las micropartículas de PLGA constituyen una buena herramienta tecnológica para vehiculizar el cannabidiol. Las micropartículas elaboradas con un ratio fármaco: polímero 10:100 presentaron un tamaño adecuado para ser administrados por vía parenteral, una elevada eficacia de encapsulación y una liberación prolongada del activo durante más de un mes. Almacenadas en refrigerador y protegidas de la luz fueron tanto física como químicamente estables durante al menos un año.

6. Las laboradas con un ratio fármaco: polímero 10:100 presentan una actividad antiproliferativa prolongada durante al menos 10 días, tras una única administración, tanto en células de cáncer de ovario como de mama. La administración de una única dosis de esta formulación demostró una eficacia antitumoral en el modelo *in ovo* de cáncer de ovario similar a la administración diaria de CBD en solución a la misma concentración (100µM).

7. Las micropartículas desarrolladas constituyen una buena estrategia para incrementar la actividad antitumoral del paclitaxel y la doxorrubicina. La administración previa de una sola dosis de micropartículas seguida de su co-administración con paclitaxel o doxorrubicina aumentó significativamente la eficacia antitumoral de estos agentes en modelos *in vitro* de cáncer de mama y ovario. La eficacia de la combinación de las micropartículas de cannabidiol y paclitaxel también se verificó en modelos *in ovo* de cáncer de ovario.

8. Las nanopartículas poliméricas de PLGA y cannabidiol constituyen una buena estrategia para vehiculizar este compuesto hasta la diana terapéutica. Las nanopartículas desarrolladas presentaron un tamaño de partícula inferior a 250 nm, una elevada eficacia de encapsulación y una liberación controlada del activo durante 96h. Estas nanopartículas

se internalizaron eficazmente en células SKOV-3.

9. Las nanopartículas cargadas con CBD demostraron un efecto antiproliferativo *in vitro* en las células SKOV-3 de cáncer de ovario. Administradas diariamente, a una concentración de CBD de 100 μM, inhibieron el crecimiento de los tumores de ovario desarrollados en la membrana corioalantoidea de embriones de pollo.

10. Las micropartículas y las nanopartículas poliméricas de CBD desarrolladas suponen una herramienta útil para aumentar la eficacia antitumoral del cannabidiol frente a células de cáncer de mama y de ovario, en monoterapia o en terapia de combinación con paclitaxel o doxorrubicina.

CONCLUSIONS

1. The endocannabinoid system has become a potential therapeutic target due to its altered expression in numerous pathological conditions and, as a consequence, cannabinoids in promising therapeutic agents. Cancer disease is one of the disorders where cannabinoids have a great deal of interest. In this field, cannabidiol (CBD) is one of the most promising cannabinoids, especially in prostate cancer, breast cancer and glioma.

2. In the range of tested concentrations, cannabidiol in solution has showed an antiproliferative effect in MCF-7 and MDA-MB-231 breast tumour cells and SKOV-3 ovarian cancer cells. The daily administration of CBD at a concentration of 100µM, inhibited the growth of highly invasive ovarian tumours that were formed on the chorioallantoic membrane of the chick fertilized eggs.

3. In ovarian cancer, the combination of CBD in solution and paclitaxel was effective. The previous administration of this cannabinoid sensitize SKOV-3 cells to paclitaxel activity. The co-administration of both actives was also effective, showing a synergistic effect. In breast cancer, the previous administration of CBD in solution sensitize MDA-MB-231 (triple negative breast cancer cells) and MCF-7 cells (oestrogen receptor positive breast cancer cells) to doxorubicin and paclitaxel. The co-administration of CBD and paclitaxel or doxorubicin was also effective. While in MCF-7 a synergistic effect was appreciated with both CBD plus doxorubicin and CBD plus paclitaxel combinations, in MDA-MB-231 cells an additive or synergistic effect was observed depending on the concentration of these antineoplastics.

4. The stability of CBD in solution is influenced by several factors such as temperature, light and oxygen. The solvent affects significantly the stability of this compound, being aqueous solutions highly unstable. The temperature is one of the most critical factors that compromises CBD stability. The proposed Arrhenius equation led the reliable estimation of the stability of this cannabinoid at different temperatures. Cannabidiol is highly sensitive to oxidation and the presence of light triggers photo-oxidative reactions. For these reasons, CBD solutions must be stored refrigerated and protected to light. The elimination of the oxygen and/or the use of antioxidants would be

advisable to improve the stability of this active.

5. The microencapsulation of CBD into PLGA microparticles could be a good strategy to vehiculizate this active. Microparticles prepared with a CBD:polymer ratio of 10:100 showed a suitable particle size to be administered by a parenteral route, a high :encapsulation efficiency and a controlled drug release for more than one month. Stored into the refrigerator and protected from light this formulation showed to be physical and chemical stable for at least one year.

6. Microparticles prepared with a CBD:polymer ratio of 10:100 showed an extended antiproliferative activity for at least 10 days after a single administration in breast and ovarian cancer cells. The administration of a single dose of this formulation exhibited an *in ovo* antitumor efficacy in ovarian cancer similar to the observed by the daily administration of CBD in solution at the same concentration (100 µM).

7. Developed microparticles were a good strategy to improve the antitumor activity of paclitaxel and doxorubicin. The previous administration of a single dose of microparticles followed by their co-administration with paclitaxel or doxorubicin significantly increased the antitumor efficacy of these agents in *in vitro* models of breast and ovarian cancer. The effect of the combination of CBD loaded microparticles and paclitaxel was also confirmed *in ovo* models of ovarian cancer.

8. The nanoencapsulation of cannabidiol into PLGA nanoparticles are a good strategy to vehiculizate this agent to the therapeutic target. The developed nanoparticles showed a particle size below 250 nm, a high encapsulation efficiency and a controlled drug release for 96 hours. These nanoparticles were efficiently internalized by SKOV-3 cells.

9. Nanoparticles loaded with CBD showed an *in vitro* antiproliferative effect in SKOV-3 ovarian cancer cells. This formulation, administered daily at a CBD concentration of 100 µM inhibited the growth of ovarian tumours formed on the chorioallantoic membrane of the chick fertilized eggs.

10. Polymeric micro and nanoparticles loaded with CBD were a good tool to increase the antitumor efficacy of cannabidiol against breast and ovarian cancer cells

administered either as monotherapy or as a part of a combination with paclitaxel or doxorubicin.