

Medicinal Chemistry & Drug Discovery

Design, Synthesis and Pharmacological Evaluation of *N*-Benzyl Linoleamide Analogues from *Tropaeolum tuberosum* as NF- κ B Inhibitors and Nrf2 ActivatorsLuis Apaza Ticona,^{*,[a, b]} Víctor Tena Pérez,^[a] Andreea Madalina Serban,^[c] Javier Sánchez-Corral,^[a] and Ángel Rumero Sánchez^[a]

Dedicated to Professor Tito Estévez Martini

Nineteen *N*-benzyl linoleamide analogues (macamide from *Tropaeolum tuberosum*) were synthesised, modifying the molecular structure (benzyl position, aromatic ring and carbonyl group). The structures were confirmed using nuclear magnetic resonance and mass spectrometry techniques. Cell viability and anti-inflammatory activity of these compounds were evaluated in three brain cell lines (C8-D1 A, Neuro-2a, and EOC 13.31). Regarding the effect on NF- κ B, several analogues had better inhibition potential than their natural precursor (*N*-benzyl linoleamide), being the *N*-(1-(3,5-dimethyl- α -methylbenzyl)-

linolethiamide the most active, with IC₅₀ values of 0.01 $\pm$ 0.01, 0.01 $\pm$ 0.01 and 0.02 $\pm$ 0.01 μ M, respectively. Finally, all analogues activated Nrf2, with the same compound being the most active, presenting EC₅₀ values of 0.03 $\pm$ 0.01, 0.07 $\pm$ 0.01 and 0.11 $\pm$ 0.02 nM, respectively. Analysing the link between chemical structure and pharmacological activity, our study shows that the changes made to *N*-benzyl linoleamide lead us to obtaining an analogue with greater anti-inflammatory activity than its precursor and with potential for the treatment of migraine.

1. Introduction

Migraine is a common disabling neurovascular disorder, affecting approximately 10–15% of the general population.^[1] Migraine attacks are characterised by recurrent, intense, throbbing and unilateral headaches.^[2] Clinical studies have shown that inflammation is one of the three mechanisms involved in migraine pathogenesis.^[3] These studies have concluded that the levels of cytokines in plasma, serum and/or urine are overexpressed in individuals with this condition.^[4]

Among the cytokines which are overexpressed in the inflammation process, NF- κ B (nuclear factor kappa-light-chain-enhancer activated B-cells) has an important role in iNOS (inducible nitric oxide synthase) and the control of the transcription of acute phase proteins, which include IL-1 β

(interleukin 1 β), IL-6 (interleukin 6) and TNF- α (tumour necrosis factor- α). NF- κ B shows a stimulating effect on adhesion molecules and antioxidant enzymes involved in inflammation.^[5] Thus, NF- κ B is a key target for the research on design of new anti-inflammatory drugs.

On the other hand, several studies have shown that the nuclear factor erythroid 2-related factor 2 (Nrf2) enzyme exerts a stimulating effect on heme oxygenase 1 (HO-1) and its metabolites that have important anti-inflammatory effects. In this sense, the elevation of the HO-1 expression leads to the inhibition of the NF- κ B signalling results, which in turn has an impact on inflammatory responses.^[6]

Moreover, both Nrf2 and NF- κ B are regulated by sensitive redox factors, and the absence of Nrf2 is associated with an increase in oxidative and nitrosative stress, increasing the NF- κ B production.^[7] Thus, the search for drugs that interact with the Nrf2 signalling pathway and, as a consequence, lead to detoxification through the modulation of the NF- κ B signalling pathways is an option for the treatment of migraine.

In this context, herbal remedies have long been used as an alternative in the prophylaxis and treatment of migraine disorders.^[8] In our study, we used *Tropaeolum tuberosum* Ruiz & Pavón (Tropaeolaceae), a native species from the Peruvian-Bolivian plateau, which can also be found in other South American countries, from Venezuela to northern Argentina. It grows at levels between 1500–4300 meters and at annual mean temperatures between 8–11 °C.^[9] Known as Mashua, it has different traditional medicinal uses. Our interest in studying this species relates to the use of its cooked tubers to treat

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soroche (from Quechua suruqchi or suruqch'i).^[10] Soroche, known as acute mountain sickness (AMS), is a disease that causes weakness, tiredness and, most importantly, a headache similar to migraine.^[11–12]

While its traditional use relates this plant species to treating headaches, recent pharmacological studies have reported that *T. tuberosum* tubers have antioxidant^[13] and anti-inflammatory activities.^[14] The compounds related to the anti-inflammatory activity of *T. tuberosum* have been reported as having a macamide structure (Figure 1). *N*-benzyl linoleamide is the most important, showing a higher anti-inflammatory activity when compared to other macamides.^[14] Likewise, it has been indicated that this macamide is found in a higher proportion in relation to the other macamides which were isolated from the *T. tuberosum* tubers.^[14]

The objective of the present work is to synthesise different *N*-benzyl linoleamide analogues for the treatment of migraine (anti-inflammatory activity), making modifications to the characteristic fragments of the *N*-benzyl linoleamide. For this study, we first determined the viability of *N*-benzyl linoleamide analogues in different brain cell lines. Subsequently, we measured the inhibition levels of the NF- κ B cytokine, since its overproduction after an inflammatory process contributes to the pathogenesis of the migraine. Finally, we measured the activation levels of Nrf2, since it has an important role in inflammatory damage by regulating the proinflammatory cytokines (NF- κ B).^[7]

For this analysis, we have taken into account the cells of the central nervous system involved in the pathological process of migraine, using the C8-D1 A (Astrocyte), Neuro-2a (Neuroblast) and EOC 13.31 (Microglia) cell lines. Likewise, the THP-1 cell line (Human Peripheral Blood Monocyte) was used as a negative control.

2. Results and discussion

Concerning the biosynthetic pathway of the *N*-benzyl linoleamide, it is generated as a result of three steps: first, the benzyl glucosinolate is hydrolysed by the myrosinase enzyme to produce benzyl isothiocyanate, which is then transformed in benzylamine; second, the lipids from the cell membrane and storage cells are hydrolysed into free fatty acids, and more specifically, linoleic acid; and third, the macamide is obtained through an amidation reaction between a benzylamine molecule and a linoleic acid molecule.^[15] However, during this process, other types of products (ester and thioester) can be formed, originating from the glucosinolates hydrolysis process.^[16] Figure 2 shows the biosynthesis of *N*-benzyl linolea-

mid and the ester and thioester type products. This type of products has not been described by previous authors working on *T. tuberosum*. Thus, we decided to analyse these compounds in order to determine if they have pharmacological potential for migraine treatment.

Analysing the molecular structure of *N*-benzyl linoleamide, we can differentiate three characteristic fragments (Figure 3): the fatty acid chain, the amide bond and the aromatic ring, which can be modified in a modular way to generate a series of analogues with better anti-inflammatory properties.

Regarding the isolated macamides from *Tropaeolum tuberosum*, previous studies have concluded that *N*-benzyl oleamide and *N*-benzyl linoleamide (Figure 1) inhibit the NF- κ B activation, with IC₅₀ values of 6.5 ± 0.75 ; 7.74 ± 0.19 and 8.37 ± 0.09 μ M (*N*-benzyl oleamide), and 2.28 ± 0.54 ; 3.66 ± 0.34 and 4.48 ± 0.29 μ M (*N*-benzyl linoleamide) in the CCD-1109Sk, MRC-5 and RWPE-1 cell lines, respectively.^[14] The difference in pharmacological activity between *N*-benzyl oleamide and *N*-benzyl linoleamide is due to the different number of double bonds of the fatty acid chain,^[14,17–18] being the *N*-benzyl linoleamide more active because of the additional double bond. This is because, as shown by mechanistic studies, the increase of double bonds in polyunsaturated fatty acids (PUFAs) leads to an increased activity in specific inflammatory disorders, through the interaction with the signalling pathway of JAK-STAT3 (Janus kinases/signal transducer and activator of transcription 3).^[19]

By performing a computational prediction of *N*-benzyl linoleamide with the SwissTargetPrediction program, we observed that this macamide exhibits increased molecule-protein interaction with cannabinoid receptors (CB1 and CB2). This result is coherent since *N*-benzyl linoleamide has an endocannabinoid type structure. This type of compounds inhibits fatty acid amide hydrolase (FAAH), which is the responsible for the degradation of endogenous endocannabinoids (anandamide), generating inflammation, pain and various disorders in the central nervous system.^[20–21] The main characteristic of these endocannabinoids is their long chain of polyunsaturated fatty acids (arachidonic acid) derived from membrane phospholipids.^[22–25] In this sense, the fatty acid chain of *N*-benzyl linoleamide is very important for NF- κ B inhibition and Nrf2 activation.

Therefore, we proceeded by modifying the amide bond and the aromatic ring of the *N*-benzyl linoleamide. *N*-benzyl linoleamide was synthesised from linoleoyl chloride and benzylamine (Scheme 1), that we used as a reference whose pharmacological activity was compared with that of the different analogues.

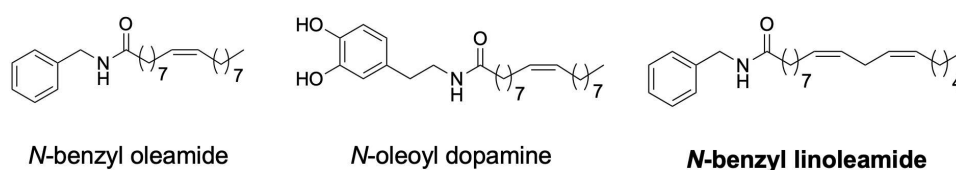


Figure 1. Macamides isolated from *T. tuberosum* tubers.

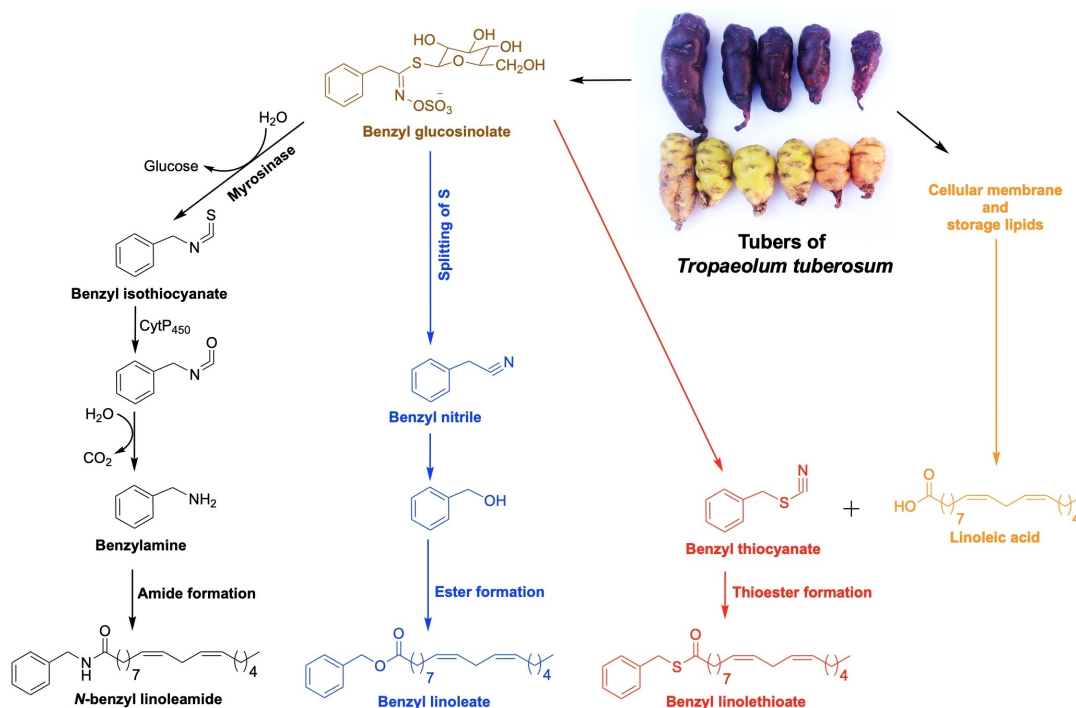


Figure 2. Biosynthetic scheme for *N*-benzyl linoleamide and other products.

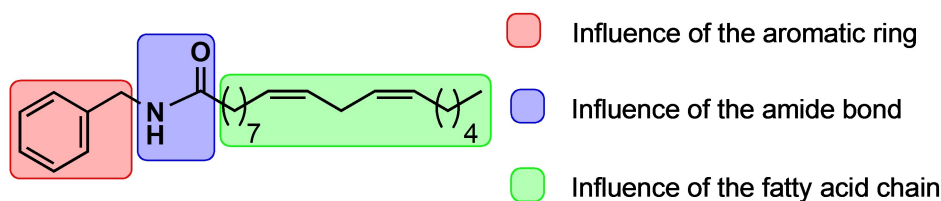
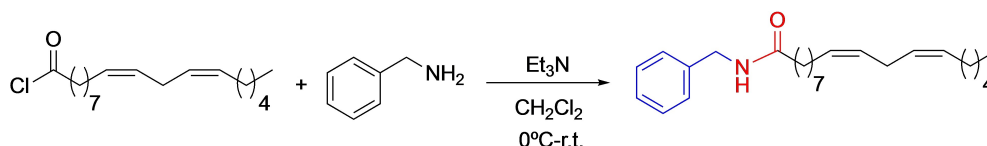


Figure 3. General structure of the *N*-benzyl linoleamide and the positions which were explored in this study.

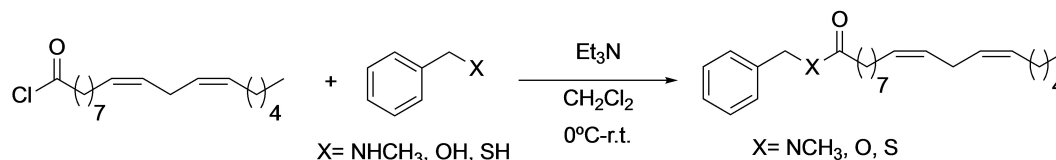


Scheme 1. Synthesis of the *N*-benzyl linoleamide.

Subsequently, we proceeded to analyse how the hydrogen of the amide of the *N*-benzyl linoleamide influences the pharmacological activity, synthesising its analogues: nitrogen-methylated (1), benzyl ester (2) and benzyl thioester (3) (Scheme 2).

Analysing the viability assay (XTT), we observed that analogues 1, 2 and 3 showed a decrease in cell viability when compared to the *N*-benzyl linoleamide that showed a CC_{50} between 90.11–93.48 μM (statistically significant, $***p < 0.001$). This decrease is partially due to the higher lipophilic capacity (cLogP, *n*-octanol/water partition coefficients) of these analogues compared to the *N*-benzyl linoleamide (Table 1).

Regarding the inhibition of the production of NF- κ B, data revealed that the tertiary amide analogue 1 showed an IC_{50} between 6–11.16 μM which is similar to *N*-benzyl linoleamide that showed an IC_{50} between 8.39–11.71 μM (statistically insignificant, $^{ns}p > 0.999$). This is due to the lipophilic capacity of analogue 1 (8.11) which is similar to the lipophilic capacity of *N*-benzyl linoleamide (7.96). In the case of the substitution of NH from the amide bond by other heteroatoms such as oxygen and sulfur, analogues 2 and 3 had a statistically significant higher NF- κ B inhibition potential than *N*-benzyl linoleamide ($***p < 0.001$), and analogue 3 had a better IC_{50} than analogue 2, between 3.8–7.05 μM (Table 2).

Scheme 2. Synthesis of *N*-benzyl linoleamide analogues 1–3.

In the Nrf2 activation assay, analogue 1 had an EC₅₀ between 27.25–38.58 nM, that is statically similar (^{ns}*p* > 0.999) to *N*-benzyl linoleamide, with an EC₅₀ between 34.66–44.33 nM. Concerning analogues 2 and 3, they showed a higher activation of Nrf2 (statistically significant, ****p* < 0.001), analogue 3 being better, with an EC₅₀ between 17.91–28.08 nM. Thus, the lipophilic capacity of the analogues influences their ability to activate Nrf2 (Table 3).

Reviewing previous works on these analogues (1–3), we found that previous authors have obtained analogue 1 synthetically and have concluded that it can be used for the reduction of blood cholesterol concentration.^[26] Regarding analogue 2, it was isolated from plant species such as *Albizia procera*,^[27] *Aloe vera*,^[28] *Laurencia brandenii*,^[29] and *Trigonella foenum grecum*,^[30] showing antimicrobial activity. Finally, there is no report concerning analogue 3 (its isolation, chemical synthesis or pharmacological activity).

Analysing our results, we can conclude that, although the substitution of the NH group from the amido bond of *N*-benzyl linoleamide by other groups or heteroatoms improves the anti-inflammatory activity (inhibition of NF-κB and activation of Nrf2), these substitutions cause also a decrease in cell viability both in the brain cell lines and the control cell line (THP-1). Therefore, we decided to proceed by carrying out the modifications in the aromatic ring.

Regarding the influence of the benzyl fragment on the *N*-benzyl linoleamide, linoleoyl chloride was reacted with the amines hydrocinanamyamine (*n* = 3), phenethylamine (*n* = 2) and aniline (*n* = 0) (Scheme 3).

Through the XTT assay, it was determined that analogues 4 and 5 decreased the viability of brain cell lines when compared to *N*-benzyl linoleamide (****p* < 0.001), showing CC₅₀ values between 47.79–56.81 μM and 58.85–65.22 μM, respectively. Only analogue 6, with a CC₅₀ between 90.03–94.1 μM, did not show cytotoxicity when compared to the *N*-benzyl linoleamide (^{ns}*p* > 0.999) (Table 1).

Regarding the anti-inflammatory activity, it was observed in the case of analogue 4 that the introduction of a propyl linker

causes a statistically significant (****p* < 0.001) increase in the inhibition of NF-κB production when compared to the *N*-benzyl linoleamide, showing an IC₅₀ between 3.3–4.87 μM (Table 2). Likewise, analogue 4 generated a higher activation of Nrf2 than *N*-benzyl linoleamide (****p* < 0.001), showing an EC₅₀ between 15.95–21.7 nM (Table 3).

However, when an ethyl linker (analogue 5) was introduced, a decrease in the inhibition of NF-κB production was observed, with IC₅₀ values between 8.3–10.19 μM and a decrease in the Nrf2 activation with EC₅₀ values between 32.19–38.34 nM (statistically significant, ****p* < 0.001 when compared to analogue 4), but these activities were similar to that of *N*-benzyl linoleamide (there is no statistically significant difference, ^{ns}*p* > 0.999) (Table 2 and 3). Finally, when aniline was used as a precursor to form analogue 6, a drastic decrease in pharmacological activity was observed, showing IC₅₀ values between 16.59–19.49 μM (inhibition of NF-κB) and EC₅₀ values between 52.35–63.55 nM (activation of Nrf2) (statistically significant, ****p* < 0.001, when compared to the *N*-benzyl linoleamide) (Tables 2 and 3).

Analysing the *n*-octanol/water partition coefficients (cLogP) of the analogues 4–6, a relationship between lipophilicity and pharmacological activity was observed, since as lipophilicity increases there is an improvement in the pharmacological activity.^[31] In this sense, analogue 4 with a lipophilic capacity of 8.6 showed a higher activity compared to the other analogues. Also, the increase in activity can be justified by the electronic distribution, in which nitrogen, being a strong activator, modifies the electronic distribution of the aromatic ring and, consequently, its interaction.^[32]

Reviewing previous works on these analogues (4–6), we found that analogues 4 and 5 were synthesised by previous authors, showing inhibitory activity *in vitro* against the MCF-7 breast cancer cells.^[34] Regarding analogue 6, there are reports on its relationship with the toxic oil syndrome (in 1981, in Spain, there was a multisystemic epidemic outbreak caused by the ingestion of a mixture of denatured cooking oil with aniline).^[35]

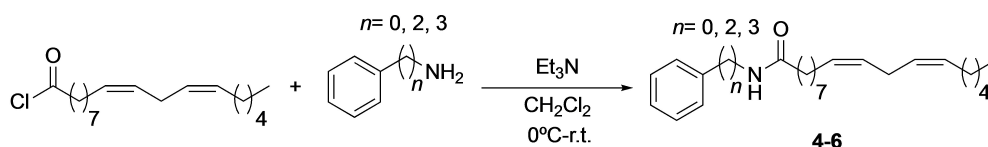
Scheme 3. Synthesis of *N*-benzyl linoleamide analogues 4–6.

Table 1. Viability of the different cell lines after treatment with synthetic *N*-benzyl linoleamide analogues.

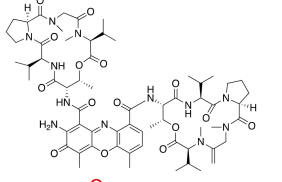
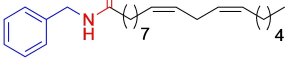
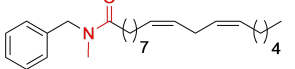
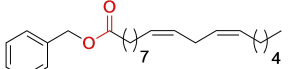
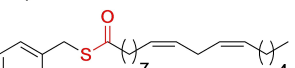
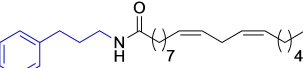
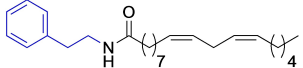
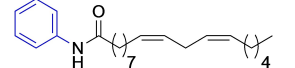
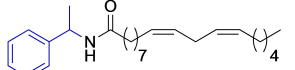
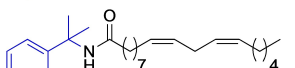
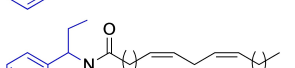
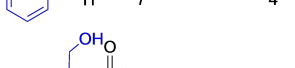
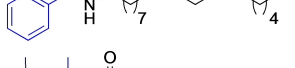
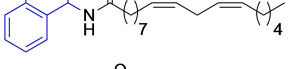
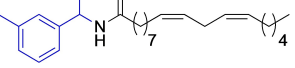
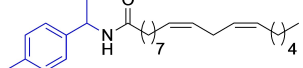
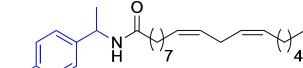
N°	Structure	Viability (CC ₅₀ μM) ^b				cLogP ^c	Yield (%)
		THP-1	C8-D1 A	Neuro-2a	EOC 13.31		
–	Untreated cells	99.46 ± 0.39	95.88 ± 0.77	97.56 ± 0.58	98.85 ± 0.45	–	–
Actinomycin D ^a		0.0083 ± 0.00047	0.0080 ± 0.00067	0.0082 ± 0.00059	0.0085 ± 0.00054	–4.03	–
A		95.07 ± 0.28	90.11 ± 0.12	91.88 ± 0.89	93.48 ± 0.88	7.96	50
1		85.17 ± 0.15	72.52 ± 0.62	73.73 ± 0.90	76.30 ± 0.80	8.11	65
2		83.52 ± 0.93	66.79 ± 0.72	67.51 ± 0.74	69.31 ± 0.20	8.10	80
3		72.72 ± 0.13	55.92 ± 0.62	58.38 ± 0.75	59.39 ± 0.93	8.17	76
4		69.81 ± 0.27	47.79 ± 0.84	52.27 ± 0.79	56.81 ± 0.19	8.60	78
5		78.49 ± 0.62	58.85 ± 0.48	59.56 ± 0.63	65.22 ± 0.55	8.28	70
6		96.72 ± 0.58	90.03 ± 0.33	90.70 ± 0.57	94.10 ± 0.93	7.82	73
7		95.41 ± 0.08	90.36 ± 0.39	91.80 ± 0.61	93.86 ± 0.49	8.20	73
8		76.71 ± 0.68	58.90 ± 0.13	59.51 ± 0.53	64.90 ± 0.23	8.33	80
9		79.17 ± 0.93	62.52 ± 0.92	63.47 ± 0.39	67.81 ± 0.58	8.29	69
10		99.65 ± 0.85	91.12 ± 0.50	91.91 ± 0.42	98.27 ± 0.80	7.29	77
11		93.86 ± 0.33	80.70 ± 0.27	83.71 ± 0.39	85.82 ± 0.94	8.20	77
12		94.59 ± 0.07	86.23 ± 0.18	86.46 ± 0.85	87.73 ± 0.59	8.31	77
13		99.95 ± 0.28	87.80 ± 0.82	88.27 ± 0.38	88.97 ± 0.67	8.31	80
14		98.41 ± 0.25	80.23 ± 0.19	82.17 ± 0.21	84.01 ± 0.85	8.00	69
15		96.27 ± 0.51	78.30 ± 0.63	81.65 ± 0.48	83.66 ± 0.18	8.14	83

Table 1. continued

Table 1. Continued							
N°	Structure	Viability (CC ₅₀ μM) ^b			cLogP ^c	Yield (%)	
		THP-1	C8-D1 A	Neuro-2a			
16		66.69 ± 0.61	48.81 ± 0.18	52.40 ± 0.42	58.20 ± 0.15	8.52	73
17		94.89 ± 0.38	83.30 ± 0.42	83.45 ± 0.20	85.70 ± 0.21	8.50	75
18		99.38 ± 0.91	88.26 ± 0.63	90.45 ± 0.16	93.70 ± 0.32	8.31	99
19		92.81 ± 0.38	83.17 ± 0.66	83.78 ± 0.15	85.38 ± 0.73	8.75	93

a = Actinomycin D (positive control). b = CC₅₀: cytotoxicity concentration 50%. b = values of cLogP were calculated by DataWarrior.^[33] The results are the means (±SD) of three separate experiments performed in triplicate. Significant diff. among means (***) $p < 0.001$ /Tukey's multiple comparisons test.

Analysing these previous references on the results of this group of analogues (4–6), we decided to discard them for future trials, since although analogue 4 showed higher pharmacological activity (inhibition of NF-κB and activation of Nrf2), it also showed higher cytotoxicity (statistically significant, *** $p < 0.001$). Regarding analogue 5, the exchange of the methyl linker of the *N*-benzyl linoleamide by an ethyl linker did not show a significant change in the pharmacological activity (inhibition of NF-κB and activation of Nrf2), therefore it was ruled out. Finally, although analogue 6 did not decrease cell viability, the reports on its toxicity and the data obtained in our own experiments (inhibition of NF-κB and activation of Nrf2) led us to the conclusion to discard it from further experiments.

Subsequently, we analysed the influence of introducing a methyl group (analogue 7), gem-dimethyl group (analogue 8), an ethyl group (analogue 9) and a hydroxyethyl group (analogue 10) in the benzyl position of the *N*-benzyl linoleamide. Table 1 shows that only analogues 8 and 9 decreased viability, with IC₅₀ of 58.9–64.9 μM and 62.52–67.81 μM, respectively (statistically significant, *** $p < 0.001$, when compared to the *N*-benzyl linoleamide). This effect is due to the lipophilic capacity of these analogues, which is similar for analogue 8 (8.33) and analogue 9 (8.29).

Analogues 7, 8 and 9 showed similar pharmacological activity (no statistically significant difference, ^{ns} $p > 0.999$), with IC₅₀ values of 4.1–7.27 μM; 3.45–7.18 μM and 3.74–7.19 μM, respectively, for the inhibition of NF-κB, and EC₅₀ values of 18.95–27.48 nM; 16.68–24.38 nM and 17.26–25.33 nM, respectively, for the activation of Nrf2. Finally, although analogue 10 did not show cytotoxicity in any of the brain cell lines (CC₅₀ values between 91.12–98.27 μM), it was the molecule with the worst pharmacological activity, with IC₅₀ values between 33.63–41.95 μM (inhibition of NF-κB) and EC₅₀ values between 69.86–84.8 nM (Table 2 and 3).

Analysing this group of analogues, we observed that the introduction of a methyl group in the benzyl position of the *N*-

benzyl linoleamide improved significantly the pharmacological activity and did not decrease cell viability. This result helped us to conclude that the geometric distribution of analogue 7 has an important influence on anti-inflammatory activity (NF-κB inhibition and Nrf2 activation). This is because by introducing a methyl group in the benzyl methylene bridge, a stereocenter is generated that can give rise to two enantiomers, with relevance in pharmacomodulation, due to the specificity of pharmacological targets towards a specific enantiomer or towards the combination of both enantiomers.^[36–38] In our case, there are studies that indicate that the *S* enantiomer of analogue 7 showed better anti-tuberculosis activity through the inhibition of *Mycobacterium tuberculosis* (statistically significant) compared to its *R* enantiomer.^[39] Likewise, the generation of stereocenters was also observed in analogues 8, 9 and 10, however there are no reports on the isolation, synthesis or activity of analogue 8. Regarding analogue 9, previous authors mentioned that it has cholesterol-lowering activity, but it is not confirmed whether this activity is provided by one of the enantiomers or the mixture of both.^[40] Finally, concerning analogue 10, previous researchers mentioned that its *S* enantiomer showed activity in the treatment of obesity and related disorders, and in the improvement of cognition.^[41] Analysing these data, we can conclude that the *S* enantiomers of our analogues, and especially of analogue 7, are responsible for their pharmacological activity.

Based on these results, we decided to analyse how the introduction of a methyl group (electron donating groups, EDG)^[42–43] in the *ortho* (11), *meta* (12) and *para* (13) positions in the aromatic ring of the analogue 7 influences the pharmacological activity. Our results show that the substitutions in the *meta* (analogue 12) and *para* (analogue 13) positions with the methyl group led to a slight decrease in cell viability, showing CC₅₀ values between 86.23–87.73 μM and 87.8–88.97 μM, respectively (not significant statistically when compared to analogue 7, ^{ns} $p > 0.999$) (Table 1). Regarding analogue 11, it

Table 2. Inhibition of NF- κ B production in different cell lines after treatment with synthetic *N*-benzyl linoleamide analogues.

N°	Structure	NF- κ B inhibition (IC ₅₀ μ M) ^b		Neuro-2a	EOC 13.31	cLogP ^c	Yield (%)
		THP-1	C8-D1 A				
–	Untreated cells	19.82 $\pm$ 0.29	18.19 $\pm$ 0.98	19.36 $\pm$ 0.26	19.54 $\pm$ 0.73	–	–
Celastrol ^a		7.47 $\pm$ 0.23	7.36 $\pm$ 0.34	7.39 $\pm$ 0.51	7.41 $\pm$ 0.11	7.08	–
A		12.51 $\pm$ 0.58	8.39 $\pm$ 0.73	8.80 $\pm$ 0.77	11.71 $\pm$ 0.59	7.96	50
1		13.63 $\pm$ 0.32	6.00 $\pm$ 0.66	6.24 $\pm$ 0.92	11.16 $\pm$ 0.49	8.11	65
2		11.60 $\pm$ 0.53	5.75 $\pm$ 0.63	5.95 $\pm$ 0.86	9.04 $\pm$ 0.35	8.10	80
3		8.49 $\pm$ 0.06	3.80 $\pm$ 0.07	4.19 $\pm$ 0.05	7.05 $\pm$ 0.90	8.17	76
4		5.43 $\pm$ 0.90	3.30 $\pm$ 0.13	3.55 $\pm$ 0.35	4.87 $\pm$ 0.51	8.60	78
5		12.36 $\pm$ 0.36	8.30 $\pm$ 0.46	8.47 $\pm$ 0.20	10.19 $\pm$ 0.56	8.28	70
6		25.61 $\pm$ 0.76	16.59 $\pm$ 0.28	16.72 $\pm$ 0.78	19.49 $\pm$ 0.71	7.82	73
7		9.61 $\pm$ 0.28	4.10 $\pm$ 0.77	4.50 $\pm$ 0.36	7.27 $\pm$ 0.77	8.20	73
8		8.98 $\pm$ 0.37	3.45 $\pm$ 0.59	3.51 $\pm$ 0.20	7.18 $\pm$ 0.93	8.33	80
9		9.24 $\pm$ 0.81	3.74 $\pm$ 0.69	3.80 $\pm$ 0.99	7.19 $\pm$ 0.64	8.29	69
10		45.65 $\pm$ 0.12	33.63 $\pm$ 0.45	34.33 $\pm$ 0.78	41.95 $\pm$ 0.66	7.29	77
11		0.46 $\pm$ 0.02	0.23 $\pm$ 0.06	0.24 $\pm$ 0.05	0.39 $\pm$ 0.05	8.20	77
12		0.41 $\pm$ 0.01	0.10 $\pm$ 0.02	0.16 $\pm$ 0.05	0.31 $\pm$ 0.01	8.31	77
13		0.35 $\pm$ 0.03	0.13 $\pm$ 0.02	0.19 $\pm$ 0.08	0.27 $\pm$ 0.09	8.31	80
14		1.16 $\pm$ 0.05	0.49 $\pm$ 0.05	0.53 $\pm$ 0.04	0.80 $\pm$ 0.03	8.00	69
15		0.69 $\pm$ 0.04	0.38 $\pm$ 0.39	0.45 $\pm$ 0.07	0.61 $\pm$ 0.05	8.14	83

Table 2. continued

N°	Structure	NF-κB inhibition (IC ₅₀ μM) ^b		Neuro-2a	EOC 13.31	cLogP ^c	Yield (%)
		THP-1	C8-D1 A				
16		0.30 ± 0.02	0.09 ± 0.05	0.13 ± 0.09	0.17 ± 0.02	8.52	73
17		0.26 ± 0.05	0.07 ± 0.01	0.11 ± 0.03	0.17 ± 0.05	8.50	73
18		0.09 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.001	8.31	99
19		0.08 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	8.75	93

a = Celastrol (positive control). b = IC₅₀: half maximal inhibitory concentration. c = values of cLogP were calculated by DataWarrior.^[33] The results are the means (± SD) of three separate experiments performed in triplicate. Significant diff. among means (***) $p < 0.001$ /Tukey's multiple comparisons test.

showed a statistically significant decrease in cell viability compared to analogue 7 (***) $p < 0.001$, with a CC₅₀ between 80.7–85.82 μM (Table 1).

Concerning pharmacological activity, analogues 11, 12 and 13 showed an IC₅₀ between 0.23–0.39 μM; 0.1–0.31 μM and 0.13–0.27 μM, respectively (inhibition of NF-κB production) and an EC₅₀ between 1.48–1.76 nM; 0.41–0.74 nM and 0.47–0.76 nM, respectively (Nrf2 activation). In both cases, the results were statistically significant compared to analogue 7 (***) $p < 0.001$ (Table 2 and 3).

Performing a review of previous work on these analogues, we found that analogues 11, 12, and 13 were reported as having cholesterol-lowering activity.^[41] However, it is unknown whether this activity is provided by one of the enantiomers or the mixture of both.

Based on our results, we decided to make substitutions with strong and moderate EDG^[44] in the *para* position (to prevent the steric effect), obtaining analogues 14 and 15. The introduction of strong (–OH) and moderate (–OCH₃) EDG showed a statistically significant decrease in cell viability (***) $p < 0.001$, compared to analogue 13, with CC₅₀ values between 80.23–84.01 μM and 78.3–83.66 μM, respectively (Table 1). Likewise, a decrease happened in the pharmacological activity of analogues 14 and 15 (statistically significant, ***) $p < 0.001$, when compared to analogue 13, showing IC₅₀ values between 0.49–0.8 μM and 0.38–0.61 μM (inhibition of NF-κB production) and EC₅₀ values between 2.59–2.72 nM and 1.03–1.6 nM (Nrf2 activation) (Table 2 and 3).

To corroborate whether weak EDG are better substituents than strong EDG, we introduced an ethyl group (weak EDG) in the *para* position (analogue 16), that allowed us to observe the effects on cell viability and pharmacological activity. The results show that analogue 16 decreases cell viability, showing CC₅₀ values between 48.81–58.2 μM (statistically significant, ***) $p < 0.001$, when compared to analogue 13 (Table 1). This effect is

due to the fact that analogue 16 has a higher lipophilic capacity (8.52) than the analogue 13 (8.31). However, concerning pharmacological activity, an improvement was observed in the inhibition of NF-κB production with IC₅₀ values between 0.09–0.17 μM and in the activation of Nrf2 with EC₅₀ values between 0.15–0.19 nM (statistically significant, ***) $p < 0.001$, when compared to analogue 13 (Table 2 and 3).

There are no previous reports on the isolation of analogues 14, 15 and 16 that would allow us to compare our results with those obtained by previous researchers.

Based on these results, we decided to study the effect of di-substitution starting from the structural analysis of the natural *N*-oleoyldopamine macamides isolated from the *T. tuberosum* tubers. This macamide inhibited the TNF-α cytokine in THP-1 cells stimulated by lipopolysaccharides, with an IC₅₀ of 3.54 ± 0.02 μM, through down-regulation of NF-κB.^[17] Analysing the structure of this macamide, we observe that it has –OH substituents in the *meta* and *para* positions of the aromatic ring (Figure 1). In this sense, based on our results on the weak EDG substituents and the information on the natural *N*-oleoyldopamine macamide, we synthesised analogue 17 (which presents methyl groups in the *meta* and *para* positions in the aromatic ring).

The results from the cell viability tests showed that there is no statistically significant difference (ⁿ $p > 0.999$) between analogue 17 (CC₅₀ between 83.3–85.7 μM) and analogue 13 (Table 1). In relation to the anti-inflammatory activity (inhibition of NF-κB and activation of Nrf2), analogue 17 showed a statistically significant difference (***) $p < 0.001$, compared to analogue 13. This effect is due to the fact that the lipophilic capacity of analogue 17 (8.5) is higher than that of analogue 13 (8.31) (Table 2 and 3). This cannot be compared to previous works because there is no prior report on analogue 17.

Finally, the next step was to analyse the importance of the acyl group for the pharmacological activity. We decided to

Table 3. Activation of Nrf2 in different cell lines after treatment with synthetic *N*-benzyl linoleamide analogues.

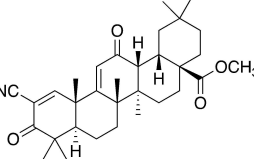
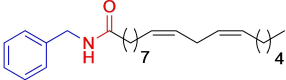
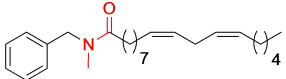
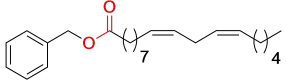
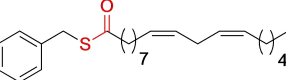
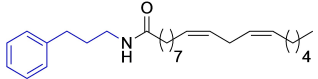
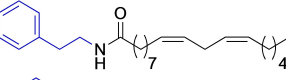
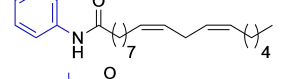
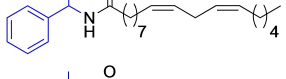
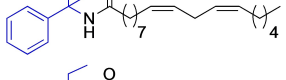
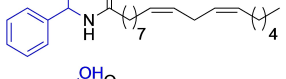
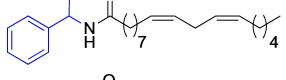
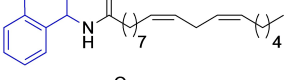
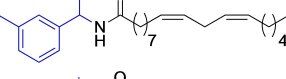
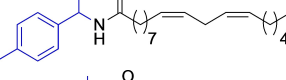
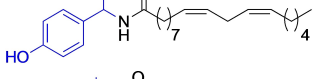
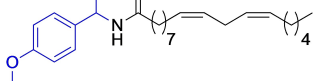
N°	Structure	Nrf2 activation (EC ₅₀ nM) ^b		Neuro-2a	EOC 13.31	cLogP ^c	Yield (%)
		THP-1	C8-D1 A				
–	Untreated cells	1.94 ± 0.28	1.17 ± 0.54	1.57 ± 0.11	1.59 ± 0.40	–	–
CDDO Methyl Ester ^a		0.13 ± 0.02	0.11 ± 0.02	0.11 ± 0.01	0.12 ± 0.03	6.22	–
A		55.17 ± 0.69	34.66 ± 0.96	35.24 ± 0.61	44.33 ± 0.56	7.96	50
1		47.83 ± 0.51	27.25 ± 0.13	27.50 ± 0.12	38.58 ± 0.75	8.11	65
2		42.18 ± 0.67	21.83 ± 0.09	25.45 ± 0.02	33.23 ± 0.67	8.10	80
3		36.40 ± 0.73	17.91 ± 0.94	18.58 ± 0.98	28.08 ± 0.79	8.17	76
4		32.76 ± 0.86	15.95 ± 0.14	16.05 ± 0.71	21.70 ± 0.58	8.60	78
5		44.04 ± 0.32	32.19 ± 0.19	32.30 ± 0.23	38.34 ± 0.11	8.28	70
6		73.44 ± 0.69	52.35 ± 0.44	54.60 ± 0.21	63.55 ± 0.52	7.82	73
7		37.55 ± 0.57	18.95 ± 0.53	20.75 ± 0.95	27.48 ± 0.45	8.20	73
8		32.51 ± 0.73	16.68 ± 0.64	17.62 ± 0.12	24.38 ± 0.98	8.33	80
9		39.13 ± 0.40	17.26 ± 0.97	19.10 ± 0.50	25.33 ± 0.95	8.29	69
10		99.89 ± 0.13	69.86 ± 0.35	70.33 ± 0.95	84.80 ± 0.79	7.29	77
11		2.09 ± 0.07	1.48 ± 0.44	1.50 ± 0.03	1.76 ± 0.21	8.20	77
12		0.91 ± 0.03	0.41 ± 0.02	0.44 ± 0.06	0.74 ± 0.01	8.31	77
13		0.92 ± 0.05	0.47 ± 0.02	0.49 ± 0.05	0.76 ± 0.89	8.31	80
14		2.98 ± 0.01	2.59 ± 0.09	2.60 ± 0.23	2.72 ± 0.95	8.00	69
15		1.88 ± 0.02	1.03 ± 0.35	1.15 ± 0.22	1.60 ± 0.66	8.14	83

Table 3. continued

N°	Structure	Nrf2 activation (EC ₅₀ nM) ^b		Neuro-2a	EOC 13.31	cLogP ^c	Yield (%)
		THP-1	C8-D1 A				
16		0.26 ± 0.01	0.15 ± 0.02	0.16 ± 0.02	0.19 ± 0.005	8.52	73
17		1.49 ± 0.05	0.31 ± 0.03	0.33 ± 0.03	0.62 ± 0.17	8.50	75
18		0.67 ± 0.06	0.22 ± 0.02	0.25 ± 0.05	0.30 ± 0.95	8.31	99
19		0.25 ± 0.07	0.03 ± 0.01	0.07 ± 0.01	0.11 ± 0.02	8.75	93

a = 2-Cyano-3,12-dioxo-oleana-1,9-(11)-dien-28-oic acid methyl ester (CDDO Methyl Ester, positive control). b = EC₅₀: half maximal effective concentration. c = values of cLogP were calculated by DataWarrior.^[33] The results are the means (± SD) of three separate experiments performed in triplicate. Significant diff. among means (***) *p* < 0.001/Tukey's multiple comparisons test.

replace the carbonyl group with a thiocarbonyl group, based on the references in the literature showing that by-products which are formed during the biosynthesis of the *N*-benzyl linoleamide have sulphur as their characteristic element in most cases.^[45] For this purpose, linolethiamides **18** and **19** were synthesised from the reaction of *N*-benzyl linoleamide analogues (**7** and **17**) and Lawesson's reagent (Scheme 4). These linolethiamides have not been isolated previously from any plant species, nor synthesised by previous researchers.

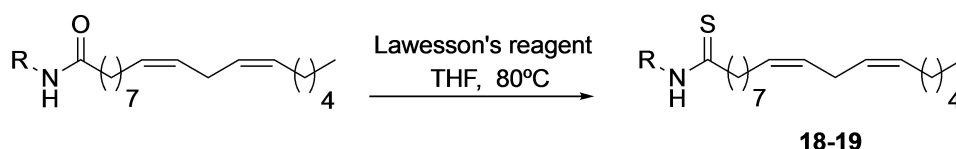
The change of the carbonyl group of analogues **7** and **17** to generate analogues **18** and **19** that present a thiocarbonyl group showed a slight decrease in cell viability (not significant statistically, ^{ns}*p* > 0.999). Concerning anti-inflammatory activity, these analogues showed a statistically significant increase (***) *p* < 0.001 in the inhibition of NF-κB (IC₅₀ of 0.03–0.04 μM and 0.01–0.02 μM, respectively) and in the activation of Nrf2 (EC₅₀ of 0.22–0.3 nM and 0.03–0.11 nM, respectively).

This effect can be justified by the lipophilic capacity, since analogues **18** and **19** showed a cLogP of 8.31 and 8.75, which is higher than that of analogues **7** and **17** that had a cLogP of 8.2 and 8.5. Moreover, the thioamide bond has higher electro-negativity than the amide bond, which allows it to adapt to the active centre of the target more easily.

Moreover, the inhibitory capacity of TNF-α-induced NF-κB by means of the analogues, especially analogue **19**, is due to

direct inhibition of the enzyme kinase IκB (largely to the IκBβ subunit).^[17,46] This mechanism is similar to the positive control (Celastrol) that blocks the IκB activity, nuclear translocation and NF-κB activation.^[47] Regarding the Nrf2 activation, our results indicate that *N*-benzyl linoleamide analogues act by inhibiting the Kelch-like ECH-associated protein 1 (KEAP1) enzyme.^[48] Since analogues are highly electrophilic molecules, they covalently modify the cysteine groups present in the KEAP1 enzyme,^[49–50] preventing the KEAP1-Nrf2 complex from forming and Nrf2 from ubiquitinating. This mechanism is similar to the positive control (CDDO Methyl Ester) that causes an electrophilic modification of KEAP1-Cys-151.^[51]

Both Nrf2 and NF-κB are regulated by sensitive redox factors and the absence of Nrf2 is associated with increased oxidative stress, leading to overproduction of NF-κB.^[52] This overproduction of NF-κB has been linked to diseases that present inflammatory processes.^[53] Various studies have shown that there is a relationship between Nrf2 and NF-κB indicating that the regulation of redox homeostasis by Nrf2 modulates the activity of NF-κB.^[54] In this sense, our results show that *N*-benzyl linoleamide analogues act by inhibiting NF-κB and activating Nrf2. Figure 4 shows the possible mechanism of action of analogues on NF-κB and Nrf2, based on previous studies that mention the interaction of these aryl amides with proinflammatory cytokines.^[46,55–59]

Scheme 4. Synthesis of the linolethiamides **18** and **19**.

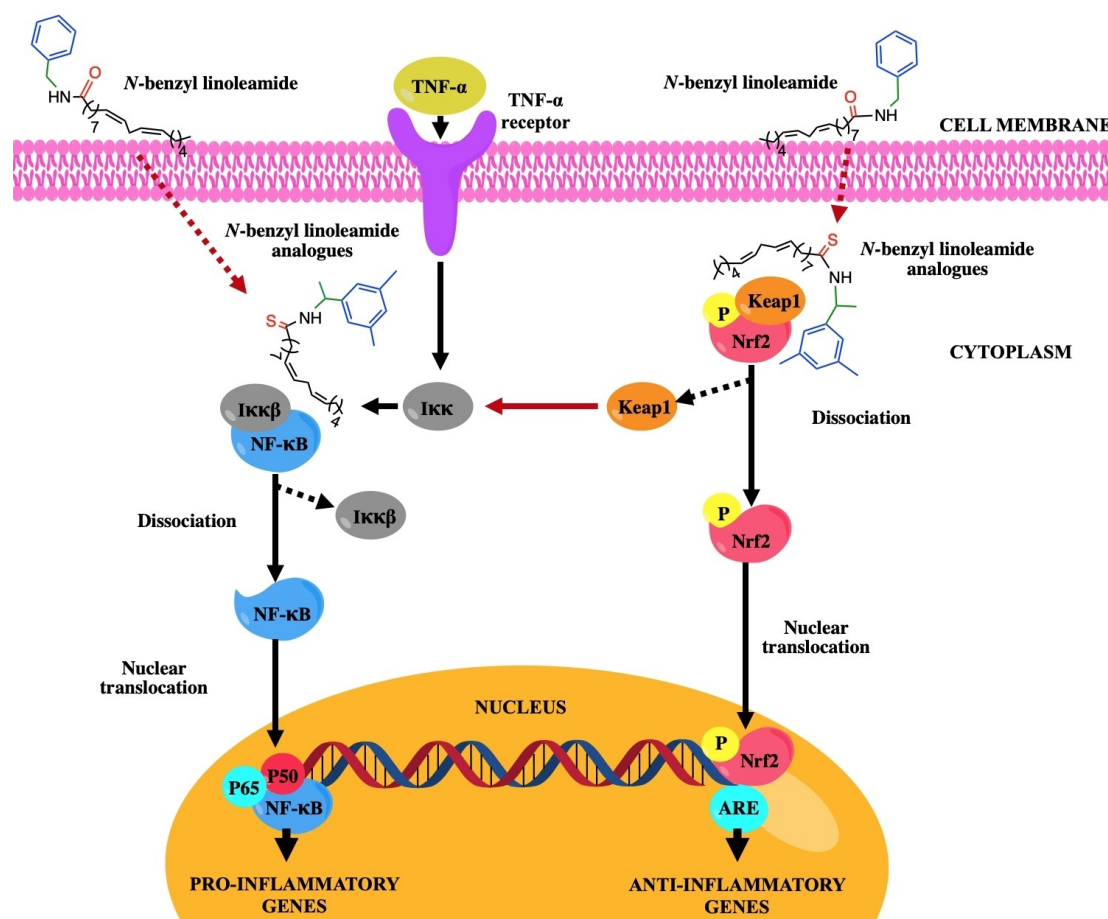


Figure 4. Mechanism of NF- κ B inhibition and Nrf2 activation of *N*-benzyl linoleamide and its analogue 19.

3. Conclusion

Through pharmacological experiments, we have shown that *N*-benzyl linoleamide analogues have better anti-inflammatory properties in the pathophysiological process of migraine than *N*-benzyl linoleamide (natural macamide present in the tubers of *T. tuberosum*). The inhibition of NF- κ B and the activation of Nrf2 in C8-D1 A, Neuro-2a and EOC 13.31 cells by means of these analogues has been tested.

Likewise, it was observed that the lipophilic capacity of these *N*-benzyl linoleamide analogues is one of the important factors that determines their anti-inflammatory activity. Our study showed that an increase in the lipophilic capacity of these analogues causes an increase in anti-inflammatory activity (acting on the proinflammatory cytokines NF- κ B and Nrf2). More specifically, in the case of analogue 19, this anti-inflammatory potential was three thousand times better than *N*-benzyl linoleamide. However, it should be mentioned that this promising anti-inflammatory activity may be due to its *S* enantiomer, as mentioned in the cases of analogues 7 and 10. In this sense, future research on this type of compounds should be oriented towards the analysis of their enantioselectivity on determined pharmacological targets.

Supporting Information Summary

^1H - and ^{13}C -NMR spectra of the *N*-benzyl linoleamide analogues analysed in this study are provided as supporting information (Figures 1S–40S).

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Macamides • NF- κ B • Nrf2 • Synthesis • *Tropaeolum tuberosum*

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