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New phenolic cinnamic acid derivatives as selective COX-2 inhibitors. Design, synthesis, biological activity and structure-activity relationships

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ABSTRACT

Selective inhibition of cyclooxygenase (COX)-2 enzyme is an important achievement when looking for potent anti-inflammatory agents, with fewer gastrointestinal side effects. In this work, a new series of cinnamic acid derivatives, namely hexylamides, have been designed, synthesized and evaluated in human blood for their inhibitory activity of COX-1 and COX-2 enzymes. From this, new structure-activity relationships were built, showing that phenolic hydroxyl groups are essential for both COX-1 and COX-2 inhibition. Furthermore, the presence of bulky hydrophobic di-*tert*-butyl groups in the phenyl ring strongly contributes for selective COX-2 inhibition. In addition, a correlation with the theoretical log *P* has been carried out, showing that lipophilicity is particularly important for COX-2 inhibition. Further, a plasma protein binding (PPB) prediction has been performed revealing that PPB seems to have no influence in the activity of the studied compounds. From the whole study, effective selective inhibitors of COX-2 were found, namely compound **9** (IC_{50} = 3.0 ± 0.3 μM), **10** (IC_{50} = 2.4 ± 0.6 μM) and **23** (IC_{50} = 1.09 ± 0.09 μM). Those can be considered starting point hit compounds for further optimization as potential non-steroidal anti-inflammatory drugs.

1. Introduction

Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) are two isoenzymes involved in the eicosanoids, prostaglandin (PG) and thromboxane, biosynthesis [1]. COX-1 is a constitutive enzyme and COX-2 expression is primarily induced during the inflammatory process [2]. Recent works also describe COX-2 as constitutively expressed in many important areas of the body, being responsible for very important functions, namely in the cardiovascular system [3–5]. COX inhibitors (CIs) diminish the biosynthesis of the referred eicosanoids and offer a therapeutic approach for the reduction of inflammatory symptoms and pain. CIs also show antipyretic, antithrombotic and analgesic effects. Some CIs in clinical use appear to be extremely potent and highly specific inhibitors of COXs. However, they still show undesirable side effects, being the most frequent, gastrointestinal complications such as the irritation of the gastric mucosa, as PGs produced by COX-1 in the gastrointestinal tract, normally have a protective role of the referred mucosa. Some examples of these inhibitors are indomethacin, diclofenac, ibuprofen and naproxen, which are depicted in Fig. 1A. In addition, other important side effects derived from COX inhibition are allergic reactions, renal and cardiovascular adverse events [5]. Concerning the gastrointestinal complications, these have been partially surpassed by COX-2 selective inhibitors such as celecoxib, rofecoxib, etoricoxib (Fig. 1B), and other members of this drug class showing that the referred selectivity constitutes an important achievement when studying CIs. In fact, COX-2 is primarily expressed in inflamed tissue [6], unlike COX-1 that is mainly expressed in the gastrointestinal tract, and, for this reason, there is much less gastric irritation related with COX-2 inhibitors and a reduced risk of gastric ulceration. However, some recent studies postulate that inhibition of both COX-1 and COX-2 is required for non-steroidal anti-inflammatory drugs (NSAID) induced gastric injury [7–9], since selective COX-1 inhibitors seem to not cause gastric damage in animal studies [10,11]. Though, the theory that inhibition of COX-1 motivates the gastrointestinal toxicity of NSAIDs in man is generally accepted and the demand for new selective COX-2 inhibitors remains a major goal to achieve. Several studies have also demonstrated that COX-2 selective inhibitors may prevent colorectal cancer [12]. Nonetheless, other important adverse effects are associated

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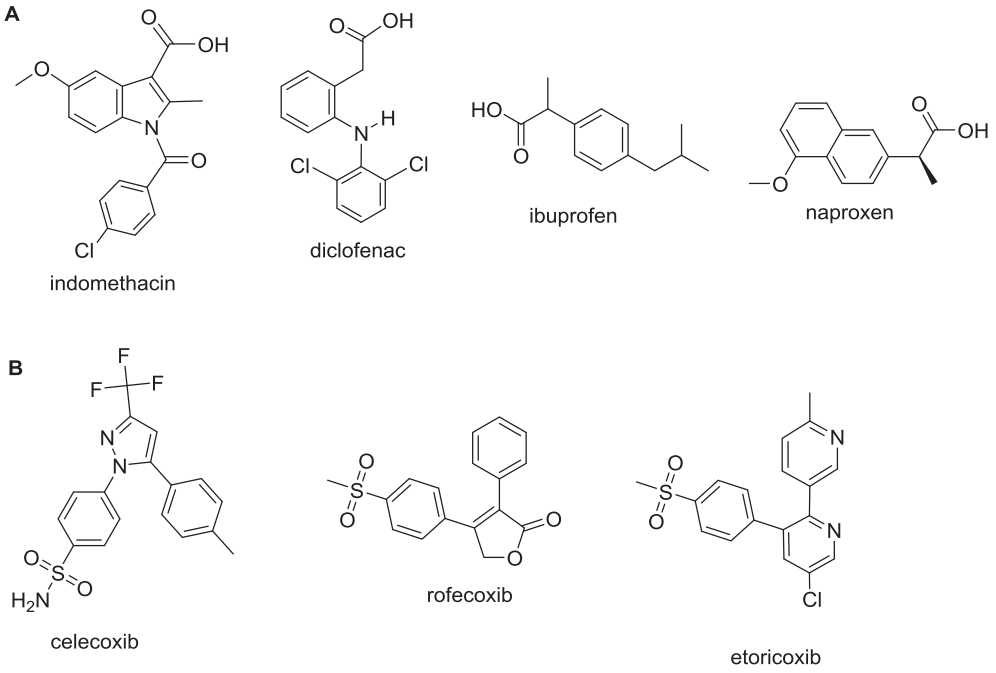


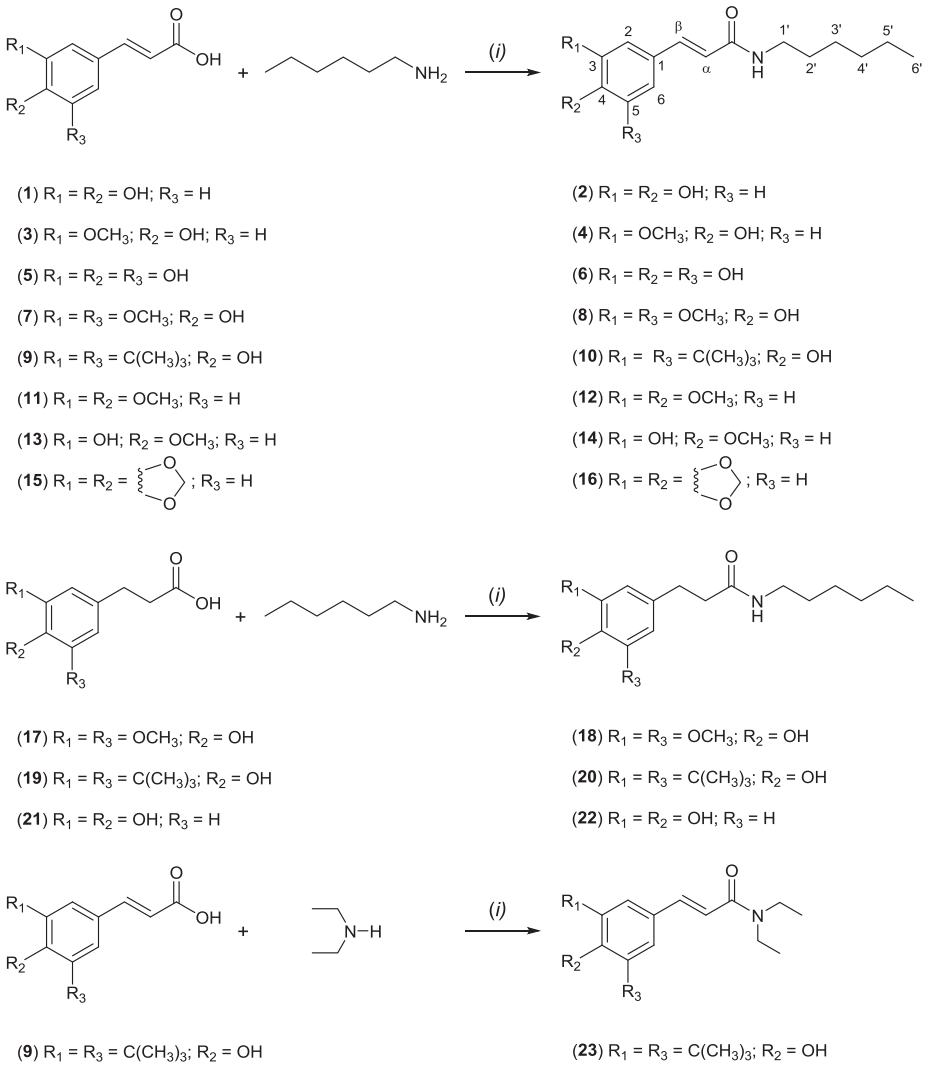
Fig. 1. (**A**) Some examples of COX-1/COX-2 inhibitors; (**B**) Some examples of selective COX-2 inhibitors.

with COX-2 inhibitors such as increased risk of renal failure and also heart attack, thrombosis, and stroke [13,14], through an increase of thromboxane accumulation. Because of this, it is essential to discover new drugs to exceed the limitations of the COX-2 selective inhibitors currently used in the clinic practice. Nowadays, there are no really safe anti-inflammatory drugs to be used as a suitable therapy with negligible gastrointestinal damage and cardiovascular toxicity. Regardless, COX-2 remains a very important therapeutic target to explore in order to find efficient treatments for the inflammation related diseases, such as the debilitating rheumatoid arthritis and osteoarthritis, and also for the prevention of colon cancer.

Phenolic cinnamic acid derivatives have been remarkable scaffolds in drug design, displaying many biological activities namely antioxidant [15,16], anticancer [16–19], and anti-inflammatory through COX-1 and/or COX-2 inhibition [20,21]. Nevertheless, no systematic structure-activity relationships (SAR) studies on COX-1 *versus* COX-2 inhibition by this type of compounds have been performed, in human blood, a more complex and physiological cellular model, that is closer to what happens in the human body [22]. Recently, we have identified a series of hydroxycinnamic acid derivatives as modulators of human neutrophils' oxidative burst, which demonstrate to have antioxidant and anti-inflammatory properties, and also the capacity to inhibit colon cancer cells proliferation [16]. In the present work, it is our intention to assess if those compounds and other new phenolic cinnamic acid derivatives have also the ability to selectively inhibit PGs production via COX-2. For this, we designed and synthesized new compounds of the referred class, and evaluated their aptitude to inhibit PGs production via COX-1 and COX-2 enzymes. Additionally, a correlation of the activities with the theoretical log *P* values and a plasma protein binding prediction were carried out and, from the whole study, new SAR were established.

2. Results and discussion

The synthesis of the hexylamides **2**, **4**, **6**, **8**, **10**, **12**, **14**, **16**, **18**, **20** and **22** (Scheme 1) was performed using a single-step reaction starting from the respective acids **1**, **3**, **5**, **7**, **9**, **11**, **13**, **15**, **17**, **19** and **21** and hexylamine, in dimethylformamide and triethylamine, in the presence of the coupling agent (benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP) in dichloromethane, at room temperature. The same reaction was used to prepare the diethylamide **23** (Scheme 1). This procedure revealed to be adequate to afford the direct amidation of both α,β-unsaturated and saturated acids, producing the desired amides in appropriate yields (37–89%) (Table 1). Then, the synthesized amides and also some of the precursor acids were tested for their capacity to inhibit PGs production via COX-1 and COX-2 enzymes (Figs. 2 and 4; Table 2) and the resulting activities were correlated with the structure of the compounds. In terms of SAR, the discussion is focused in three main structural features, namely: carboxylic acid function *versus* amide group; aromatic substitution pattern; and presence *versus* absence of a α,β-double bond in the aromatic side chain. In addition, SAR are discussed for COX-1 and COX-2 independently and also comparatively. In this way, looking at Fig. 2 and Table 2 it is possible to check that the parent carboxylic acids do not show activity against COX-1, such as **1** and **3**, or just show low activity, as observed for **5**, **9** and **19** at the highest tested concentration (100 μM). On the contrary, the respective amide derivatives **2**, **4**, **6**, **10** and **23** (**20** is apparently an exception) show significant activity against COX-1. One possible reason for these results may be the low lipophilicity of carboxylic acids when comparing with the respective hexylamides, which makes difficult to cross the membranes of blood cells. Among the carboxylic acids, **9** and **19** with superior log *P*(s) (Table 2) are effectively more active than **1** and **3**. The carboxylic acid **5**, with the lowest log *P* (Table 2) among the studied compounds, showed some activity, which may be due to the fact that it can cross the blood cell membranes through some kind of active transport, as previously described by the authors.[16] At this point, one can state that the presence of a lipophilic amide, such as hexylamide or diethylamide, is better than a carboxylic acid group for COX-1 inhibition. Further, comparing the hexyamide **10** (IC_{50} = 24 ± 5 μM) with the diethylamide **23** (IC_{50} = 4.3 ± 0.3 μM), it seems that a teriary bulky amide is superior to a secondary linear amide for COX-1 inhibition. However, the higher lipophilicity of the amides relatively to the carboxylic acids, does not explain all the observed activities for the referred compounds. The aromatic substitution pattern also seems to be very important for the activity. In fact, one main conclusion is that the presence of at least one phenolic group appears to be crucial for COX-1 inhibition, since the dimethoxy derivative **12** and the methylenedioxy derivative **16**



Scheme 1. Synthesis of *n*-hexylamides (secondary amides) and a diethylamide (tertiary amide) of hydroxycinnamic acids. Reagents and conditions: (*i*) DMF, TEA, BOP, dichloromethane, rt.

Table 1
Reaction time (h) and yield (%) for the synthesised compounds.

Compound	Reaction time (h)	Yield (%)
2	4	65
4	4	70
6	5 h 30 min	39
8	2	37
10	3	81
12	5	76
14	5	43
16	5	66
18	1	56
20	3	37
22	6	89
23	6	56

revealed to be not active against COX-1. In addition, comparing COX-1 inhibitory activity of the disubstituted hexylamides **2**, **4** and **14**, all of them with similar log *P* (Table 2), it is possible to conclude that the simultaneous presence of a methoxyl group and a hydroxyl group in the aromatic ring, as in **4** and **14**, is better than the presence of two hydroxyl groups, as in **2**. In fact, **4** and **14** even show around 50% of inhibiton at 12.5 μM while **2** only show 30 ± 8% of inhibition at the same concentration (Fig. 2). The position of the methoxyl group, among the *meta* and *para* positions, relatively to the amide chain, has little influence in the COX-1 inhibition, since **4** (*meta*-methoxyl group) and **14** (*para*-methoxyl group) show similar activities, although the presence of the methoxyl group in the *meta* position is preferable. Considering the trisubstituted hexylamides **6**, **8** and **10**, one can say that the 3,5-di-*tert*-butyl-4-hydroxyl aromatic substitution pattern, present in **10**, is the more convenient, since this compound shows an IC_{50} value of 24 ± 5 μM followed by the 3,5-dimethoxy-4-hydroxyl **8** (IC_{50} = 45 ± 4 μM), and the 3,4,5-trihydroxyl **6** (IC_{50} = 87 ± 2 μM) (Table 2). Comparing the trisubstituted hexylamides **18** and **20**, which do not present α,β-double bond in the aromatic side chain, the 3,5-dimethoxy-4-hydroxyl aromatic substitution pattern, present in **18** (IC_{50} = 11 ± 2 μM), is now the more adequate for COX-1 inhibition. Regarding the presence *versus* absence of a α,β-double bond in the aromatic side chain of the hexylamide derivatives, one can assess the influence of this particular feature in COX-1 inhibition, by comparing the activities of the pairs **2**/**22**, **8**/**18**, **9**/**19** and **10**/**20**. In general, it is possible to conclude that the presence of this feature influence COX-1 inhibition. However, this influence may be positive (**10**/**20**), or negative (**8**/**18**, **9**/**19**, **2**/**22**) depending on the substitution pattern of the aromatic ring. Actually, there are more situations where the absence of the double bond is beneficial. In summary, the most important SAR for COX-1 inhibition by the studied compounds are the presence of an amide group and disubstituted aromatic rings with one hydroxyl group

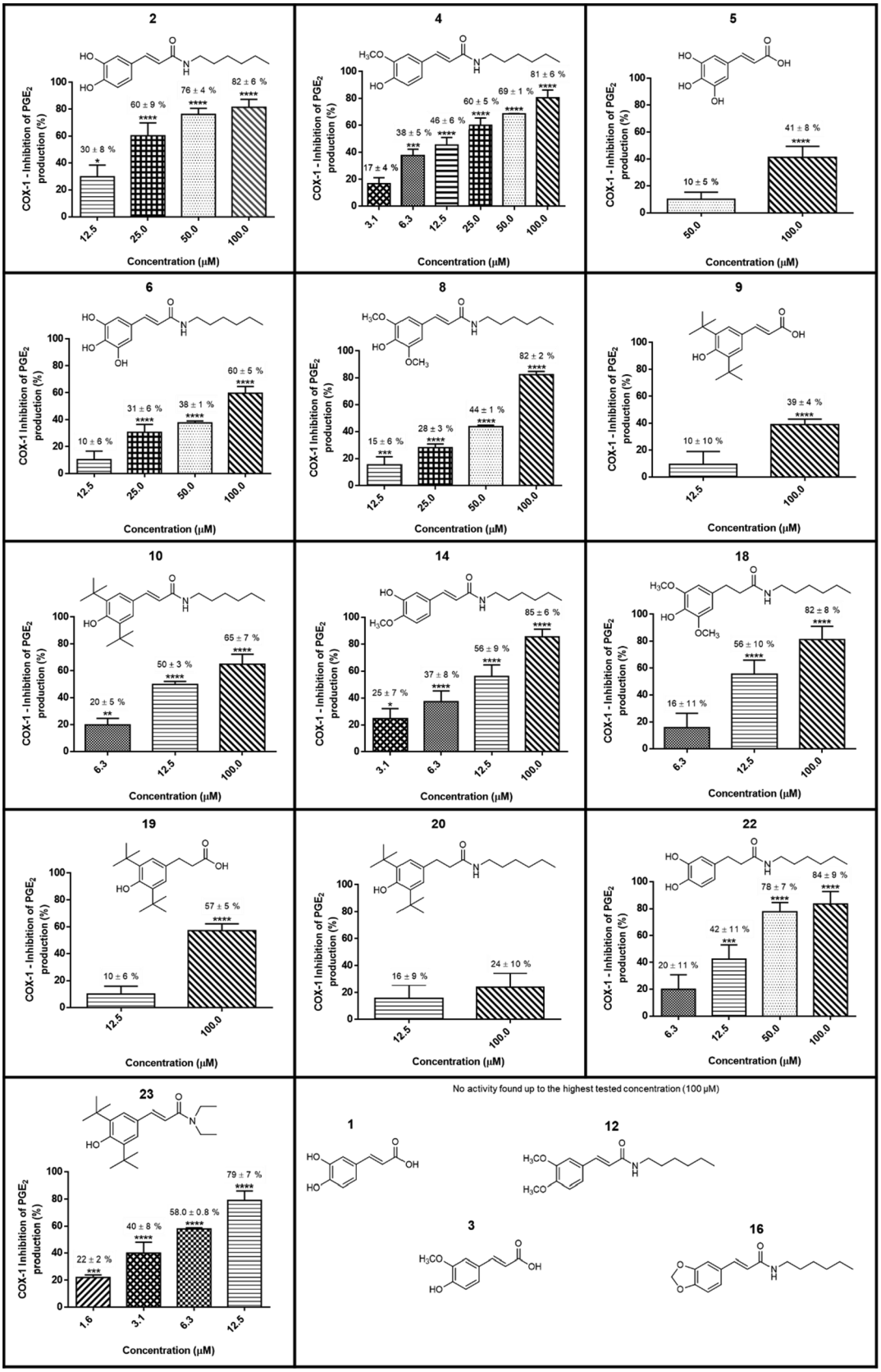


Fig. 2. Inhibition of PGE_2 production via COX-1, in human whole blood by the indicated studied compounds determined by EIA. Each value represents mean ± SEM of at least three experiments. ****p < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.5 compared with the stimulated control (TXBSI/ calcium ionophore A23187). Indomethacin was used as positive control and presented an inhibitory activity of 91 ± 4%, for 1 μM.

Table 2
Inhibition (%) of PGE_2 production, via COX-1 and COX-2, IC_{50}, log *P*, and ADME parameters prediction, for the studied compounds.

Compound	COX-1		COX-2		COX-1/COX-2 ratio	log *P*[a]	PPB[b] (%)	Caco-2[c] (nm/s)
	IC_{50} (mean ± SEM)	Inhibition (%, mean ± SEM)*	IC_{50} (mean ± SEM)	Inhibition (%, mean ± SEM)*				
1	N.A.	N.A.	N.A.	N.A.	–	1.15	40.3	21.1076
2	24 ± 3	82 ± 6	12.7 ± 0.5	99.0 ± 0.7	1.9	2.81	85.4	21.8728
3	N.A.	N.A.	N.A.	N.A.	–	1.42	50.4	21.1177
4	14 ± 2	81 ± 6	20 ± 1	98.3 ± 0.9	0.7	3.08	84.6	34.0661
5	> 100	41 ± 8	N.A.	N.A.	–	0.76	35.8	21.1069
6	87 ± 2	60 ± 5	30 ± 9	74 ± 3	2.9	2.43	82.5	20.5539
8	45 ± 4	82 ± 2	19 ± 5	91 ± 5	2.4	2.95	80.6	38.8512
9	> 100	39 ± 4	3.0 ± 0.3	93 ± 7	> 33	4.95	100.0	21.2638
10	24 ± 5	65 ± 7	2.4 ± 0.6	99.4 ± 0.6	10.0	6.61	97.2	33.5845
12	N.A.	N.A.	N.A.	N.A.	–	2.91	84.9	55.4088
14	9 ± 2	85 ± 6	21 ± 2	79 ± 7	0.4	2.63	84.2	34.1678
16	N.A.	N.A.	N.A.	N.A.	–	3.37	84.7	48.453
18	11 ± 2	82 ± 8	62 ± 6	90 ± 7	0.2	2.97	83.2	39.1876
19	97 ± 2	57 ± 5	N.A.	N.A.	–	4.97	100.0	21.2721
20	> 100	24 ± 10	76 ± 4	57 ± 2	> 1.3	6.63	100.0	33.7775
22	15 ± 3	84 ± 9	13.4 ± 0.5	98.0 ± 0.9	1.1	2.83	90.5	21.9176
23	4.3 ± 0.3	79 ± 7	1.09 ± 0.09	85 ± 4	3.9	5.45	100.0	42.5118
Indomethacin	–	91 ± 4	–	–	–	3.69	89.6[d]	20.0313
Celecoxib	–	–	–	71 ± 1	–	4.34	91.1[e]	0.4994

N.A. – Non active, up to the highest tested concentration (100 μM).
SEM – standard error of the mean.
* The values represent the percentage of inhibition ± SEM for the highest tested concentration (100 μM for all compounds, except for compound **23** which was 12.5 μM for COX-1 and 3.1 μM for COX-2, and indomethacin and celecoxib, which was 1 μM).
[a] Theoretical values calculated from ChemBioDraw 14.0 Software.
[b] Plasma Protein Binding values predicted by the PreADMET web-based application.
[c] In vitro Caco-2 cell permeability predicted by the PreADMET web-based application.
[d] Literature value for PPB of indomethacin: 90% [24].
[e] Literature value for PPB of celecoxib: 97% [25].

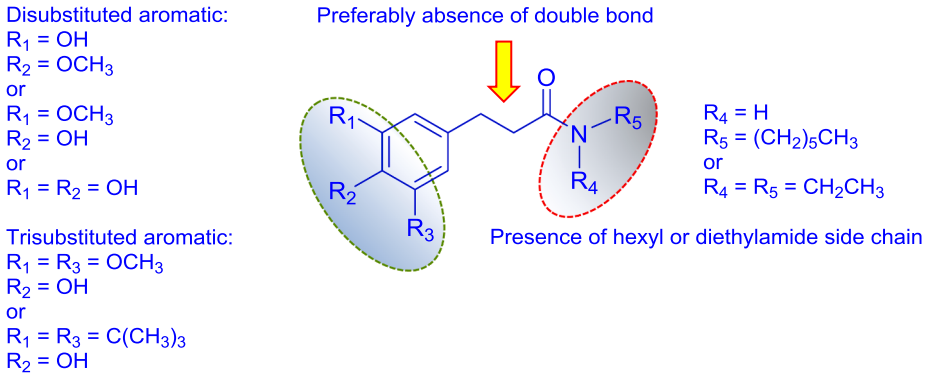


Fig. 3. Principal structural features for COX-1 inhibition.

and one methoxyl group or two hydroxyl groups, preferably without α,β-double bond (Fig. 3). These features are combined in **4, 14** and **22**. In addition, the absence of a α,β-double bond along with a trisubstituted aromatic ring with two methoxyl groups and one hydroxyl group seems to favour the activity (Fig. 3). These features are combined in compound **18**. Further, the 3,5-di-*tert*-butyl-4-hydroxyl aromatic substitution pattern combined with the α,β-double bond along with the tertiary diethylamide group, features that are present in compound **23** (IC_{50} = 4.3 ± 0.3 μM), seems to be the better combination to achieve higher COX-1 inhibition, since **23** was the best COX-1 inhibitor, in this work.

Concerning COX-2 inhibition, it is possible to notice that carboxylic acids **1**, **3**, **5** and **19** do not show activity against COX-2 (Fig. 4, Table 2). Interestingly, the carboxylic acid **9** demonstrates to be a very potent inhibitor of COX-2 reaching an IC_{50} value of 3.0 ± 0.3 μM. On the contrary to the majority of carboxylic acids, their corresponding hexylamides **2**, **4**, **6** and **20** are effectively active against COX-2. In the case of amides **10** and **23**, they are even more potent than the corresponding carboxylic acid **9**, reaching IC_{50} values of 2.4 ± 0.6 and 1.09 ± 0.09 μM, respectively. As in COX-1 inhibition, one can state that the amide group is better than a carboxylic acid group for COX-2 inhibition and that a teriary bulky amide as in **23** is superior to a secondary linear amide as in **10** for COX-2 inhibition. Once more, the low lipophilicity of carboxylic acids **1**, **3** and **5** should be responsible for its low COX-2 inhibition. Nevertheless, other reasons than lipophilicity will account for the great COX-2 inhibition of carboxylic acid **9**, as discussed below. By analyzing the aromatic substitution pattern one can notice that the presence of at least one phenolic group seems to be also crucial for COX-2 inhibition, since the methylenedioxy derivative **16** and the dimethoxy derivative **12** are again not active against COX-2. In addition, comparing COX-2 inhibitory activity of the disubstituted hexylamides **2**, **4** and **14**, it appears that, in this case, the presence of two hydroxyl groups (cathecol) in the aromatic ring as in **2**, is more advantageous than the simultaneous presence of one hydroxyl group

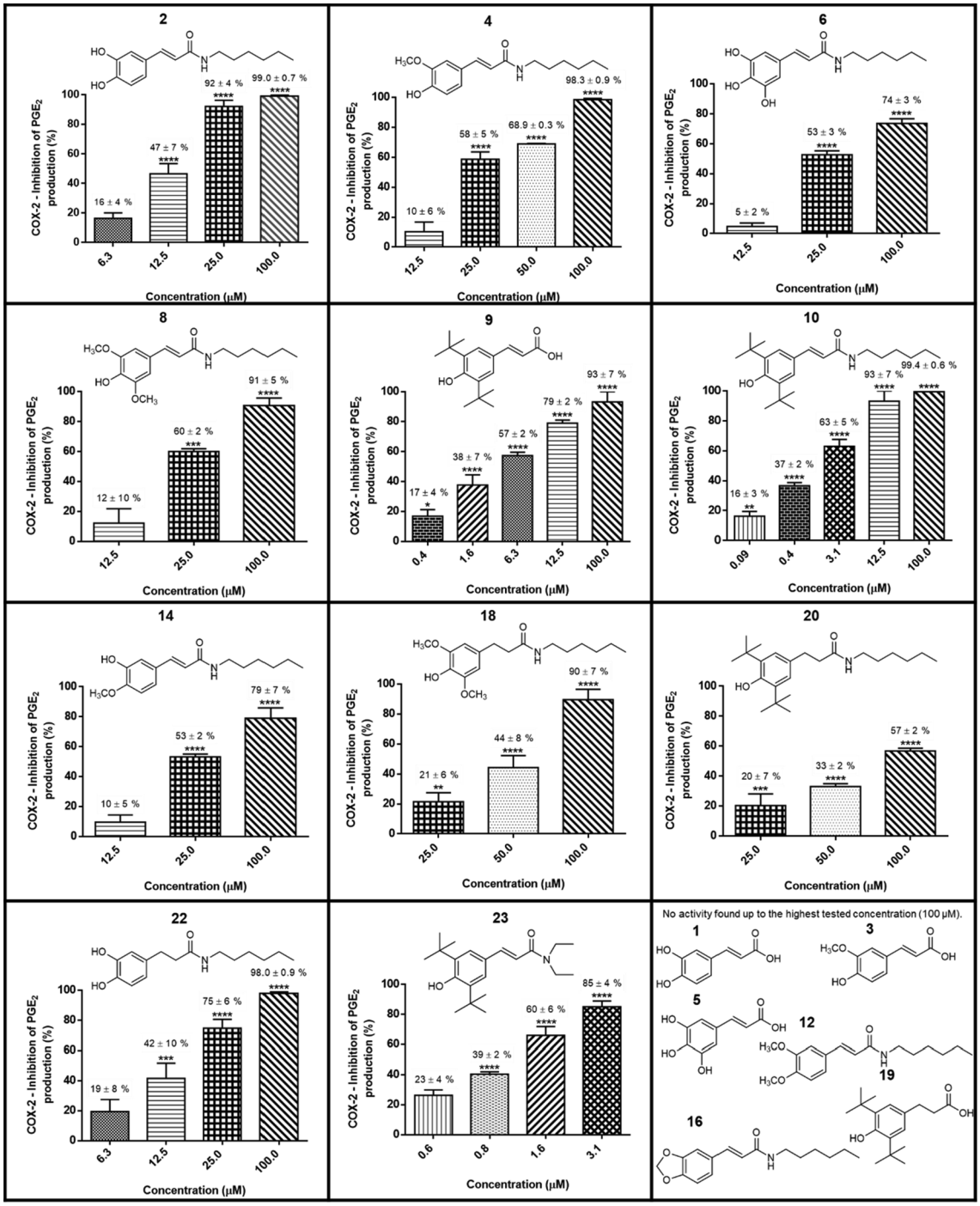


Fig. 4. Inhibition of PGE_2 production via COX-2, in human whole blood by the indicated studied compounds determined by EIA. Each value represents mean ± SEM of at least three experiments. ****p < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.5 compared with the stimulated control (TXBSI/LPS). Celecoxib was used as positive control and presented an inhibitory activity of 71 ± 1%, for 1 μM.

and one methoxyl group, as in **4** and **14**. In fact, **2** shows an IC_{50} value of 12.7 ± 0.5 μM, against 20 ± 1 μM for **4** and 21 ± 2 μM for **14** (Table 2). Again, the position of the methoxyl group, among the *meta* and *para* positions relatively to the amide chain, does not seem to influence the COX-2 inhibitory activity, since **4** (*meta*-methoxyl group) and **14** (*para*-methoxyl group) show similar activities. Considering the trisubstituted hexylamides **6**, **8** and **10**, one can say that, in this case, the aromatic substitution pattern has great influence in COX-2 inhibition. In fact, the 3,5-di-*tert*-butyl-4-hydroxyl aromatic substitution pattern, present in **10**, seems to be very appropriate for COX-2 inhibition. The 3,5-dimethoxyl-4-hydroxyl aromatic substitution pattern, present in **8**, is the second best approach and the 3,4,5-trihydroxyl, present in **6**, is the less suitable aromatic substitution. These results can be correlated with the active site of COX-2. In fact, it is known that the most significant difference between COX-1 and COX-2 isoenzymes is the presence of an isoleucine residue, at position 523 in COX-1, instead of a valine residue in COX-2. The smaller size valine residue permits a better access to a hydrophobic side-pocket in COX-2, allowing molecules with hydrophobic groups to better interact with its active site [13]. This can explain the great COX-2 inhibitory activity of the carboxylic acid **9** and even better of the hexylamide **10** and diethylamide **23**, all of them bearing two hydrophobic *tert*-butyl groups in the aromatic ring. These

groups confer great lipophilicity to the referred compounds, which present log *P* values of 4.95, 6.61 and 5.45 (Table 2), respectively. Besides the *tert*-butyl groups in the aromatic ring, compound **23** presents a bulky hydrophobic diethylamine group that can also contribute to favor the access and the interaction with the active site of COX-2. Based on this approach, it can be explained why compound **8**, with less hydrophobic methoxyl groups and with log *P* of 2.95 (Table 2), show lower COX-2 inhibitory activity, followed by **6**, with hydrophilic hydroxyl groups and with log *P* of 2.43 (Table 2). Regarding the presence *versus* absence of a α,β-double bond in the aromatic side chain of the hexylamide derivatives, one can assess the influence of this particular feature in COX-2 inhibition, by comparing the activities of the pairs **2**/**22**, **8**/**18**, **9**/**19** and **10**/**20**. Generally, and on the contrary to what was noticed for COX-1, for COX-2 inhibition the presence of the double bond in the side chain is very beneficial, since the trisubstituted compounds with the double bond, **8**, **9** and **10** are more active than the corresponding compounds without the double bond, **18**, **19** and **20**, respectively. For the disubstituted pair **2**/**22**, this difference is not evident, showing both compounds similar activities, although **2** is slightly better. In summary, the most important SAR for COX-2 inhibition by the studied compounds are: presence of an amide group; disubstituted aromatic rings with two hydroxyl groups, preferably with α,β-double bond; more important, trisubstituted aromatic rings with 3,5-di-*tert*-butyl-4-hydroxyl aromatic substitution, definitely with a double bond in the side chain of either the acid or the amides (Fig. 5). In the case of disubstituted compounds, these features are combined in caffeic acid hexylamide **2** (IC_{50} = 12.7 ± 0.5 μM) and hydrocaffeic acid hexylamide **22** (IC_{50} = 13.4 ± 0.5 μM). In the case of trisubstituted compounds, the referred features are combined in compounds **9**, **10**, and **23** which are the best COX-2 inhibitors studied in this work, reaching IC_{50} values of 3.0 ± 0.3, 2.4 ± 0.6 and 1.09 ± 0.09 μM (Table 2).

More remarkable is the comparison between COX-1 and COX-2 inhibition by the studied compounds. From this, we can assess if there is some selectivity for either COX-1 or COX-2 inhibition. In fact, the carboxylic acids **5** and **19** show a slight inhibition, but total selectivity for COX-1, since they are not active against COX-2 (Figs. 2 and 4, Table 2). Also, compounds **4**, and mainly **14**, and **18** are selective COX-1 inhibitors. Moreover, the carboxylic acid **9**, the hexylamide **10**, and the diethylamide **23**, besides being very powerful compounds in COX-2 inhibition, are very selective for the referred enzyme with COX-1/COX-2 ratio values of > 33, 10.0, and 3.9 respectively, demonstrating their higher affinity to COX-2 (Table 2). This is in agreement with established SAR for the COX inhibitor flurbiprofen, where chemical modifications in the phenyl group are capable of inducing steric constraint resulting in an increased selectivity for COX-2 [23]. Compounds **9**, **10**, and **23** bearing two bulky *tert*-butyl groups in their phenyl rings are capable of inducing the abovementioned steric restrictions when interacting with COX-2 resulting in very effective and selective COX-2 inhibitors.

Finally, as the present study was performed in human whole blood, a plasma protein binding (PPB) prediction for the studied compounds was done using the PreADMET web-based application (Table 2). The positive controls tested, indomethacin and celecoxib, presented PPB values of 89.5% and 91.1%, respectively, really similar to the ones found in the literature, around 90% and 97%, respectively [24,25]. It was observed that the most active compounds bind considerably to plasma proteins. However, the same was observed for the reference compounds indomethacin and celecoxib, showing that apparently, the plasma protein binding has no influence in the activity of the studied compounds. An *in vitro* Caco-2 cell permeability prediction was also carried out, for the studied compounds, using the PreADMET web-based application. All the compounds analyzed seem to have capacity to cross the membranes of Caco-2 cells in a similar or superior way to indomethacin, being the amide derivatives more able to do so than the parent carboxylic acids. This is in accordance with the predicted log *P* and justifies once more, the superior COX-1 and COX-2 inhibition showed by the amide derivatives relatively to the correspondent carboxylic acids. Based on all the work we can consider compounds **9**, **10**, and **23** hit compounds for further optimization and a starting point for the preparation of selective COX-2 inhibitors as potential non-steroidal anti-inflammatory drugs.

3. Material and methods

3.1. Chemistry

Reactions were controlled by thin layer chromatography (TLC) using silica gel 60 HF254 plates. Column chromatography was performed using silica gel 60 (0.063–0.200 mm). Melting points (Mps) were determined on a Reichert Thermopan hot block apparatus, except for compounds **12** and **14**, which were measured in a Büchi Melting Point B-540 apparatus. Mp values were not corrected. IR spectra were recorded on a Jasco 420FT/IR spectrometer, except for compounds **12**, **14**, **22**, and **23** which IR spectra were recorded on a Perkin Elmer Spectrum 400 FT-IR/FT-NIR spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz, respectively, on a Varian Unity 400, using DMSO-d_6 as solvent. Peaks positions are given in parts per million (ppm) using the residual non-deuterated solvent as the internal standard. Data are reported as follows: chemical shift (ppm), integrated intensity, multiplicity (indicated as: s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet and combination thereof), coupling constants (*J*) values in Hertz (Hz) and corresponding nucleus. Caffeic acid **1**, ferulic acid **3** and 3,4-dimethoxycinnamic acid **11** were purchased from Sigma-Aldrich (Schnelldorf, Germany); 3,4,5-trihydroxycinnamic acid **5**, 4-hydroxy-3,5-dimethoxycinnamic acid **7** and 3-(4-hydroxy-3,5-dimethoxyphenyl)propionic acid **17** from Apin Chemicals Limited (Abingdon, Oxon, United Kingdom); 3,5-di-*tert*-butyl-4-hydroxycinnamic acid **9**, 3,4-(methylenedioxy)cinnamic acid **15** and 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propionic acid **19**, from Alfa Aesar (Karlsruhe, Germany); 3-hydroxy-4-methoxycinnamic acid **13** from Carbosynth Limited (Berkshire, United Kingdom); and 3-(3,4-dihydroxyphenyl)propionic acid **21** from Honeywell Fluka (Göterborg, Sweden).

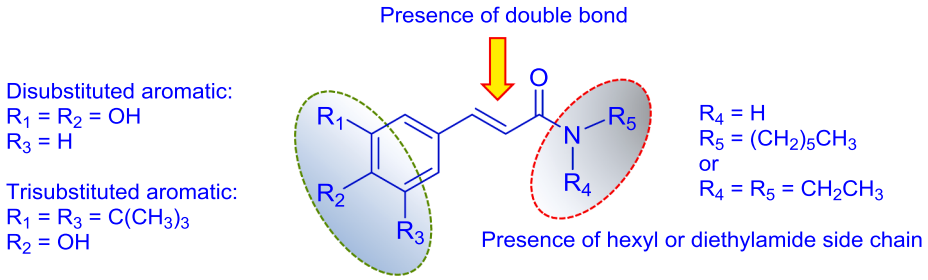


Fig. 5. Principal structural features for COX-2 inhibition. In the case of the disubstituted aromatic with R_1 = R_2 = OH, the compound without double bond (22) is also very active. In the case of the trisubstituted aromatic, the acid (compound **9**) is also very active.

3.2. General procedure to obtain the hexylamides **2**, **4**, **6**, **8**, **10**, **12**, **14**, **16**, **18**, **20**, **22** and **23**

Carboxylic acids **1**, **3**, **5**, **7**, **9**, **11**, **13**, **15**, **17**, **19** and **21** (Scheme 1) were dissolved in dimethylformamide (DMF) and triethylamine (TEA). The solution was then cooled in an ice-water bath and hexylamine or diethylamine was added, followed by a solution of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) in dichloromethane. The mixture was stirred at 0 °C for 30 min and then at room temperature for specific periods of time. Dichloromethane was removed under reduced pressure and the remaining solution was diluted with water (100 mL). The aqueous phase was extracted with ethyl acetate (2x 100 mL) and the organic phases were washed with 1 N HCl (2x 100 mL), water (2x 100 mL), $NaHCO_3$ 5% (3x 100 mL) and water (2x 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated. The obtained residues were purified by silica gel column chromatography or crystallization yielding the corresponding hexylamides **2**, **4**, **6**, **8**, **10**, **12**, **14**, **16**, **18**, **20**, **22** and **23**.

3.3. N-Hexyl-3-(3,4-dihydroxyphenyl)-2-propenamide (**2**)

Obtained as described before [15].

3.4. N-Hexyl-3-(4-hydroxy-3-methoxyphenyl)-2-propenamide (**4**)

Obtained as described before [15].

3.5. N-Hexyl-3-(3,4,5-trihydroxyphenyl)-2-propenamide (**6**)

Obtained as described before [16].

3.6. N-Hexyl-3-(4-hydroxy-3,5-dimethoxyphenyl)-2-propenamide (**8**)

Compound **7** (1.12 g, 5 mmol); DMF (10 mL); TEA (0.7 mL); hexylamine (0.66 mL, 5 mmol); BOP (2.21 g, 5 mmol); CH_2Cl_2 (10 mL); reaction time: 2 h; ethyl acetate crystallization; 37% yield. $Mp_{(ethyl\ acetate)}$ 121–123 °C. IR (ATR) ν_{max} cm^{-1}: 3523 (O—H), 3279 (N—H), 1653 (C═O), 1210 (C—O), 1113 (C—O). ^1H NMR (400 MHz, DMSO-d_6) δ : 0.85 (3H, t, J = 6.9, CH_3), 1.23–1.27 (6H, m, CH_2, (C-3′-C-5′)), 1.40–1.45 (2H, m, CH_2 (C-2′)), 3.14 (2H, dt, J = 5.4, 6.6, CH_2(C-1′)), 3.78 (6H, s, OCH_3 (C-3 and C-5)), 6.47 (1H, d, J = 15.6, CH(α)), 6.83 (2H, s, CH (C-2 and C-6)), 7.31 (1H, d, J = 15.6, CH(β)), 7.96 (1H, t, J = 5.4, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 14.0 (CH_3), 22.2 (C-5′), 26.2 (C-4′), 29.2 (C-3′), 31.1 (C-2′), 38.8 (C-1′), 56.0 (−OCH_3 C-3 and C-5), 105.2 (C-2 and C-6), 119.5 (C-α), 125.4 (C-1), 137.3, 139.3 (C-β), 148.1, 165.4 (C═O).

3.7. N-Hexyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)-2-propenamide (**10**)

Compound **9** (2.76 g, 10 mmol); DMF (20 mL); TEA (1.4 mL); hexylamine (1.32 mL, 10 mmol); BOP (4.42 g, 10 mmol); CH_2Cl_2 (20 mL); reaction time: 3 h; ethyl acetate crystallization; 81% yield. $Mp_{(ethyl\ acetate)}$ 173–174 °C. IR (ATR) ν_{max} cm^{-1}: 3523 (O—H), 3280 (N—H), 1651 (C═O), 1218 (C—O), 1113 (C—O). ^1H NMR (400 MHz, DMSO-d_6) δ: 0.86 (3H, t, J = 6.9, CH_3), 1.26–1.29 (6H, m, CH_2 (C-3′-C-5′)), 1.36–1.44 (2H, m, CH_2 (C-2′)), 1.39 (18H, s, $(CH_3)_3$), 3.12–3.16 (2H, m, CH_2 (C-1′)), 6.45 (1H, d, J = 15.7, CH(α)), 7.28 (1H, br s, OH), 7.29 (2H, s, CH (C-2 and C-6)), 7.32 (1H, d, J = 15.7, CH(β)), 7.92 (1H, t, J = 5.5, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ : 13.8 (CH_3), 21.9 (C-5′), 26.1 (C-4′), 29.0 (C-3′), 30.1 (C($\underline{C}H_3)_3$), 30.9 (C-2′), 34.4 ($\underline{C}(CH_3)_3$), 38.5 (C-1′), 118.9 (C-1), 124.1 (C-2), 126.2 (C-1), 139.1 (C-3 and C-5), 139.2 (C-β), 155.3 (C-4), 165.1 (C═O).

3.8. N-Hexyl-3-(3,4-dimethoxyphenyl)-2-propenamide (**12**)

Compound **11** (500.0 mg, 2.40 mmol); DMF (5.5 mL); TEA (0.34 mL); hexylamine (0.32 mL, 2.40 mmol); BOP (1.06 g, 2.40 mmol); CH_2Cl_2 (5 mL); reaction time: 5 h; ethyl acetate crystallization; 76% yield. $Mp_{(ethyl\ acetate)}$ 95–97 °C. IR (ATR) ν_{max} cm^{-1}: 3283 (N—H), 1650 (C═O), 1136 (C—O). ^1H NMR (400 MHz, DMSO-d_6) δ : 0.87 (3H, t, J = 6.5, CH_3), 1.27–1.31 (6H, m, CH_2 (C-3′-C-5′)), 1.40–1.45 (2H, m, CH_2 (C-2′)), 3.12–3.17 (2H, m, CH_2 (C-1′)), 3.78 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 6.49 (1H, d, J = 15.7, CH(α)), 6.97 (1H, d, J = 8.3, CH (C-6)), 7.09 (1H, dd, $J_{5,6}$ = 8.3, $J_{5,2}$ = 1.5, CH (C-5)), 7.14 (1H, d, $J_{2,5}$ = 1.5, CH (C-2)), 7.33 (1H, d, J = 15.7, CH(β)), 7.95 (1H, t, J = 5.5, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ : 13.8 (CH_3), 21.9 (C-5′), 26.1 (C-4′), 29.1 (C-3′), 30.9 (C-2′), 38.5 (C-1′), 55.3 (OCH_3), 55.4 (OCH_3), 109.8 (C-α), 111.7 (C-6), 120.0 (C-5), 121.2 (C-2), 127.7 (C-1), 138.3 (C-β), 148.8 (C-2), 149.9 (C-3), 164.9 (C═O).

3.9. N-Hexyl-3-(3-hydroxy-4-methoxyphenyl)-2-propenamide (**14**)

Compound **13** (500.0 mg, 2.57 mmol); DMF (6 mL); TEA (0.36 mL); hexylamine (0.34 mL, 2.57 mmol); BOP (1.14 g, 2.57 mmol); CH_2Cl_2 (6 mL); reaction time: 5 h; column chromatography (hexane/ethyl acetate); 43% yield. $Mp_{(ethyl\ acetate)}$ 102–103 °C. IR (ATR) ν_{max} cm^{-1}: 3321 (O—H and N—H), 1642 (C═O), 1126 (C—O). ^1H NMR (400 MHz, DMSO-d_6) δ : 0.86 (3H, t, J = 6.8, CH_3), 1.27–1.29 (6H, m, CH_2 (C-3′-C-5′)), 1.39–1.45 (2H, m, CH_2 (C-2′)), 3.12–3.16 (2H, m, CH_2 (C-1′)), 3.79 (3H, s, OCH_3), 6.38 (1H, d, J = 15.7, CH(α)),6.93–6.97 (2H, m, CH (C-5 and C-6)), 6.96 (1H, br s, CH (C-2)), 7.25 (1H, d, J = 15.7, CH(β)), 7.96 (1H, t, J = 5.5, NH), 9.15 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ : 13.8 (CH_3), 21.9 (C-5′), 26.1 (C-4′), 29.1 (C-3′), 30.9 (C-2′), 38.5 (C-1′), 55.4 (OCH_3), 111.9 (C-α), 113.2 (C-5), 119.6 (C-6), 120.0 (C-2), 127.7 (C-1), 138.4 (C-β), 146.6 (C-4), 148.9 (C-3), 164.9 (C═O).

3.10. N-Hexyl-3-(3,4-methylenedioxyphenyl)-2-propenamide (**16**)

Compound **15** (500.0 mg, 2.60 mmol); DMF (6 mL); TEA (0.37 mL); hexylamine (0.34 mL, 2.60 mmol); BOP (1.15 g, 2.60 mmol); CH_2Cl_2 (6 mL); reaction time: 5 h; ethyl acetate crystallization; 66% yield. $Mp_{(ethyl\ acetate)}$ 80–81 °C. IR (ATR) ν_{max} cm^{-1}: 3305 (N—H), 1647 (C═O), 1037 (C—O). ^1H NMR (400 MHz, DMSO-d_6) δ : 0.86 (3H, t, J = 7, CH_3), 1.26–1.30 (6H, m, CH_2 (C-3′-C-5′)), 1.40–1.45 (2H, m, CH_2 (C-2′)), 3.12–3.16 (2H, m, CH_2 (C-1′)), 6.06 (2H, s, O-CH_2-O), 6.45 (1H, d, J = 15.7, −CH(α)), 6.93 (1H, d, J = 7.65, CH (C-6)), 7.05 (1H, dd, $J_{5,6}$ = 7.7, $J_{5,2}$ = 1.6, CH (C-5)), 7.12 (1H, d, $J_{2,5}$ = 1.6, −CH (C-2)), 7.31 (1H, d, J = 15.7, CH(β)), 7.95 (1H, t, J = 5.5, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ : 13.8 (CH_3), 21.9 (C-5′), 26.1 (C-4′), 29.0 (C-3′), 30.9 (C-2′), 38.6 (C-1′), 101.3 (O-CH_2-O), 106.0 (C-α), 108.4 (C-6), 120.4 (C-5), 122.9 (C-2), 129.3 (C-1), 138.0 (C-β), 147.8 (C-4), 148.2 (C-3), 164.8 (C═O).

3.11. N-Hexyl-3-(4-hydroxy-3,4-dimethoxyphenyl)propanamide (**18**)

Compound **17** (1.13 g, 5 mmol); DMF (10 mL); TEA (0.7 mL); hexylamine (0.66 mL, 5 mmol); BOP (2.21 g, 5 mmol); CH_2Cl_2 (10 mL); reaction time: 1 h; ethyl acetate crystallization; 56% yield. $Mp_{(ethyl\ acetate)}$ 93–94 °C. IR (ATR) ν_{max} cm^{-1}: 3319 (N—H), 1643 (C═O), 1225 (C—O), 1125 (C—O). ^1H NMR (400 MHz, DMSO-d_6) δ : 0.85 (3H, t, J = 6.9, CH_3), 1.20–1.26 (6H, m, CH_2 (C-3′-C-5′)), 1.33–1.38 (2H, m, CH_2 (C-2′)), 2.34 (2H, t, J = 7.8, CH_2(α)), 2.71 (2H, t, J = 7.8, CH_2(β)), 3.02 (2H, dt, J = 5.4, 6.0, CH_2(C-1′)), 3.72 (6H, s, CH_3 (C-3 and C-5)), 6.44 (2H, s, CH (C-2 and C-6), 7.76 (1H, t, J = 5.4, NH), 8.04 (1H, s, OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ : 13.9 (CH_3), 22.0 (C-5′), 26.1 (C-4′), 29.1 (C-3′), 31.0 (C-2′), 31.3, 37.4, 38.4 (C-1′), 55.8 (2 × O$\underline{C}H_3$), 105.5 (C-2 and C-6), 131.3 (C-1), 133.6, 147.8 (C-3 and C-5), 171.3 (C═O).

3.12. N-Hexyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propanamide (**20**)

Compound **19** (2.78 g, 10 mmol); DMF (20 mL); TEA (1.4 mL);

hexylamine (1.32 mL, 10 mmol); BOP (4.42 mg, 10 mmol); CH_2Cl_2 (20 mL); reaction time: 3 h; ethyl acetate crystallization; 37% yield. $Mp_{(ethyl\ acetate)}$ 77–78 °C. IR (ATR) ν_{max} cm^{-1}: 3284 (N—H), 1619 (C═O), 1205 (C—O). ^1H NMR (400 MHz, DMSO-d_6) δ : 0.85 (3H, t, J = 6.9, CH_3), 1.23–1.26 (8H, m, CH_2 (C-2′-C-5′)), 1.35 (18H, s, $(CH_3)_3$), 2.28 (2H, t, J = 7.8, CH_2 (α)), 2.68 (2H, t, J = 7.8, CH_2 (β)),3.00–3.02 (2H, m, CH_2 (C-1′)), 6.7 (1H, s, OH), 6.89 (1H, s, CH (C-2 and C-6)), 7.75 (1H, t, J = 5.5, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ : 13.8 (CH_3), 21.9 (C-5′), 25.9 (C-4′), 29.1 (C-3′), 30.3 (C($\underline{C}H_3)_3$), 30.9 (C-2′), 31.1, 34.3 ($\underline{C}(CH_3)_3$), 37.4, 38.4 (C-1′), 124.0 (C-2 and C-6), 132.1 (C-1), 138.9 (C-3 and C-5), 151.7 (C-4), 171.2 (C═O).

3.13. N-Hexyl-3-(3,4-dihydroxyphenyl)propanamide (22)

Compound **21** (500.8 mg, 2.7 mmol); DMF (5.5 mL); TEA (0.38 mL); hexylamine (0.36 mL, 2.7 mmol); BOP (1.19 g, 2.7 mmol); CH_2Cl_2 (5.4 mL); reaction time: 6 h; column chromatography (hexane/ethyl acetate); 89% yield. $Mp_{(ethyl\ acetate)}$ 65–67 °C. IR (ATR) ν_{max} cm^{-1}: 3486 (O—H), 3369 (N—H), 1606 (C═O), 1189 (C—O), 1108 (C—O) ^1H NMR (400 MHz, DMSO-d_6) δ : 0.86 (3H, t, J = 6.9, CH_3), 1.21–1.27 (6H, m, CH_2 (C-3′-C-5′)), 1.33–1.36 (2H, m, CH_2 (C-2′)), 2.24 (2H, t, J = 7.7, CH_2 (α)), 2.60 (2H, t, J = 7.7, CH_2 (β)),2.97–3.02 (2H, m, CH_2 (C-1′)), 6.41 (1H, dd, $J_{5,6}$ = 7.97, $J_{5,2}$ = 2.0, CH (C-5)), 6.55 (1H, d, $J_{2,5}$ = 2.0, CH (C-2)), 6.59 (1H, dd, $J_{6,5}$ = 8.0, CH (C-6)), 7.71 (1H, t, J = 5.4, NH), 8.58 (1H, s, OH), 8.67 (1H, s, OH). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 13.8 (CH_3), 21.9 (C-5′), 25.9 (C-4′), 29.0 (C-3′), 30.6 (C-2′), 30.9, 37.5, 38.3 (C-1′), 115.2 (C-5), 115.6 (C-2), 118.6 (C-6), 132.1 (C-1), 143.2, 144.8, 171.2 (C═O).

3.14. N,N-Diethyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)-2-propenamide (23)

Compound **9** (500.2 mg, 1.81 mmol); DMF (4.2 mL); TEA (0.25 mL); diethylamine (0.19 mL, 1.81 mmol); BOP (800.7 mg, 1.81 mmol); CH_2Cl_2 (4.2 mL); reaction time: 6 h; ethyl acetate crystallization; 56% yield. $Mp_{(ethyl\ acetate)}$ 161–164 °C. IR (ATR) ν_{max} cm^{-1}: 3217 (O—H), 1638 (C═O), 1261 (C—O), 1191 (C—N). ^1H NMR (400 MHz, DMSO-d_6) δ : 1.07 (3H, t, J = 6.3, CH_3), 1.15 (3H, t, J = 5.6, CH_3), 1.40 (18H, s, $(CH_3)_3$), 3.37–3.39 (2H, m, CH_2 (C-1′)), 3.49–3.54 (2H, m, CH_2 (C-1′)), 6.80 (1H, d, J = 15.3, CH(α)), 7.34 (2H, s, CH (C-2 and C-6)), 7.43 (1H, d, J = 15.3, CH(β)). ^{13}C NMR (100 MHz, DMSO-d_6) δ : 13.2 (CH_3), 15.0 (CH_3), 30.1 (C($\underline{C}H_3)_3$), 34.4 ($\underline{C}(CH_3)_3$), 40.1 (C-1′), 41.3 (C-1′), 115.1 (C-α), 124.5 (C-2 and C-6), 126.4 (C-1), 139.0 (C-3 and C-5), 142.3 (C-β), 155.6 (C-4), 164.9 (C═O).

3.15. Biological assays

The following reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, USA): dimethylsulfoxide (DMSO), acetylsalicylic acid, gentamicin sulfate, cremophor® EL, Dulbecco's phosphate-buffered saline (DPBS), lipopolysaccharides from *Escherichia coli* 026:B6 (LPS), calcium ionophore (A23187). Ethanol absolute was purchased from Fisher Chemical (Loughborough, Leic, UK). The thromboxane synthase inhibitor (TXBSI) was synthesized as described [26]. The "PGE2 Enzyme Immunoassay (EIA) Kit" was obtained from Enzo Life Sciences (Lausen, Switzerland).

3.16. Human whole blood assay

Venous blood was collected by antecubital venipuncture into heparin-Li^+ vacuum tubes from healthy human volunteers, following informed consent. The human whole blood assays to assess the inhibition of PGE_2 production - via COX-1 and -2 were performed as previously reported [27,28].

3.17. COX-1 assay

Briefly, the collected blood (500 µL) was placed in microtubes and incubated in a water bath at 37 °C with TXBSI (1 µM, in DPBS) and the tested compounds (3.1–100 µM, in DMSO), for 15 min. Then, the calcium ionophore A23187 (12.2 µg/mL, in ethanol) was added and the mixture was incubated for another 15 min. After, the samples were placed on ice, for 5 min. The samples were subsequently centrifuged at 1000*g* for 20 min, at 4 °C. The supernatant was then collected and stored at −20 °C until use. The quantity of solvents used did not have inhibitory effects and neither affected the cellular viability (data not shown).

3.18. COX-2 assay

Briefly, the collected human whole blood (800 µL) was placed in a six-wells plate and incubated in a humidified incubator at 37 °C with TXBSI (1 µM, in DPBS-gentamicin), the acetylsalicylic acid (10 µg/mL, in DPBS-gentamicin), and the tested compounds [0.08–100 µM, in DMSO/chremophor/ethanol 1% (1:10)], for 15 min. Then, LPS (10 µg/mL, in DPBS-gentamicin) was added and the mixture was incubated for 5 h. After this period of time, DPBS-gentamicin buffer (1 mL) was added to the samples and they were then placed on ice, for 10 min. The samples were subsequently centrifuged at 1000*g* for 15 min, at 4 °C. The supernatant was then collected and stored at −20 °C until use. The quantity of solvents used did not have inhibitory effects and neither affected the cellular viability (data not shown).

3.19. Determination of PGE_2 production

The above mentioned commercial EIA kit was used to determine the amount of PGE_2 in the samples (thawed plasma supernatants), according to the manufacturer's instructions, as an indicator of COX-1 and -2 activities. COX inhibitors, indomethacin (1 µM) and celecoxib (1 µM), were used as positive controls. Results are expressed as the percent inhibition of control PGE_2 production. Each study corresponds to at least three independent experiments.

4. Statistical analysis

GraphPad Prism™ (version 6.0; GraphPad Software, San Diego, CA, USA) was used to perform the statistical analysis. Results are expressed as mean ± standard error of the mean (SEM). Statistical comparison between groups was estimated using the one-way analysis of variance (ANOVA), followed by the Bonferroni's post-hoc test. In all cases, p-values lower than 0.05 were considered as statistically significant.

5. Note

The molecular structure of COX-2 used in the graphical abstract was created by using the PDB ID: 3LN1 (**Structure of celecoxib bound at the COX-2 active site**; Wang, J.L., Limburg, D., Graneto, M.J., Springer, J., Hamper, J.R., Liao, S., Pawlitz, J.L., Kurumbail, R.G., Maziasz, T., Talley, J.J., Kiefer, J.R., Carter, J.; (2010) Bioorg Med Chem Lett **20** 7159–7163), the NGL Viewer (A.S. Rose, A.R. Bradley, Y. Valasatava, J.D. Duarte, A. Prlić, P.W. Rose (2018) NGL viewer: web-based molecular graphics for large complexes. *Bioinformatics* (doi: https://doi.org/10.1093/bioinformatics/bty419), and RCSB PDB (www.rcsb.org).

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