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Original article

Investigations of new lead structures for the design of novel cyclooxygenase-2 inhibitors

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Abstract

On the basis of molecular modelling studies, five new compounds were synthesised and studied in an attempt to design new lead structures as selective COX-2 inhibitors. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: NSAIDs; Cyclooxygenase (COX-1, COX-2); Selective COX-2 inhibitors; Thromboxane B_2

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen or indomethacin are widely used in the treatment of pain and inflammatory diseases [1]. Their clinical efficacy is closely related to their ability to inhibit both COX-1 and COX-2 isoforms of the enzyme cyclooxygenase (COX) also referred to as prostaglandin H_2 synthase since it catalyses the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) [2]. The constitutive COX-1 isoform is mainly responsible for the synthesis of prostaglandins which exert cytoprotective effect in the gastrointestinal tract and control renal function in the kidneys, whereas, the inducible COX-2 is selectively activated by pro-inflammatory stimuli cytokines (IL-1) and growth factors (FGF, PDGF) and facilitates the release of prostaglandins involved in the inflammatory process [3]. These observations suggest that selective COX-2 inhibitors could provide antiinflammatory, analgesic and antipyretic drugs devoid of the unwanted side effects such as ulcers and renal failure associated with the classical nonselective NSAIDs [4]. Selective COX-2 inhibition could also be an important strategy to prevent or treat a number of cancers [5,6] and to delay or slow the clinical expression of Alzheimer's disease [7,8]. Therefore, considerable efforts have been made to discover selective COX-2 inhibitors, which are structurally related to two general classes, the diarylheterocycles and the methanesulphonanilides. Two compounds in the first class, celecoxib [9] and rofecoxib [10] and one in the second, nimesulid, have recently been marketed for the treatment of acute pain, osteoarthritis and rheumatoid arthritis (Fig. 1). Their clinical efficacy was shown similar to that of classical NSAIDs and was associated with a greater gastrointestinal safety [11]. More recently, the structures of some non selective NSAIDs such as indomethacin, zomepirac, aspirin and flurbiprofen have been successfully converted into COX-2 selective inhibitors [12]. In order to facilitate the design and to predict the activity of new selective COX-2 inhibitors, we have previously performed a three-dimensional quantitative structure-activity relationship study (3D-QSAR) [13] using comparative molecular field analysis (CoMFA) [14]. We obtained a predictive model from an extensive series of various derivatives known as selective COX-2 inhibitors. This robust model has been used to design novel selective COX-2 inhibitors and to predict their biological activities. Five compounds were then selected according to their predicted COX-2 inhibitory potency and their

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chemical accessibility (Table 1). We report here, the synthesis and the pharmacological evaluation of these derivatives, which can be considered as new leads for chemical optimisation.

2. Chemistry

Compounds listed in Table 1 were synthesised according to Figs. 2–5.

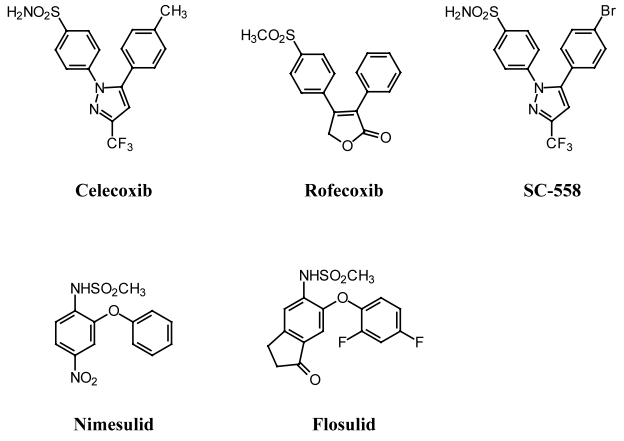
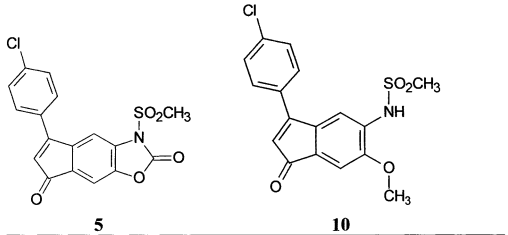
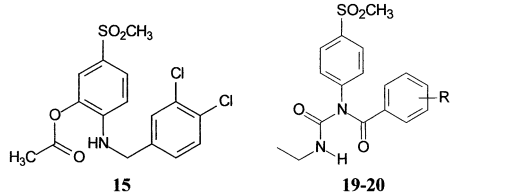


Fig. 1. Structures of selective COX-2 inhibitors.

Table 1
Predicted and experimental IC_{50} values of studied compounds



Compound		Predicted IC_{50} (μM)	Experimental [a] IC_{50} (μM)		COX-1/COX-2 ratio
		COX-2	COX-1	COX-2	
5		0.002	> 5	0.96	> 5.2
10		0.27	26.00	0.37	70



Compound	R	Predicted IC_{50} (μM)	Experimental [a] IC_{50} (μM)		COX-1/COX-2 ratio
		COX-2	COX-1	COX-2	
15	-	0.03	24.00	0.54	44
19	4-F	0.50	4.16	0.27	15.4
20	3,5-diF	0.66	170.00	0.57	298
aspirin			13.70	98.10	0.1
ibuprofen			31.40	81.40	0.4
nimesulid			297.00	1.52	195.4

[a] Experimental IC_{50} values represent the mean values of 3 determinations.

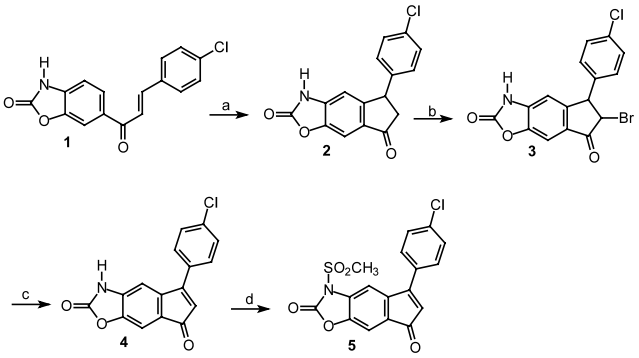


Fig. 2. (a) PPA. (b) Br_2, $MeCO_2H$, chloroform. (c) $N(Et)_3$, acetone. (d) $MeSO_2Cl$, pyridine.

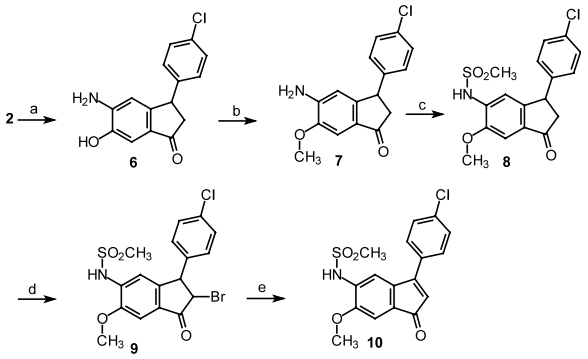


Fig. 3. (a) NaOH, MeOH, H_2O. (b) i, NaOEt; ii, MeI, DMF. (c) $MeSO_2Cl$, pyridine. (d) $CuBr_2$, AcOEt, chloroform. (e) $N(C_2H_5)_3$, acetone.

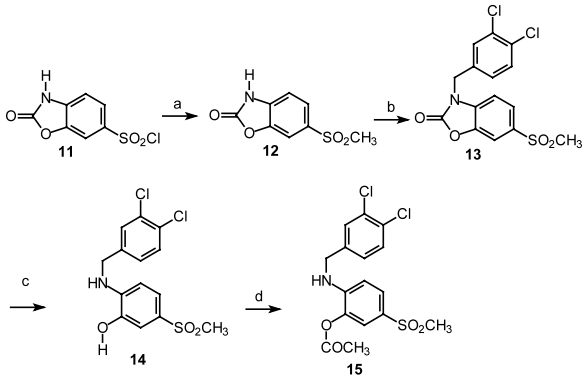


Fig. 4. (a) Na_2SO_3, $NaHCO_3$, $ClCH_2CO_2H$; (b) $ClCH_2C_6H_3Cl_2(3,4)$, K_2CO_3, acetone; (c) NaOH, MeOH, H_2O; (d) $(Ac)_2O$, pyridine, methylene chloride.

The synthesis of indenone **5** is shown in Fig. 2. Intramolecular cyclisation of chalcone **1** [15] was achieved by treatment with polyphosphoric acid [16] at 120 °C affording indanone **2** in 30% yield. The reaction of **2** with bromine in acetic acid–chloroform gave compound **3**. Dehydrobromination of **3** with triethylamine in acetone proceeded at reflux, giving **4**. Treatment of **4** with methanesulphonyl chloride in pyridine gave **5** in 76% yield.

Fig. 3 illustrates the synthesis of compound **10**. The oxazolinone ring of **2** was opened using sodium hydroxide in methanol–water medium. Selective alkylation of the phenolic group of **6** by methyl iodide in the presence of sodium ethoxide in DMF gave **7**. *N*-sulphonation of **7** using methanesulphonyl chloride in pyridine afforded **8**. The indenone **10** was obtained in two-step process. First, generation of the brominated compound **9** with copper (II) bromide in ethyl acetate–chloroform, and second, dehydrobromination with triethylamine in acetone.

Access to compound **15** is presented in Fig. 4. From compound **11** [17], the sulphone **12** was obtained by action of sodium sulphite and chloroacetic acid in water [18]. *N*-alkylation with 3,4-dichlorobenzyl chloride in the presence of potassium carbonate in DMF gave **13**. Ring opening of the oxazolinonic ring afforded compound **14**, which by treatment with acetic anhydride in methylene chloride in the presence of pyridine led to the aminoester **15**.

The synthesis of compounds **19**–**20** is outlined in Fig. 5, starting from 1-ethyl-3-(4-methylsulphanylphenyl)-urea (**16**), which was prepared as previously described [19]. According to standard methods, compound **16** was *N*-acylated with the appropriate benzoyl chloride in methylene chloride to give derivatives **17**–**18**. Finally, compounds **19**–**20** were obtained by oxidation of the appropriate compounds **17**–**18** with oxone in acetone.

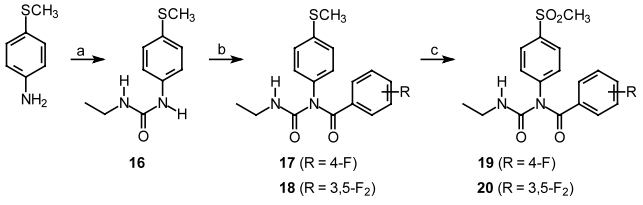


Fig. 5. (a) EtNCO, THF; (b) $ClCOC_6H_4F(4)$ or $ClCOC_6H_3F_2(3,5)$, pyridine, CH_2Cl_2; (c) oxone, acetone.

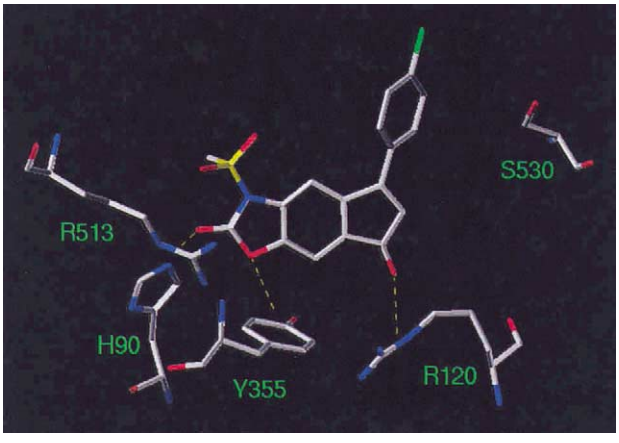


Fig. 6. Compound **5** docked into the COX-2 active site. H-bonds are shown (dashed lines).

3. Pharmacology

The ability of compounds **5**, **10**, **15**, **19** and **20** to inhibit both COX-1 and COX-2 isoforms was determined in a cell assay using mononuclear cells (monocytes, lymphocytes and platelets) isolated from human whole blood by density gradient centrifugation [20]. Their activities against the two isoforms were evaluated measuring the production of thromboxane B_2 (TxB_2) [21] by a specific radioimmunoassay. Western blot analyses were previously carried out to detect the presence of both isoforms. To assess the accuracy of the model, different inhibitors with distinct selectivity for COX-1 versus COX-2 were used as reference substances: aspirin [22,23], ibuprofen [24,25] and nimesulid [26,27]. The cytotoxicity of our compounds was also estimated measuring lactate dehydrogenase activity [28].

4. Results and discussion

The five compounds which were synthesised on account of their activity values predicted in our previous 3D-QSAR model [13] are reported in Table 1. They present some common structural features of most COX-2 inhibitors, namely a phenylmethylsulphonyl or a methanesulphonamide group in the vicinity of another phenyl ring generally substituted by halogens or lower alkyl groups [29,30]. Compounds **5** and **10** can be considered as structural analogues of nimesulid and flosulid whereas **15**, **19** and **20** are related to opened diarylheterocycles. In our 3D-QSAR model these compounds exhibit predicted IC_{50} values lower than 1 μM whereas the predicted IC_{50} values of celecoxib is 0.03 μM. Compound **15** was, therefore, predicted as active as celecoxib and compound **5** ten fold more potent.

The design of these compounds was facilitated by the availability of the X-ray crystal structure of murine COX-2 bound with SC-558, a selective COX-2 inhibitor [31]. The SC-558 conformation extracted from the X-ray crystallographic inhibitor-enzyme complex was used to direct the design of compounds **15**, **19** and **20**, assuming that this conformation represents the most probable bioactive conformation of the diarylheterocycle derivatives at the enzyme level site. The flexible docking of **5**, the most interesting derivative according to its predicted activity value, was performed into the enzyme active site using the FlexiDock module of SYBYL [32]. This study suggests that hydrogen bonding occurs between **5** and three residues of the enzyme active site, His90, Arg120 and Tyr355 (Fig. 6). Compound **5** binds to the positive-charged Arg120 in the COX-2 nonselective binding site. Its methylsulphonyl group occupies the COX-2 side-pocket without interacting with the hydrophilic residues His90 or Arg513

[29]. However, we notice that sp^2 and sp^3 oxygen atoms of the benzoxazolinone ring accept H-bonds from His90 and Tyr355, respectively. This study has also shown that **5** could be correctly fitted on the bioactive conformation of SC-558 when positioned in the protein cavity.

The results of the experimental IC_{50} values obtained with both COX-1 and COX-2 are summarised in Table 1. The reference compound nimesulid is around 200 times more potent against COX-2 than COX-1, whereas aspirin and ibuprofen are ca. equipotent. These results are in agreement with previous published data [24–27]. As for references, our compounds were able to inhibit the TxB_2 production by stimulated human mononuclear cells in a dose-dependent manner. The experimental values are in good agreement with the predicted ones except for compound **5**, which theoretically was the best inhibitor. A possible explanation for this exception could be related with the test used in this study and the physicochemical properties of this compound. As a matter of fact: (1) the COX-1 IC50 value of **5** could not be accurately evaluated because of its lack of solubility. (2) In our 3D-QSAR model, the inhibitory values of the selected compounds had been obtained from the same biological method using the recombinant enzyme [33]. (3) In the current study we preferred a whole cell test since it takes into account the penetration of the compounds through the membranes.

Compounds **10**, **15** and **19** were found to be almost three to four times more potent than nimesulid in terms of COX-2 inhibition but their COX-1/COX-2 ratios were lower than this reference. Compound **20** emerges as the most promising one since it exhibits a COX-1/COX-2 ratio value of 298 and a COX-2 inhibitory activity of 0.57 μM, higher than that of the reference inhibitor. For all compounds tested, we have also checked the lack of cytotoxicity ($< 5\%$) dosing lactate dehydrogenase activity.

5. Conclusions

In conclusion, we have described the synthesis of five novel selective COX-2 inhibitors that we designed on the basis of a 3D-QSAR study. Their biological evaluation using a whole cell assay has confirmed the predictive power of our 3D-QSAR model since they were found at least as potent as nimesulid against COX-2. Nevertheless only compound **20** has shown an interesting selectivity with a COX-1/COX-2 ratio reaching 298. These results allow us to select it as a lead compound in view to develop a new series of potent and selective COX-2 inhibitors. Moreover, compound **5** that was the best predicted one cannot be dissolved to a concentration higher 5 μM. Its lack of solubility did not allow us to accurately determine its selectivity (COX-1/COX-2 ratio). Furthermore, we think that this could also explain the great difference between its COX-2 predicted and experimental inhibitory values. Nevertheless, the high COX-2 predicted value for this compound needs and justify further chemical and pharmacological stu-dies, which are developed after present time.

6. Experimental

6.1. Chemistry

Melting points (m.p.) were determined on a Büchi SMP-20 capillary apparatus and are uncorrected. IR spectra were recorded on a Vector 22 Brucker spectrophotometer. ^{1}H-NMR spectra were recorded on a AC 300 Brucker spectrometer. Chemical shifts are reported in δ units (parts per million) relative to $(Me)_4Si$. Elemental analyses for final substances were performed by CNRS Laboratories (Vernaison, France). Obtained results were within 0.4% of the theoretical values.

6.1.1. 5-(4-Chlorophenyl)-3H-indano[5,6-d]oxazol-2,7-dione (2)

Compound **1** (39 g, 130 mmol) and PPA (400 g) were heated at 120 °C during 1 h. The reaction mixture was quenched with ice water and the precipitate was filtered, washed with water and dried. The obtained solid was refluxed in chloroform (200 mL) for 2 h. After filtration and evaporation of the organic phase, the residue was recrystallised from acetonitrile (12 g, 30%): m.p. 250–251 °C; IR ν NH 3200 cm^{-1}, CO 1775, 1670 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.55 (dd, 1H, $J = 3.79$, 18.90 Hz), 3.22 (dd, 1H, $J =$ 7.56, 18.90 Hz), 4.65 (dd, 1H, $J = 3.79$, 7.56 Hz), 6.79 (s, 1H), 7.22 (d, 1H, $J = 8.64$ Hz), 7.38 (d, 1H, $J = 8.64$ Hz), 7.50 (s, 1H), 12.00 (br s, 1H, exchangeable with D_2O).

6.1.2. 6-Bromo-5-(4-chlorophenyl)-3H-indano[5,6-d]-oxazol-2,7-dione (3)

To a cooled suspension of compound **2** (3.0 g, 10 mmol) in 60 mL of a mixture of chloroform–acetic acid (1/1) was added dropwise under stirring a solution of bromine (0.52 mL, 10 mmol) in chloroform (20 mL). The reaction mixture was stirred at r.t. for 12 h. Solvents were evaporated under reduced pressure and the solid was triturated with petroleum ether. The precipitate was dried and recrystallised from acetonitrile (1.63 g, 43%): m.p. 235–236 °C; IR ν NH 3140 cm^{-1}, CO 1770, 1670 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 4.81 (d, 1H, $J = 4.29$ Hz), 5.12 (d, 1H, $J = 4.29$ Hz), 6.54 (s, 1H), 7.21 (d, 2H, $J = 8.37$ Hz), 7.46 (d, 2H, $J = 8.37$ Hz), 7.68 (s, 1H), 12.25 (br s, 1H, exchangeable with D_2O).

*6.1.3. 5-(4-Chlorophenyl)-3H-indeno[5,6-d]oxazol-2,7-dione (**4**)*

To a solution of compound **3** (1.0 g, 2.64 mmol) in acetone (30 mL) was added triethylamine (1.47 mL, 11 mmol). The reaction mixture was refluxed for 12 h and acetone was evaporated under reduced pressure. The solid was triturated with water (100 mL) and acidified with an aqueous solution of 1 M HCl. The precipitate was filtered, washed with water, dried and recrystallised from dioxane (0.48 g, 61%): m.p. > 270 °C; IR *v* NH 3209 cm^{-1}, CO 1815, 1690 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 6.19 (s, 1H), 7.01 (s, 1H), 7.38 (s, 1H), 7.61 (d, 2H, $J = 8.57$ Hz), 7.74 (d, 2H, $J = 8.57$ Hz), 12.04 (br s, 1H, exchangeable with D_2O).

*6.1.4. 5-(4-Chlorophenyl)-3-methanesulphonyl-3H-indeno[5,6-d]oxazol-2,7-dione (**5**)*

To a suspension of compound **4** (2.0 g, 6.7 mmol) in pyridine (10 mL) was added slowly methanesulphonyl chloride (0.70 mL, 8.7 mmol). The reaction mixture was stirred for 12 h at room temperature (r.t.), poured into water and acidified with an aqueous solution of 3 M HCl. The precipitate was filtered, washed with water, dried and recrystallised from acetonitrile (1.91 g, 76%): m.p. 222–223 °C; IR *v* CO 1804, 1708 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.73 (s, 3H), 6.34 (s, 1H), 7.54 (s, 1H), 7.68 (s, 1H), 7.69 (d, 2H, $J = 8.72$ Hz), 7.78 (d, 2H, $J = 8.72$ Hz). Anal. Calc. for $C_{17}H_{10}ClNO_5S$: C, 54.34; H, 2.68; N, 3.73. Found: C, 54.16; H, 2.66; N, 3.85%.

*6.1.5. 5-Amino-3-(4-chlorophenyl)-6-hydroxyindan-1-one (**6**)*

To a solution of compound **2** (10.0 g, 34 mmol) in methanol (120 mL) was added a solution of sodium hydroxide (20 g, 500 mmol) in water (120 mL). The reaction mixture was refluxed for 24 h. After cooling, the solution was acidified with an aqueous solution of 6M HCl, filtered and basified with a saturated solution of $NaHCO_3$. The precipitate was filtered, washed with water, dried and recrystallised from EtOAc(4.65 g, 50%): m.p. 260–261 °C; IR *v* OH 3360 cm^{-1}, NH_2 3260 cm^{-1}, CO 1650 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.29 (dd, 1H, $J = 2.99$, 18.55 Hz), 2.95 (dd, 1H, $J = 7.77$, 18.55 Hz), 4.36 (dd, 1H, $J = 2.99$, 7.77 Hz), 5.73 (br s, 2H, exchangeable with D_2O), 6.25 (s, 1H), 6.85 (s, 1H), 7.14 (d, 2H, $J = 8.38$ Hz), 7.34 (d, 2H, $J = 8.38$ Hz), 9.72 (br s, 1H, exchangeable with D_2O).

*6.1.6. 5-Amino-3-(4-chlorophenyl)-6-methoxyindan-1-one, hydrochloride (**7**)*

To a freshly prepared solution of sodium (0.44 g, 0.0189 at. gr) in absolute ethanol (120 mL) was added **6** (4.3 g, 15.7 mmol). The reaction mixture was stirred at r.t. during 1 h and then evaporated under reduced pressure. The residue was dissolved in dimethylformamide (30 mL) and methyl iodide (1.18 mL, 18.9 mmol) was added slowly. After stirring for 1 h at r.t., the reaction mixture was poured into water (200 mL) and acidified with an aqueous solution of 6 M HCl. The precipitate was filtered, washed with water, dried and recrystallised from EtOAc (3.21 g, 63%): m.p. 100–110 °C; IR *v* NH_3^+ 3000–2600 cm^{-1}, CO 1700 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.55 (dd, 1H, $J = 3.20$, 18.87 Hz), 3.16 (dd, 1H, $J = 7.60$, 18.87 Hz), 3.86 (s, 3H), 4.43 (dd, 1H, $J = 3.20$, 7.60 Hz), 6.37 (s, 1H), 6.93–7.01 (m, 4H, NH_3^+ exchangeable with D_2O), 7.14–7.28 (m, 4H).

*6.1.7. N-[3-(4-Chlorophenyl)-6-methoxy-1-oxo-indan-5-yl]methanesulphonamide (**8**)*

To a suspension of compound **7** (0.3 g, 0.93 mmol) in pyridine (3 mL) was added slowly methanesulphonyl chloride (0.1 mL, 1.2 mmol). The reaction mixture was stirred for 12 h at r.t., poured into water and acidified with an aqueous solution of 3 M HCl. The precipitate was filtered, washed with water, dried and recrystallised from EtOAc (0.20 g, 60%): m.p. 169–170 °C; IR *v* NH 3340 cm^{-1}, CO 1700 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.60 (dd, 1H, $J = 3.46$, 18.61 Hz), 3.20 (dd, 1H, $J = 7.79$, 18.61 Hz), 3.51 (s, 3H), 3.99 (s, 3H), 4.53 (dd, 1H, H_3, $J = 3.46$, 7.79 Hz), 7.21 (s, 1H), 7.24 (s, 1H), 7.26 (d, 2H, $J = 8.66$ Hz), 7.52 (d, 2H, $J = 8.66$ Hz), 10.95 (br s, 1H, exchangeable with D_2O).

*6.1.8. N-[2-Bromo-3-(4-Chlorophenyl)-6-methoxy-1-oxo-indan-5-yl]methanesulphonamide (**9**)*

Compound **8** (4.3 g, 11.8 mmol) and copper(II) bromide (3.2 g, 14.1 mmol) in 120 mL of a mixture of chloroform–EtOAc(1/1) were refluxed for 12 h. The reaction mixture was filtered and concentrated under reduced pressure. The precipitate was filtered, dried and recrystallised from ethanol (2.6 g, 45%): m.p. 182–184 °C; IR *v* NH 3262 cm^{-1}, CO 1720 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.09 (s, 3H), 3.94 (s, 3H), 4.75 (d, 1H, $J = 4.42$ Hz), 5.02 (d, 1H, $J = 4.42$ Hz), 6.54 (s, 1H), 7.21 (d, 2H, $J = 8.37$ Hz), 7.46 (d, 2H, $J = 8.37$ Hz), 7.68 (s, 1H), 12.25 (br s, 1H, exchangeable with D_2O).

*6.1.9. N-[3-(4-Chlorophenyl)-6-methoxy-1-oxo-1H-inden-5-yl]methanesulphonamide (**10**)*

To a solution of compound **9** (2.6 g, 5.85 mmol) in acetone (80 mL) was added triethylamine (3.3 mL, 23.4 mmol). The reaction mixture was stirred at r.t. for 72 h and then evaporated under reduced pressure. An aqueous (aq.) 1 M HCl solution was added and the precipitate was filtered, washed with water, dried and recrystallised from acetonitrile (0.40 g, 19%): m.p. 195–197 °C; IR *v* NH 3247 cm^{-1}, CO 1699 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.04 (s, 3H), 3.92 (s,

3H), 6.20 (s, 1H), 7.28 (s, 1H), 7.39 (s, 1H), 7.38 (s, 1H), 7.65 (d, 2H, $J = 8.53$ Hz), 7.77 (d, 2H, $J = 8.53$ Hz), 9.29 (br s, 1H, exchangeable with D_2O). Anal. $C_{17}H_{14}ClNO_4S$: C, 56.12; H, 3.87; N, 3.85. Found: C, 56.35; H, 3.86; N, 3.72%.

6.1.10. 6-Methanesulphonyl-2(3H)-benzoxazolone ***(12)***

To a solution of Na_2SO_3 (0.63 g, 5.0 mmol) and $NaHCO_3$ (1.26 g, 15.0 mmol) in water (60 mL) was added at 75 °C portionwise and over 10 min compound **11** (1.0 g, 5.0 mmol). Heating was continued for 1 h and chloroacetic acid (0.7 g, 7.5 mmol) was added. The resulting solution was refluxed for 48 h. After cooling the precipitate was collected by filtration, dried and recrystallised from acetonitrile (0.34 g, 32%): m.p. > 270 °C; IR ν NH 3271 cm^{-1}, CO 1788 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.35 (s, 3H), 7.30 (d, 1H, $J = 8.27$ Hz), 7.72 (d, 1H, $J = 8.27$ Hz), 7.85 (s, 1H), 12.23 (br s, 1H, exchangeable with D_2O).

6.1.11. 3-(3,4-Dichlorobenzyl)-6-methanesulphonyl-2(3H)-benzoxazolone ***(13)***

A mixture of compound **12** (1.6 g, 7.5 mmol), K_2CO_3 (3.1 g, 22.5 mmol) in dimethylformamide (10 mL) was heated at 70 °C during 1 h. 3, 4-Dichlorobenzyl chloride chloride (1.25 mL, 9 mmol) was added and heating was continued for an additional 2 h. After cooling, the reaction mixture was poured into water and acidified with an aqueous solution of 6 M HCl. The precipitate was filtered, washed with water, dried and recrystallised from toluene from acetonitrile (2.74 g, 98%): m.p. 211–212 °C; IR ν CO 1760 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.22 (s, 3H), 5.12 (s, 2H), 7.38 (d, 1H, $J = 8.25$ Hz), 7.47 (d, 1H, $J = 8.26$ Hz), 7.62 (d, 1H, $J = 8.26$ Hz), 7.76–7.80 (m, 2H), 7.93 (s, 1H).

6.1.12. 2-(3,4-Dichlorobenzylamino)-5-methanesulphonyl-phenol ***(14)***

This compound was prepared following the procedure described for compound **6**. From compound **13** (3.1 g, 8.3 mmol), sodium hydroxide (1.15 g, 29 mmol), methanol (150 mL) and water (50 mL). Recrystallisation from acetonitrile (2.0 g, 71%): m.p. 173 °C; IR ν OH 3410 cm^{-1}; NH 3400 ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.00 (s, 3H), 4.45 (d, 2H, $J = 5.70$ Hz), 6.42 (d, 1H, $J = 8.80$ Hz), 6.55 (br s, 1H, exchangeable with D_2O), 7.10–7.15 (m, 2H), 7.32 (dd, 1H, $J = 2.07$, 8.28 Hz), 7.55 (d, 1H, $J = 8.28$ Hz), 7.60 (s, 1H), 10.20 (br s, 1H, exchangeable with D_2O).

6.1.13. 2-(3,4-Dichlorobenzylamino)-5-methanesulphonylphenyl acetate ***(15)***

To a solution of compound **14** (1.0 g, 2.9 mmol) in methylene chloride (40 mL) were added pyridine (0.73 mL, 8.7 mmol) and acetic anhydride (0.4 mL, 4.3 mmol). The reaction mixture was stirred at r.t. for 12 h, quenched with water and extracted with methylene chloride. The organic layer was separated, washed with a 2 M HCl solution, dried over $MgSO_4$ and evaporated under reduced pressure. The solid was triturated with diethylether, filtered and recrystallised from toluene (0.34 g, 30%): m.p. 127–128 °C; IR ν NH 3393 cm^{-1}, CO 1764 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.36 (s, 3H), 3.00 (s, 3H), 4.40 (d, 2H, $J = 5.60$ Hz), 6.66 (d, 1H, $J = 8.38$ Hz), 7.11 (br s, 1H, 1H exchangeable with D_2O), 7.28 (dd, 1H, $J = 1.20$, 8.38 Hz), 7.48–7.50 (m, 2H), 7.58–7.64 (m, 2H). Anal. $C_{16}H_{15}Cl_2NO_4S$: C, 49.48; H, 3.89; N, 3.60. Found: C, 49.62; H, 3.76; N, 3.56%.

6.1.14. 1-(4-Fluorobenzoyl-1-(4-methylsulphanylphenyl)-3-ethylurea ***(17)***

To a cooled solution of compound **16** (0.8 g, 3.8 mmol) in dichloromethane (60 mL) were added pyridine (1.3 mL, 15.3 mmol) and slowly 4-fluorobenzoyl chloride (0.55 mL, 4.6 mmol). The reaction mixture was refluxed for 12 h, poured into water and acidified with an aq. 1 M HCl solution. The organic layer was washed with water, dried over $MgSO_4$ and evaporated under reduced pressure. The residue was recrystallised from cyclohexane (0.34 g, 27%): m.p. 143–145 °C; IR ν NH 3310 cm^{-1}, CO 1683, 1632 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 1.28 (t, 3H, $J = 7.44$ Hz), 2.42 (s, 3H), 3.44 (m, 1H), 6.86–7.31 (m, 4H), 8.13–8.19 (m, 4H), 8.95 (m, 1H, exchangeable with D_2O).

6.1.15. 1-(3,5-Difluorobenzoyl)-1-(4-methylsulphanylphenyl)-3-ethylurea ***(18)***

This compound was prepared following the procedure described for compound **17**. From compound **16** (1.0 g, 4.8 mmol), pyridine (1.2 mL, 14.4 mmol) and 3,5-difluorobenzoyl chloride (0.8 mL, 5.76 mmol). Recrystallisation from cyclohexane (0.8 g, 48%): m.p. 119–121 °C; IR ν NH 3313 cm^{-1}, CO 1712, 1660 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 1.27 (t, 3H, $J = 7.37$ Hz), 2.44 (s, 3H), 3.40 (m, 1H), 6.69–6.82 (m, 3H), 7.02 (d, 2H, $J = 8.42$ Hz), 7.12 (d, 2H, $J = 8.42$ Hz), 8.72 (m, 1H, exchangeable with D_2O).

6.1.16. 1-(4-Fluorobenzoyl)-1-(4-methanesulphonylphenyl)-3-ethylurea ***(19)***

To a solution of compound **17** (0.17 g, 0.50 mmol) in acetone (5 mL) was added a solution of oxone (1.2 g, 1.8 mmol) in 10 mL water. After stirring for 1 h at r.t., the reaction mixture was diluted with water and extracted with EtOAc. The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was recrystallised from EtOAc (0.11 g, 60%): m.p. 130–131 °C; IR ν NH 3312 cm^{-1}, CO 1711, 1664 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.26 (m, 3H, $J = 7.11$ Hz), 3.02 (s, 3H), 3.40 (m, 2H), 6.87–6.92 (m, 2H), 7.24–7.29 (m, 2H) 7.35 (d, 2H, $J = 8.23$ Hz), 7.84

(d, 2H, $J = 8.23$ Hz), 8.88 (m, 1H, exchangeable with D_2O). Anal. $C_{17}H_{17}FN_2O_4S$: C, 56.04; H, 4.70; N, 7.68. Found: C, 56.12; H, 4.75; N, 7.75%.

6.1.17. 1-(3,5-Difluorobenzoyl)-1-(4-methanesulphonylphenyl)-3-ethylurea (**20**)

This compound was prepared following the procedure described for compound **19**. From **18** (0.5 g, 1.43 mmol), acetone (20 mL) and oxone (3.2 g, 5.2 mmol) in 25 mL water. Purification by column chromatography (EtOAc–cyclohexane: 6/4) (0.48 g, 88%): m.p. 118–120 °C; IR ν NH 3312 cm^{-1}, CO 1711, 1664 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.26 (m, 3H, $J = 7.28$ Hz), 3.02 (s, 3H), 3.42 (m, 2H), 6.74–6.79 (m, 3H), 7.36 (d, 2H, $J = 8.33$ Hz), 7.87 (d, 2H, $J = 8.33$ Hz), 8.73 (m, 1H, exchangeable with D_2O). Anal. $C_{17}H_{16}F_2N_2O_4S$: C, 53.40; H, 4.21; N, 7.32. Found: C, 53.42; H, 4.32; N, 7.43%.

6.2. Pharmacology

6.2.1. Chemical products

Buffer compounds and chemical products were purchased from Sigma Chemical (St Louis, MO, USA). Primary and secondary antibodies were purchased from Tebu (Le Perray en Yvelines, France). ECL Western blotting detection reagents and Hyperfilm ECL were purchased from Amersham International plc (Buckinghamshire, UK).

6.2.2. Cell separation technique

Mononuclear cells were isolated as described in the first step of PMN preparation [20]. Once 15 mL fresh by drawn human heparinised blood, obtained from healthy donors, has been diluted 1:2 with a 0.1 M phosphate buffer saline, pH 7.4, 10 mL of Histopaque®-1077 was placed at the bottom of a conical tube. After centrifugation ($400 \times g$ for 30 min at 20 °C), the upper layer, composed of monocytes, lymphocytes and platelets, was recovered. The suspension was centrifuged ($400 \times g$ for 10 min at 4 °C), and the pellet was resuspended in a Hanks'HEPES buffer pH 7.4. Cell viability, determined by blue Trypan exclusion, was over 95%. The cells were kept at 4 °C until use.

6.2.3. Assessment of the cytotoxicity of the compounds

The cytotoxicity of the different inhibitors was estimated by dosing lactate deshydrogenase activity in the cellular supernatant 5×10^6 cells were incubated in 1 mL of Hank's Hepes buffer at 37 °C with several concentrations of the compound. The suspension was centrifuged ($400 \times g$ for 10 min at 4 °C), all supernatants were separated, the pellet of the reference tube was resuspended with 1 mL of water and shaken for 5 min to lyse the cells. This tube was centrifuged ($400 \times g$ for 10 min at 4 °C), and the supernatant was also separated. The enzymatic activity of lactate deshydrogenase was measured at 30 °C by enzymatic kinetic read at 340 nm after adding 0.2 mM NADH and 1.6 mM pyruvate, as described in manufacturer's protocol [28]. The percentage of lactate deshydrogenase released was estimated in comparison with the maximal activity of this enzyme.

6.2.4. In vitro COX-1 assay

The lymphocytes and the monocytes were preincubated with the inhibition, if necessary, then they were incubated with calcium ionophore 50 μM A 23187 and 50 μM arachidonic acid for 1 h at 37 °C. At the end of the incubation, the tubes were centrifuged ($400 \times g$ for 10 min at 4 °C). The TxB_2 was extracted and measured from the supernatant as described later.

6.2.5. In vitro COX-2 assay

When the lymphocytes and the monocytes had been incubated with 500 μM acetyl salicylic acid for 30 min to block the catalytic site of COX-1, the compound was eliminated with several washings. The cells were placed in contact with the inhibitors, if necessary, and incubated with 8 μg mL^{-1} lipopolysaccharide and 50 μM arachidonic acid for 3 h at 37 °C. Cell suspensions were centrifuged ($400 \times g$ for 10 min at 4 °C) and TxB_2 was extracted and measured from the supernatant as described later.

6.2.6. Extraction of thromboxane B_2 [34]

Two milliliter of acetone were added to 1 mL of supernatant, and the tubes were shaken for 2 min. They were then centrifuged ($400 \times g$ for 10 min at 4 °C). The supernatants were transferred to a separate tube and 2 mL of hexane were added. The tubes were shaken for 2 min and then centrifuged ($400 \times g$ for 5 min at 4 °C). The upper layers were kept and the pH of each lower layer was adjusted to 3.0–4.0 with 1 M citric acid solution. Following this, 2 mL of chloroform was added, the tubes were shaken for 2 min, and centrifuged ($400 \times g$ for 5 min at 4 °C). The lower chloroform layers containing TxB_2, were separated, and the top layer was processed, for another extraction, with 2 mL of chloroform. The two chloroform extracts were combined. The tubes were dried under nitrogen, and TxB_2 was assayed as described by the manufacturer's protocol.

6.2.7. Statistical studies

The data obtained were subjected to statistical analysis using a non-parametric test (Student's), where P-values < 0.05 were considered significant versus reference.

6.3. Molecular modelling

Molecular modelling studies were performed using SYBYL software version 6.4 [32] running on Silicon Graphics workstations. The geometry of all compounds was subsequently optimised using the Tripos force field [35] including the electrostatic term calculated from Gasteiger and Hückel atomic charges. The method of Powell available in Maximin2 procedure was used for energy minimisation until the gradient value was smaller than 0.001 kcal mol^{-1} $Å^{-1}$. The structure of the COX-2 enzyme was obtained from its complexed X-ray crystal structure with SC-558 available in the RCSB Protein Data Bank (1CX2) [31]. Flexible docking of compounds into the enzyme active site was performed using the FlexiDock module of SYBYL. The complexes were energy-minimised using the Powell method available in Maximin2 procedure with the MMFF94 force field [36] and a dielectric constant of 4.0 until the gradient value reached 0.05 kcal mol^{-1} $Å^{-1}$. The anneal function was used defining around the inhibitor a hot region (10 Å) and an interesting region (15 Å).

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