Designing Multitarget Anti-inflammatory Agents: Chemical Modulation of the Lumiracoxib Structure toward Dual Thromboxane Antagonists-COX-2 Inhibitors.

UNIVERSITÀ DEGLI STUDI DI TORINO

Designing multitarget anti-inflammatory agents: chemical modulation of lumiracoxib structure toward dual thromboxane antagonists-COX-2 inhibitors

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Abstract

*A series of lumiracoxib derivatives were designed to explore the influence of isosteric substitution on balancing COX-2 inhibition and thromboxane A_2 prostanoid (TP) receptor antagonism. The compounds were synthesized through a copper-catalyzed coupling procedure and characterized for their pK_a values. TP receptor antagonism was assessed on human platelets; COX-2 inhibition was determined on human isolated monocytes and human whole blood. TPα receptor binding of the most promising compounds was evaluated through radioligand-binding assays. Some of the isosteric substitutions at the carboxylic acid group afforded compounds with improved TP receptor antagonism (**18**, **20**, **27**, **31**); of these, the tetrazole derivative **18** retained good COX-2 inhibitory activity and selectivity. The identification of **18** acting as a balanced dual-acting compound, in human whole blood, and the SARs analysis of the synthesized lumiracoxib derivatives, might give a contribution to the rational design of a new class of cardioprotective anti-inflammatory agents.*

Introduction

Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) display antiinflammatory, analgesic, and antipyretic activities, and are the most widely used drugs. Their action mechanism is mainly connected with their capacity to inhibit the COX-enzyme involved in transforming arachidonic acid (AA) into prostanoids (prostaglandins PGs, thromboxane A_2 TXA_2, and prostacyclin PGI_2).[1] Two isoforms of this enzyme are known: COX-1 and COX-2. The former is constitutively expressed in most tissues and generates PGs involved primarily in 'housekeeping' functions, i.e. gastric cytoprotection and haemostatic integrity. COX-2 is highly regulated and its expression can be induced in response to inflammatory stimuli, although it is expressed constitutively in the brain, kidney, and some types of endothelial cells.[2]

Classical NSAIDs inhibit both isoforms, albeit with different relative potencies depending on their structure. This inhibition of COX-1, which is present in the gastric mucosa where it induces formation of gastroprotective PGE_2, combined with local damage caused directly by the drug, are responsible for NSAIDs gastrotoxic effects; these comprise gastric discomfort and severe effects including ulcers, bleeding and perforation.[3-5] A number of strategies have been proposed to reduce NSAID-induced gastroduodenal damage: co-therapy with various gastroprotectants including zinc (Zn) compounds, administration of NSAIDs chemically pre-associated with phosphatidylcholine (PC), complex formation of Zn-NSAIDs, nitric oxide (NO) or hydrogen sulphide (H_2S) releasing NSAIDs, and dual inhibitors of COX and 5-LOX (5-lipoxygenase).[5,6]

Identification and characterisation of the COX-2 isoform in inflammatory cells made it possible to design a new class of NSAIDs: the so called COXIBs.[7-9] These compounds are selective inhibitors of this isoform, and consequently display antiinflammatory activity and reduced gastrotoxicity compared with the classical NSAIDs. Celecoxib (Celebrex) **1** and rofecoxib (Vioxx) **2** were the first two products of this class to enter therapeutic use (Figure 1). After the launch of the COXIBs, however, increasing evidence of cardiovascular risk emerged for these compounds, leading to withdrawal of rofecoxib and valdecoxib from the market.[10] Cardiovascular risk is now considered to be of general concern with long-

term therapy not only with COXIBs, but also with traditional NSAIDs.[11] According to the “imbalance theory”, cardiotoxicity is the result of these drugs inducing a shift of the intricate prostanoid balance toward the platelet aggregation stimulator and vasoconstrictor TXA_2, and away from the platelet aggregation inhibitor and vasodilator PGI_2.[12] Indeed, celecoxib and rofecoxib have been found to induce a significant reduction in the urinary excretion of 2,3-dinor 6-keto $PGF_{1\alpha}$, the principal PGI_2 metabolite, and a predictive index of its vascular non-renal generation.[13] There is now great interest in designing new anti-inflammatory drugs that combine the anti-inflammatory activity of COXIBs with a cardioprotective component. While this could be achieved by combining existing drugs, the co-administration of two different molecules might not be the best pharmacological approach. One strategy that has received particular attention is the realization of hybrid drugs (multitarget drugs) in which a selective COX-2 inhibitor is combined with moieties able to release nitric oxide (NO-COXIBs).[14-18] This messenger is known to display multiple actions at the level of cardiovascular system: vasodilation, inhibition of platelet aggregation, modulation of platelet- and leucocyte-adhesion to the endothelium, regulation of vascular smooth-muscle cell proliferation.[19] Consequently, it may be expected to resolve or ameliorate the cardiovascular issues raised by common COXIBs.

Recently, a strategy has been proposed whereby hybrid structures are developed that combine the ability to selectively inhibit COX-2 enzyme and thromboxane prostanoid (TP) receptor antagonism.[10, 20] Only in humans, TP_α and TP_β isoform expression is a product of mRNA splicing, with TP_α expression the default.[21] These isoforms possess different tail lengths, the β isoform tail being longer than that of the α isoform. Activation of TP receptors induces platelet aggregation, constriction of vascular smooth-muscle cells, as well as mitogenesis and hypertrophy of vascular smooth-muscle cells. TXA_2 formation is increased in thrombotic disorders and has been implicated in a variety of cardiovascular diseases.[22]

Considering that the clinical efficacy of aspirin in cardiovascular syndromes is believed to be due to its inhibition of platelet TXA_2 synthesis, antagonism of TP receptors may be expected to provide similar anti-thrombotic protection. Indeed, terutroban (**3**) (figure 1), an oral selective antagonist of TP receptors in platelets and in the vessel wall, showed non-inferiority compared to aspirin in the secondary prevention of cardiovascular ischemic events in patients with a non-cardioembolic cerebral ischemic event.[23] Recently, our findings have shown that lumiracoxib (**4**), a well known potent and selective COX-2 inhibitor (Figure 1), also displays competitive TP receptor antagonist properties; however, these two activities are unfortunately not well balanced, the former largely prevailing over the latter.[24] Although after its introduction lumiracoxib was withdrawn from the market, owing to adverse liver toxicity,[25] it represents a good lead for further manipulation.

In order to be effective, a hybrid drug must display the desired activities in the same concentration range.[26] This study describes an attempt to obtain new COXIBs with an in vitro improved balancing of COX-2 inhibition and TP receptor antagonism with respect to lumiracoxib by substituting the carboxylic function present in this lead with non-classical isosteres of acid groups. This approach was recently used to modulate TP receptor antagonists by substituting the carboxylic function with different cyclopentane 1,3-dione moieties.[27]

The synthesis, structural and physico-chemical characterization of these new products, their ability to inhibit COX-1 and COX-2 enzymes, and their antagonist properties versus TP receptor, are reported and discussed; a brief insight into SARs is also presented.

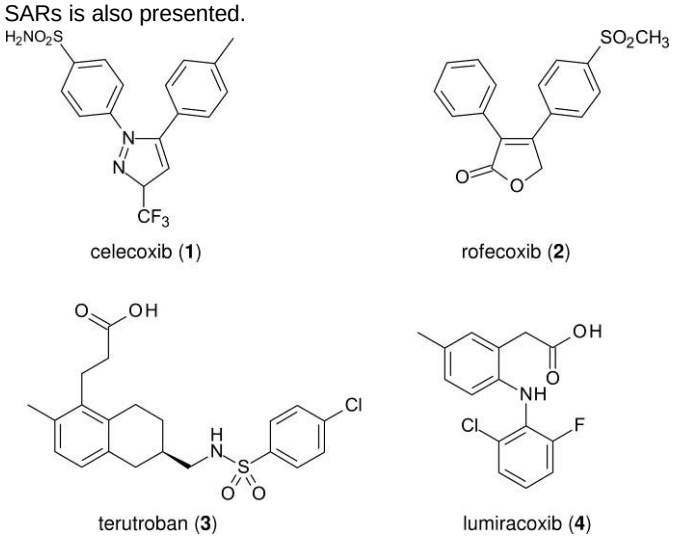


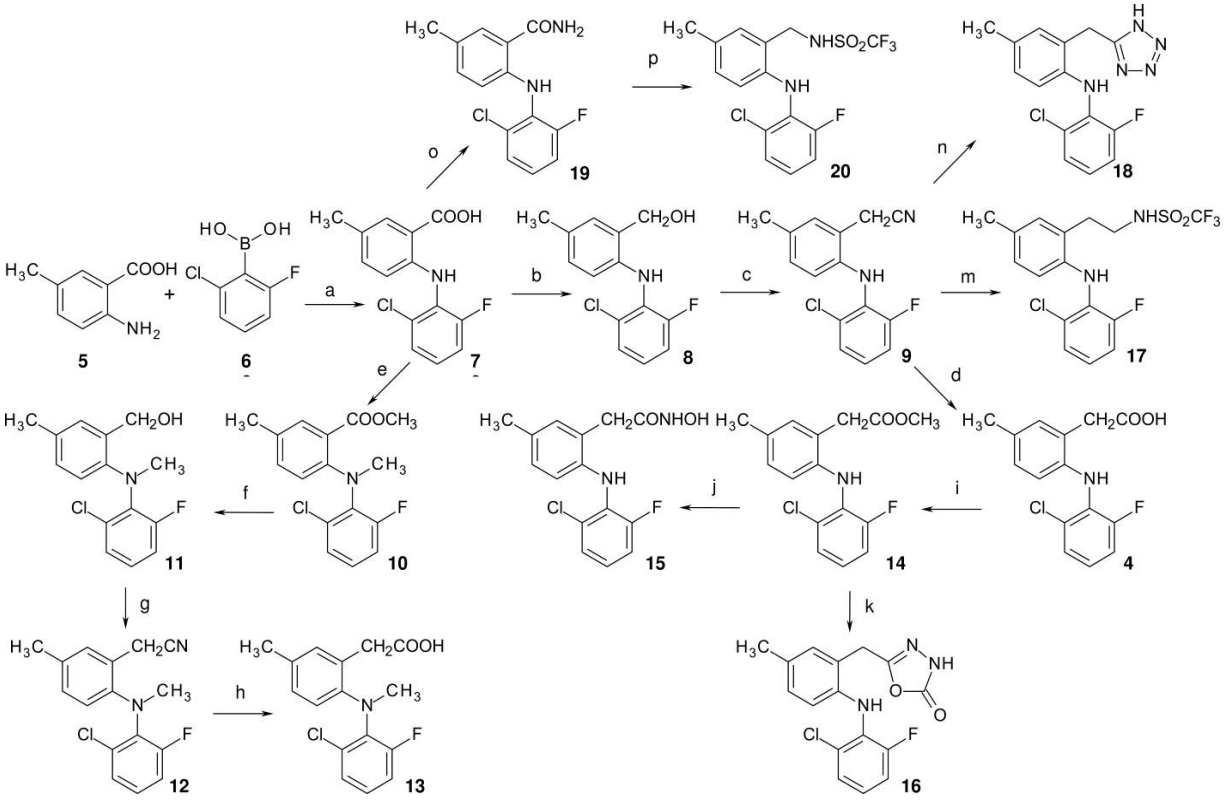
Figure 1. Structures of celecoxib, rofecoxib, terutroban and lumiracoxib.

Results and Discussion

Chemistry

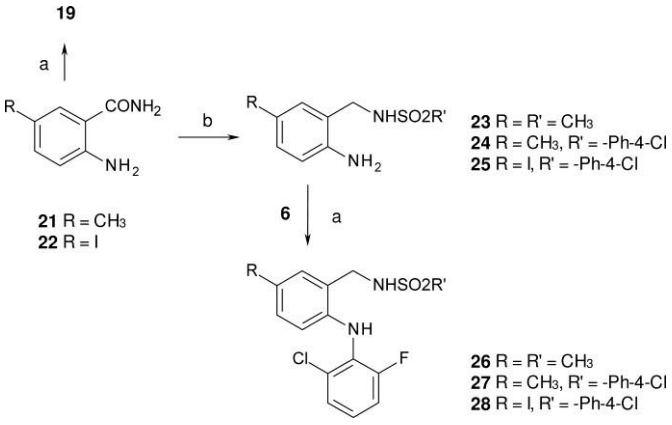
The carboxylic function present in the lead was replaced with a number of non-classical isoster groups[28]: hydroxamic function (comp. **15**), differently substituted reversed sulfonamido moieties (comps. **17**, **20**, **26**, **27**, **31**), 1,3,4-oxadiazol-2(3*H*)-one and tetrazole planar rings (comps. **16**, **18**). Lumiracoxib (**4**) and its *N*-methyl derivative **13** were also considered as references. The synthesis of the latter two compounds is shown in Scheme 1, together with that of models **15**-**18**, **20**. The common starting compound to obtain these structures was the commercially available 2-amino-5-methylbenzoic acid (**5**), which was coupled with 2-chloro-6-fluorophenylboronic acid (**6**) in the presence of 1,8-diazabicylo[5,4,0]undec-7-ene (DBU) and a stoichiometric amount of copper acetate in dioxane solution (Chan-Lam coupling[29]). The resulting acid **7** deriving from aryl carbon-nitrogen bond formation was reduced to the alcohol **8** using $BH_3 \cdot SMe_2$ complex. This intermediate was treated with pyridine/$SOCl_2$ to give the corresponding chloride that, without isolation or characterization, was immediately transformed into the nitrile **9** by action of KCN in DMSO. This is a key product for obtaining target compounds **17** and **18**. The former arises from the reduction of **9**, by the complex $BH_3 \cdot THF$ in refluxing THF and subsequent sulfonylation using trifluoromethansulfonic anhydride in the presence of Et_3N; the latter is obtained by action of NaN_3 in DMF.

To prepare the target compounds **15**, **16**, the nitrile **9** was hydrolyzed into lumiracoxib (**4**) by action of $Ba(OH)_2$. The sequence of reactions to obtain **4** from **5** is a new synthetic route to prepare this drug. Treatment of **4** with dicylohexylcarbodiimide (DCC) in the presence of a catalytic amount of 4dimethylaminopyridine (DMAP) in methanol afforded the methyl ester **14;** in this case it was necessary to add DMAP before the coupling agent, in order to avoid cyclization of **4** to the corresponding *N*-aryl oxindole. The ester **14** afforded the desired hydroxamic acid **15** by treatment with an excess of NH_2OH, in the presence of a catalytic amount of KCN. To obtain the final product **16**, the ester **14** was converted into the corresponding hydrazide by action of NH_2NH_2 in ethanol. This intermediate was purified by flash chromatography, and without characterization was cyclized to the desired product by overnight treatment with carbonyldiimidazole (CDI) at room temperature in dry THF. To prepare the *N*-methyl analogue of lumiracoxib **13**, the acid **7** was treated with NaH in DMF, and then with an excess of methyl iodide to obtain the *N*-methylated ester **10**, that was reduced with $LiAlH_4$ at room temperature (to avoid defluorination) to the corresponding alcohol **11**. This latter product was converted into the nitrile **12**, using the same procedure adopted for the preparation of **9** from **8**. To prepare the final trifluormethylsulfonylaminomethyl substituted product **20**, the acid **7** was converted into the amide **19** by consecutive action of $SOCl_2$ and of aqueous ammonia. Treatment of this intermediate with $LiAlH_4$ in dioxane at room temperature, and then with trifluoromethansulfonic anhydride in the presence of Et_3N, afforded the target compound.



Scheme 1. *Reagents and conditions*: a) DBU, $Cu(OAc)_2$, dioxane, 25°C; b) BH_3□SMe_2, dry THF, reflux; c) $SOCl_2$, Pyr, dry THF, 0°C to RT, then KCN, DMSO, 50°C; d) $Ba(OH)_2$, dioxane/H_2O, N_2, reflux, 30 h; e) NaH, dry DMF, MeI, ; f) $LiAlH_4$, dry THF, RT; g) $SOCl_2$, Pyr, dry THF, 0°C to RT, then, KCN, DMSO 50°C; h) NaOH 10%, EtOH, reflux; i) DMAP, MeOH, DCC; j) NH_2OH, KCN cat, MeOH/THF; k) NH_2NH_2, H_2O / EtOH, reflux 1.5 h, then CDI, THF dry, RT, 12 h; m) BH_3, THF dry, reflux, then $(CF_3SO_2)_2O/Et_3N$, 0°C to RT, 1h; n) NaN_3, NH_4Cl, DMF dry, 120°C, 18 h; o) $SOCl_2$, NH_3aq; p) $LiAlH_4$, $AlCl_3$,dry THF reflux, 1h, then $(CF_3SO_2)_2O/Et_3N$, 0°C to RT, 1h.

The pathway followed for the synthesis of the final sulfonamides **26**, **27**, is depicted in Scheme 2.

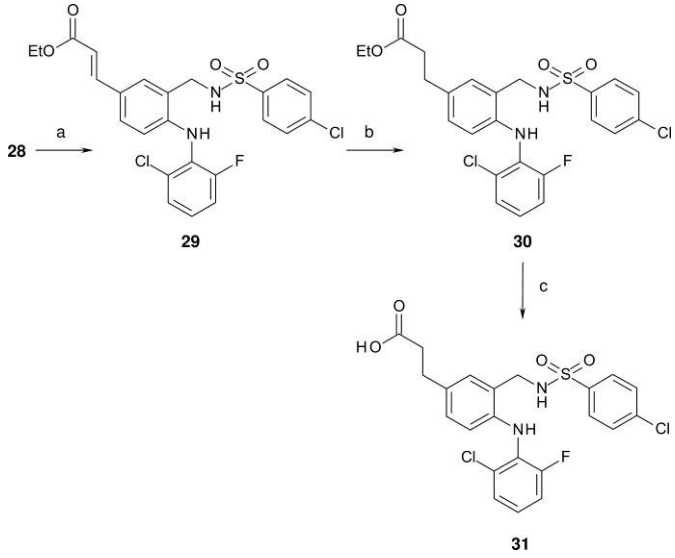


Scheme 2. *Reagents and conditions*: a) $Cu(OAc)_2$, DBU, dioxane, RT; b) BH_3□THF, reflux, 3 h, then ClO_2SR', Et_3N.

The 2-amino-5-methylbenzamide (**21**), synthesized as previously described[30] was reduced with BH_3·THF complex in refluxing THF, and then sulfonylated in the presence of Et_3N, with methanesulfonyl chloride or *p*-chlorobenzenesulfonyl chloride, respectively. The resulting intermediates **23** and **24** were transformed into the desired compounds **26** and **27** by Chan-Lam coupling following the procedure used to prepare **7** from **5**. Compound **21** was also used for direct synthesis of **19** through Chan-Lam coupling.

The iodo-substituted product **28** was prepared starting from the 2amino-5-iodobenzamide (**22**)[31] which was first reduced to the intermediate **25**, then coupled to the 2-chloro-6-fluoro phenylboronic moiety with a procedure similar to that adopted to obtain the products **26**, **27**.

To prepare the final model **31** (Scheme 3), the iodo-substituted diarylamine **28** was subjected to Pd-catalyzed Heck coupling with ethylacrylate in the presence of bis(dibenzylideneacetone)$Pd^{(0)}$ ($Pd(dba)_2$) to give **29**. Reduction of the double bond present in **29** with H_2,Pd/C gave **30**, which, in turn, afforded the desired final product by alkaline hydrolysis.



Scheme 3. *Reagents and conditions*: a) Ethyl acrylate, Et_3N, PPh_3, $Pd(dba)_2$, DMF, 120°C, 20 h; b) H_2 Pd/C 10%, 1 bar, EtOH, RT, 2 h; c) NaOH 10%, EtOH, 80°C, 1 h.

Dissociation Constants

The dissociation constants (pK_a's) were determined using a Sirius GLpK_a automated potentiometric system. Due to low water solubility of the products, the measurements were carried out in water containing methanol as co-solvent, in percentages ranging from 20% to 60%. The aqueous pK_a values were determined by extrapolation to 0% methanol, following the Yasuda-Shedlovsky procedure,[32] and are shown in Table 1 together with the corresponding ionization degree (ID) values at physiological pH. Lumiracoxib (**4**) and its *N*-methyl analogue **13** are sufficiently strong acids to be more than 99% ionized at physiological pH. Among the products considered, only the tetrazole derivative **18** behaves similarly. Conversely, the sulfonamide groups present in **26**, **27**, **31** exist at this pH in the undissociated form. Their dissociation constants are too high to be detectable through the pH-metric method. By contrast, the carboxylic group present in the lateral chain of **31** is about 99% ionized. The introduction on the $NHSO_2$ moiety of the strong electron-withdrawing group CF_3 gives rise to the stronger acid **20**, which is largely dissociated at physiological pH (ID = 83%). As expected, the higher homologue **17** is weaker than **20**, but still exists at this pH, in equilibrium between dissociated and undissociated forms, with prevalence of the former (ID= 69%). In the case of the weaker acids **15** and **16**, the undissociated form prevails (ID= 3% and ID = 49%, respectively).

Pharmacology and SARs

All products synthesized and lumiracoxib (**4**), as well as its *N*-methyl analogue **13,** taken as references, were assessed for their ability to act as TP receptor antagonists, on washed platelets from healthy human volunteers, in which the TP_α isoform is extensively expressed.[33] When washed platelet samples were challenged with U-46619, a well-known TXA_2 stable analogue,[34] concentration-dependent platelet aggregation occurred. It had previously been observed that the aggregatory response of this agonist is fully independent of endogenous TXA_2.[24] We previously demonstrated that incubation with increasing concentrations (20-100 μM) of lumiracoxib (**4**) inhibited the aggregation of washed human platelets, causing a rightward shift of the concentration-response curve of U-46619, typical of competitive antagonism.[24] Interestingly, neither the selective COX-2 inhibitor celecoxib, nor the non-selective inhibitor flurbiprofen, inhibited the aggregation evoked by U-46619 (data not shown). To determine the anti-aggregatory potency of the compounds under

investigation, washed platelets were incubated with increasing concentrations of the products (20-100 μM), and then treated with 0.5 μM U-46619, a concentration that induces maximal aggregation.[24] The extent of the resulting aggregation was detected by the Born-turbidimetric assay. Table 1 shows the anti-aggregatory potencies, expressed as IC_{50} values. Analysis of the data indicates that N-methylation of **4**, which gives rise to **13**, a product with ID similar to that of **4**, largely suppresses the TP receptor antagonist properties of the lead. This means that the NH moiety is essential to inhibit the U-46619-mediated aggregatory response. Since N-methylation abolishes the possibility of an intramolecular hydrogen bond between the secondary amine and the deprotonated carboxylic acid, this might suggest that the molecular conformation stabilized by this bond is important for the interaction of **4** with the receptor. Among the products containing the sulfonamido moiety, the $NHSO_2CH_3$ substituted compound **26** did not display any TP_α antagonism when tested at the maximal 100 μM concentration. Unlike **4**, at physiological pH this product exists in the undissociated form, which might indicate that the presence of a negative charge on the acid function is one of the essential requisites for activity. Indeed, when a CF_3 group, endowed with a strong inductive electron withdrawing effect, is substituted for the methyl group to give **20**, which is partly ionized (ID = 83%) at physiological pH, an antagonist six times more potent than **4** is obtained. The increase in the length of the lateral chain, and the decrease in ID (ID = 69%), appear to be the principal determinants of the low activity of the sulfonamide **17** compared to **20**. Introduction of the p-chlorophenyl moiety on the $NHSO_2$ group gives rise to **27**. In spite of this product being undissociated at physiological pH, it is an antagonist five times more potent than **4**. At the present state the complete 3D structure for human TP receptor is not available. Very recently an attempt to design dual TP receptor/COX-2 inhibitors based on modeling studies has been published.[35] according to this model the potent TP antagonist SQ 34,550 establish an electrostatic interaction with R^+295. The binding mode of sulfonamide derived ligands to the TP receptor has not been identified, in this case we can only speculate that for derivative **27** the interaction with R^+295 is maintained through sulfonamide-mediated H-bonding and the p-chlorophenyl moiety could be allocated in an hydrophobic pocket in the spatial proximity of the charged centre. The introduction, in the place of the methyl group in **27**, of a propionic acid chain, which is present in terutroban (**3**), gives rise to **31**, the most potent TP receptor antagonist among all the products studied. The tetrazole derivative **18**, which displays an acidic profile similar to that of **4**, is an antagonist slightly more potent than this latter, while the 1,3,4-oxadiazol-2(3*H*)-one derivative **16**, interestingly, triggers a feeble agonist response. Finally, the low antagonist activity of the hydroxamic acid **15** is in keeping with its low ID (ID = 3%). The ability of **4**, **18**, **20**, **27**, and **31** to compete for the orthosteric binding site, labelled by the specific antagonist [^{3}H]-SQ29,548 in HEK293 cells transiently transfected with wild-type human TP_α receptor, was confirmed in standard radioligand binding studies (see Experimental Section). Transfection conditions were adjusted to obtain binding capacities in the range 0.5 to 1 pmol mg^{-1} protein, values comparable to receptor expression in human platelets. Mixed-type curves of [^{3}H]-SQ29,548 and heterologous competition curves of the compounds were monophasic, fitting a single-site model. The data indicated typical binding parameters for the interaction of SQ29,548 with the TP_α receptor, as reported elsewhere.[36] No detectable binding in mixed-type curve of [^{3}H]SQ29,548 was observed when cells were transfected with the empty vector (data not shown). Calculated affinities are reported in Table 1. The results obtained are in full agreement with platelet aggregation findings, with **31** being the most potent antagonist of the series, **20** and **27** approximately comparable in the micromolar range, and the lead **4** similar to its tetrazole derivative **18**.

The capacity of the products under study to act as COX-2 inhibitors was first determined on isolated human monocytes suspended in Hank's Balanced Salt Solution (HBSS) (pH = 7.1 - 7.4). After stimulation of COX-2 expression with LPS, the PGE_2 produced was determined by enzyme immunoassay (EIA); the results are reported in Table 1. The most active compounds were the lead **4** (IC_{50} = 0.0033 μM) and its tetrazole analogue **18** (IC_{50} = 0.0096 μM); in this case the potencies of the products fell in the nM range, and the potency of **4** was only about three times that of **18**. The hydroxamic acid derivative **15** (IC_{50} = 0.025 μM) and the trifluoromethylsulfonyl substituted compound **20** (IC_{50} = 0.476 μM) displayed good COX-2 inhibitory activity in these conditions. As expected, the *N*-methylated analogue of lumiracoxib **13** had lower COX-2 antagonism (IC_{50} = 0.131 μM) than **4**. COX-2 inhibition was then evaluated in whole blood pretreated with aspirin. In these conditions, among the tested compounds, only the tetrazole derivative **18** was capable of inhibiting COX-2 enzyme in a concentration-dependent manner (Figure 2A), with a potency (IC_{50}) about 60 times lower than that of the lead **4** (Table 1). The differences between the results obtained working in whole blood or in buffer solution are likely due to protein binding, which may occur in whole blood.[37] In order to check whether **18** retained COX-2 selectivity, its ability to inhibit the COX-1 enzyme was determined on whole human blood in the absence of anticoagulating agents, assayed via its ability to inhibit TXB_2 production (EIA detection) in comparison to lumiracoxib **4**. As determined from the concentration-response curve (Figure 2B), the product had an IC_{50} value of 206 μM ± 12% CV (lumiracoxib, IC_{50} = 68 μM ± 18% CV), thus retaining 22-fold COX-2 vs. COX-1 selectivity.

Overall, the data obtained show that, although some of the newly synthesized compounds (**18**, **20**, **27**, **31**) possess TP receptor antagonism similar to or better than that shown by the lead compound, only the lumiracoxib tetrazole derivative **18** showed the promising profile of a dual TP receptor antagonist and COX-2 selective inhibitor. To confirm this, **18** was further investigated in another pharmacological model, and antagonism to the TP receptor was determined in isolated rat aortic rings stimulated with U-46619. The rat aortic rings were pretreated with indomethacin to block the COX response. Cumulative concentration-response curves for U-46619 were established in the absence (control) or in the presence of either lumiracoxib (**4**) or compound **18** (Figure 3) added to the organ bath fluid 20 min before the concentration-response curves for U-46619 were determined. All responses were expressed as percent of maximum contraction, induced by U-46619 (3 μM). As shown in Figure 3, compound **18** was indeed able to inhibit the rat aortic ring contraction induced by

U-46619 in a concentrationdependent manner, showing a slightly more potent antagonism (about 3-fold) than that shown by the reference compound (apparent U-46619 EC_{50} = 0.51 μM □ 12%CV and 0.19 μM □ 9%CV in the presence of 60 μM of **18** and **4**, respectively) Taken together, the above results indicate that the attempt to obtain products whose TP receptor antagonist/COX-2 inhibitor properties are better balanced than **4** was successful in the case of compound **18**, in which the acid tetrazole moiety is present. This product displays good TP receptor antagonist and COX-2 inhibitor potencies, evaluated respectively on human platelets and on human monocytes in plasma, within the same concentration range (IC_{50}TP/IC_{50}COX-2 = 1.4, versus IC_{50}TP/IC_{50}COX-2 = 154.3 for the lead **4**). The balance is lost when the COX-2 inhibition is evaluated on isolated monocytes in buffer solution, a condition in which protein binding does not play any role. This situation is reversed in the case of the trifluoromethylsulfonamido substituted product **20**, which acts as a fairly well balanced hybrid drug when its COX-2 inhibitory potency is evaluated in buffer (IC_{50}TP/IC_{50}COX-2 = 7).

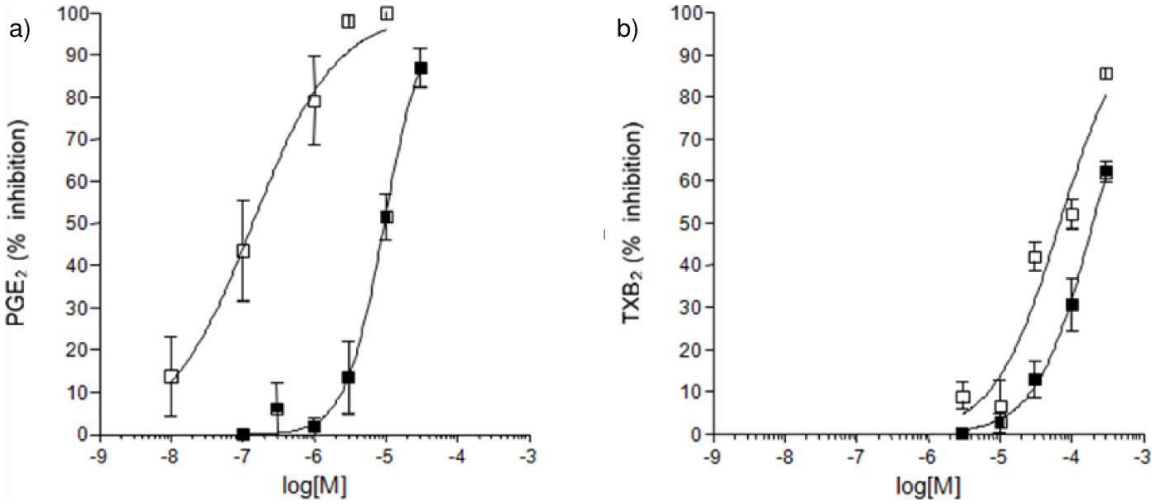


Figure 2. Evaluation of COX-2 selectivity of compounds **4** (■) and **18** (□) by assay in whole blood. a) COX-2 activity was assessed following pretreatment with 10 μg/ml aspirin and overnight treatment with 10 μg mL^{-1} LPS, and measured by release of PGE_2 (EIA) in plasma. b) COX-1 activity was measured in terms of release of TXB_2 (metabolite of TXA_2) from platelets during clotting. Data are expressed as % inhibition of PGE_2 or TXB_2 release versus untreated controls. Error bars represent mean ± SE of at least three independent experiments each performed in duplicate. Curves were computer generated from the simultaneous analysis of several independent experiments.

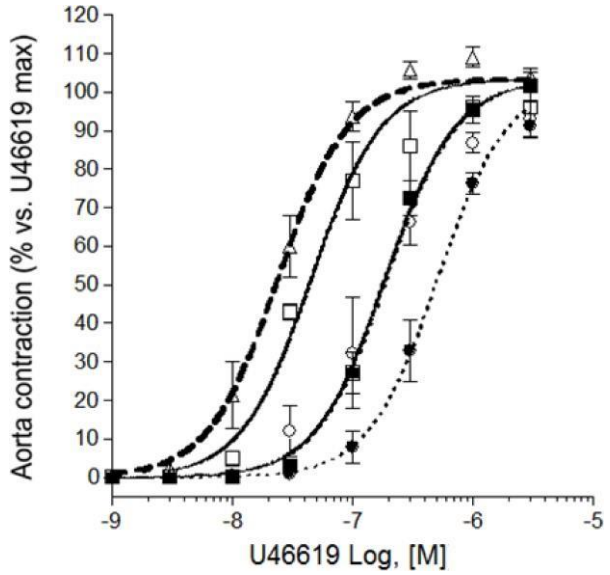


Figure 3. Evaluation of TP receptor antagonism in isolated rat aortic rings pretreated with 10 μM indomethacin and contracted with U-46619 in the presence of the indicated compounds **4** 20 μM (□), **4** 60 μM (■), **18** 20 μM (○), **18** 60 μM (●), or vehicle alone (□). Error bars represent mean ± SE of at least three independent experiments. Curves were computer generated from the simultaneous analysis of several independent experiments.

Table 1. Thromboxane A_2 antagonism, binding at TPα receptor, COX-2 inhibitory activity and dissociation constants for synthesized compounds **13**, **15**-**18**, **20**, **26**, **27**, **31** and lumiracoxib (**4**).

Compound	TXA_2 antagonism	TPα receptor binding	COX-2 inhibition		Dissociation constants
	Washed platelets IC_{50} (μM) ± %CV [a]	Ki (μM) ± %CV [b]	Isolated monocytes IC_{50} (μM) ± %CV [c]	Whole blood IC_{50} (μM) ± %CV [c]	p*Ka*[d]
Lumiracoxib (4)	21.3 ± 10	73.5 ± 54	0.0033 ± 21	0.138 ± 58	4.15 ± 0.03
13	10% inhibition (60 μM)	nt	0.131 ± 92	inactive	2.88 ± 0.04 5.20 ± 0.01
15	10% inhibition (60 μM)	nt	0.0251 ± 95	inactive	8.93 ± 0.01
16	inactive[e]	nt	inactive	inactive	7.41 ± 0.01
17	20% inhibition (30 μM)	nt	inactive	inactive	7.06 ± 0.02
18	12.8 ± 5	61 ± 45	0.0096 ± 26	8.9 ± 26	4.85 ± 0.01
20	3.37 ± 16	1.4 ± 20	0.476 ± 66	Inactive	6.70 ± 0.01
26	inactive	nt	inactive	inactive	>11
27	3.86 ± 22	6.5 ± 95	inactive	inactive	>11
31	1.56 ± 12	0.6 ± 13	inactive	inactive	5.40 ± 0.01 >11

[a] Determined by measuring inhibition of human washed platelet aggregation stimulated using 0.5 μM U-46619 as TXA_2 agonist; [b] Determined by measuring competition of the specific antagonist [^{3}H]SQ29,548 from the human TPα receptor in recombinant cells. [c] Determined by measuring inhibition of PGE_2 production in human monocytes stimulated with LPS; [d] Determined by potentiometry (GlpK_a apparatus); MeOH was used as cosolvent in percentages ranging from 20 to 60 (%Wt) according to the solubility of compounds; extrapolation to zero % cosolvent was calculated by the Yasuda-Shedlovsky procedure; [e] weak partial agonism was evidenced at 60 μM; nt = not tested.

Conclusions

In an effort to obtain products with better-balanced TP receptor antagonist and COX-2 inhibitor properties than lumiracoxib, a number of acid groups were substituted for the carboxylic moiety in the lead. These acid groups included the hydroxamic function, differently substituted reversed sulfonamido moieties, 1,3,4oxadiazol-2(3*H*)-one and tetrazole planar rings. Most of these substitutions gave rise to products either devoid of TP antagonist properties or endowed with more potent antagonist activity than lumiracoxib. In particular, the substitution of carboxylic acid with appropriate sulfonamido moieties generated the most potent antagonists. Conversely, all the isostere substitutions afforded products inactive as COX-2 inhibitors when evaluated on human monocytes in whole blood, with the sole exception of the tetrazole substituted compound **18**. This product displays good potency both as a TP receptor antagonist and as a COX-2 inhibitor, within the same concentration range (IC_{50}TP/IC_{50}COX-2 = 1.4, versus IC_{50}TP/IC_{50}COX-2 = 154.3 for the lead **4**). Since it retains 22-fold COX-2 versus COX-1 selectivity, it deserves additional in vivo studies as a new COXIB, potentially endowed with reduced cardiotoxicity. Future work will address the chemical modulation of other molecular portion either of the lead **4** or of the tetrazole **18** in order to improve potency on both targets and to prevent expectable toxicity. In carrying out chemical modulation of different critical residues (e.g. 5-methyl substituent) we will take into account modeling studies on well characterized COX-2 enzyme[38] and of those recently published on TP receptor from a primate model.[35]

Experimental Section

Chemistry

General: Melting points (mp) were measured with a capillary apparatus (*Büchi 540*). Melting points with decomposition were determined after introduction of the sample into the bath at a temperature 10°C lower than the melting point; heating rate was 3°C min^{-1}. All compounds were routinely checked by ^{1}H and ^{13}C-NMR (*Bruker Avance 300*) and mass spectrometry (*Finnigan-Mat TSQ-700*). The following abbreviations are used to indicate the peak multiplicity: s = singlet; d = doublet; t = triplet; m = multiplet, br = broad. Flash column chromatography was run on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM) using the eluents indicated. Thin layer chromatography (TLC) was run on 5 x 20 cm plates with a 0.25 mm layer thickness (Fluka). Anhydrous magnesium sulfate was used as drying agent for the organic phases. The new compounds were analysed (C, H, N) by REDOX (Monza) and by Service de Microanalyse, Université de Genève, Genève (CH); the results are within ± 0.4% of theoretical values. Ethyl acetate, acetonitrile, dicloromethane, ethanol, methanol and petroleum ether (b.p. 4070°C) were used without further purification. Dry dicloromethane was obtained by refluxing with P_2O_5 under nitrogen, distilled and stored with molecular sieves (4Å). Dry acetonitrile was obtained by refluxing with CaH_2 under nitrogen, distilled and stored with molecular

sieves (4Å). Tetrahydrofuran (THF) was distilled immediately before use from Na and benzophenone. Dioxane was freshly distilled before use.

2-[(2-chloro-6-fluorophenyl)amino]-5-methyl-benzoic acid (7): To a stirred solution of 5-methylanthranilic acid (1g; 6.6 mmol) in distilled dioxane (50 mL), DBU (3 mL; 19 mmol; 3 eq) and finely powdered $Cu(OAc)_2$ monohydrate (1.32 g; 6.6 mmol, 1 eq) were added. To this stirred mixture, 2-chloro,6-fluoro phenyl boronic acid (1.21 g; 6.9 mmol;1.05 eq) in distilled dioxane (10 mL) was added dropwise. After the addition was complete, the reaction mixture was stirred at RT for 8 h, then two portions of 2-chloro,6-fluoro phenyl boronic acid (0.28 g; 1.6 mmol; 0.25 eq) were added, until the reaction reached completion (TLC). The mixture was treated with pH 4.5 NaOAc/AcOH buffer (70 mL) then with 0.3 M EDTA tetrasodium (20 mL) and extracted with EtOAc (2 × 50 mL). The organic layer was washed with 1N HCl (2 × 25 mL) and brine (25 mL), dried ($MgSO_4$) and evaporated under reduced pressure to give 1.68 g (91%) of a cream coloured solid (**7**) pure by NMR. An analytical sample was obtained by recrystallization from EtOH. White solid; mp: 236-237°C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.23 (s, 3H, CH_3), 6.35 (dd, J = 8.4, 3.9 Hz, 1H, ArH_3), 7.20 (d, J = 8.4 Hz, 1H, ArH_4), 7.28-7.49 (m, 3H, $ArH_{3',4',5'}$), 7.73 (s, 1H, ArH_6), 9.32 (s, 1H, NH), 13.17 ppm (s, br, 1H, COOH); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 19.76 (s, CH_3), 112 (s, C_1), 113.29 (d, $J_{C\text{-}F}$ = 3 Hz, C_3), 115.49 (d, $J_{C\text{-}F}$ = 20.5 Hz, $C_{5'}$), 125.8 (d, $J_{C\text{-}F}$ = 3.3 Hz, $C_{3'}$), 125.83 (s, C_1), 125.93 (s, C $J_{C\text{-}F}$ = 14.8 Hz, $C_{1'}$), 126.37 (s, C_5), 126.85 (d, $J_{C\text{-}F}$ = 9.2 Hz, $C_{4'}$), 131.09 (s, C_5), 131.16 (s, C_4), 131.13 (d, $J_{C\text{-}F}$ = 4.8 Hz, $C_{2'}$), 134.9 (s, C_6), 128.8 (d,), 144.58 (d, $J_{C\text{-}F}$ = 1 Hz, C_2), 157.74 ppm (d, $J_{C\text{-}F}$ = 247 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 280-282 $[M+H]^+$. Anal. calcd. for $C_{14}H_{11}ClFNO_2$: C 60.12, H 3.96, N 5.01; found: C 59.93, H 3.86, N 4.89.

2-[(2-chloro-6-fluorophenyl)amino]-5-methyl-benzenemethanol
(8): To a solution of $BH_3{\cdot}SMe_2$ (1.88 g; 2.39 mL; 24.8 mmol) in dry THF (30 mL) in a flame-dried 3-neck flask kept at 0°C under nitrogen, a solution of **7** (1.39 g; 4.9 mmol) in dry THF (20 mL) was added dropwise. After addition was complete, the reaction mixture was refluxed for 6 h. Excess borane was quenched with ice/water, THF was evaporated under reduced pressure and the aqueous layer saturated with Na_2CO_3. The aqueous layer was transferred to a separating funnel and extracted with Et_2O (2 × 20 mL). The combined organic layers were washed with water (25 mL) and brine (25 mL), dried ($MgSO_4$) and evaporated under reduced pressure to give the crude product as an oil. The crude material was purified by FC eluting with PE containing 10% EtOAc to afford 1.66 g (93%) of the desired product (**8**) as a white solid. Mp: 93°C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.21 (s, 3H, CH_3), 4.56 (d, J = 5.4 Hz, 2H, CH_2), 5.47 (t, J = 5.4 Hz, 1H, OH), 6.34 (dd, J = 8.1, 3.9 Hz, 1H, ArH_3), 6.77-7.51 ppm (m, 6H, $ArH_{4,6}$, $ArH_{3',4',5'}$, NH); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 20.11 (s, CH_3), 61.96 (s, CH_2), 114.26 (d, $J_{C\text{-}F}$ = 3.1 Hz, C_3), 116.1 (d, $J_{C\text{-}F}$ = 20 Hz, $C_{5'}$), 123.76 (d, $J_{C\text{-}F}$ = 8.8 Hz, $C_{4'}$), 125.66 (d, $J_{C\text{-}F}$ = 3.2 Hz, $C_{3'}$), 127.86 (s, C_4), 127.88 (d, $J_{C\text{-}F}$ = 14.8 Hz, $C_{2'}$), 128.19 (d, $J_{C\text{-}F}$ = 14 Hz, $C_{1'}$), 128.42 (s, C_1^*), 128.65 (s, C_5^*), 128.73 (s, C_6), 139.82 (d, $J_{C\text{-}F}$ = 1 Hz, C_2), 155.8 ppm (d, $J_{C\text{-}F}$ = 246 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 266-268 $[M+H]^+$. *Assignment might be reversed.

2-[(2-chloro-6-fluorophenyl)amino]-5-methylbenzene acetonitrile (9): In a flame-dried 250 mL three-neck flask, pyridine (1.69 mL, 20 mmol;) was added to a stirred solution of **8** (1 g; 3.7 mmol,) in dry THF (25 mL) at 0°C. To the mixture kept at 0°C, thionyl chloride (1.69 mL; 23.4 mmol; 6.2 eq) in dry THF (25 mL) was added dropwise, keeping the temperature below 5°C. After addition was complete (TLC), the reaction mixture was treated with ice then with 2N HCl (20 mL). The mixture was then extracted with EtOAc (3 × 30 mL), the organic layers were washed with 2N HCl (2 × 20 mL) then with water (30 mL) and brine (30 mL), dried ($MgSO_4$) and evaporated under reduced pressure at RT, to leave an orange coloured solid which, owing to its instability, was used immediately in the next step. KCN (1.8 g; 27.6 mmol;) was added to the resulting chloride (1.05 g; 3.7 mmol) in dry DMSO (20 mL), stirred under nitrogen. The reaction mixture was heated to 40°C for 1.5 h. After the reaction was complete, the mixture was treated with ice (the orange-red reaction mixture turned to a yellow solution) and extracted with EtOAc (3 × 30 mL). The combined organic layers were washed with 5N HCl (3 × 30 mL), water (30 mL), and brine (30 mL), then dried ($MgSO_4$) and evaporated under reduced pressure to give the crude product as a yellow solid. Purification by flash chromatography over silica gel, eluting with PE containing 5% EtOAc, afforded the pure cyanide (**9**) (0.7 g; 69%) as a white solid. Mp: 77 – 78°C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.21 (s, 3H, CH_3), 4.02 (d. 2H, CH_2), 6.34 (d, J = 8.1 Hz, 1H, ArH_3), 6.93 (d, J = 8.1 Hz, 1H, ArH_4), 7.05 (s, 1H, NH), 7.08-7.34 ppm (m, 4H, ArH_6, $ArH_{3',4',5'}$); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 19.6 (s, CH_2), 20.4 (s, CH_3), 115.49 (d, $J_{C\text{-}F}$ = 20 Hz, $C_{5'}$), 117.4 (d, $J_{C\text{-}F}$ = 1.1 Hz , C_3), 119.28 (s, CN), 120.7 (s, C_1), 124.96 (d, $J_{C\text{-}F}$ = 8.7 Hz, $C_{4'}$), 126.41 (d, $J_{C\text{-}F}$ = 3.2 Hz, $C_{3'}$), 129.43 (s, C_4), 129.57(d, $J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$), 130.24 (d, $J_{C\text{-}F}$ = 13.2 Hz , $C_{1'}$), 130.39 (s, C_5), 130.68 (s, C_6), 140 (s, C_2), 156.68 ppm (d, $J_{C\text{-}F}$ = 245 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 275-277 $[M+H]^+$.

2-[(2-chloro-6-fluorophenyl)amino]-5-methyl-benzeneacetic acid (4): To a stirred suspension of $Ba(OH)_2$ (0.093 g; 0.54 mmol) in water (10 mL), a solution of **9** (0.05 g; 0.18 mmol) in 1,4-dioxane (5 mL) was added and the reaction mixture refluxed for 30 h. The mixture was acidified with 1N HCl and extracted with EtOAc (3 × 20 mL); the combined organic layers were washed with water (20 mL) then brine (15 mL), dried (Na_2SO_4) and evaporated under reduced pressure to afford the crude product. The product was purified by FC, eluting with CH_2Cl_2 containing 1% MeOH to give 0.05 g (quant.) of the title product (**4**) as a white solid. An analytical sample was obtained by recrystallisation from EtOH/water. MS and NMR data are consistent with that of an original sample.[39]

2-[(2-chloro-6-fluorophenyl)amino]-5-methyl-benzoic acid, methyl ester (10): A solution of **7** (0.1 g; 0.36 mmol) was added dropwise to a suspension of NaH 60% in mineral oil (41mg; 1.07 mmol) in dry DMF (3 mL) kept at 0°C. Iodomethane (0.15 mL; 1.07 mmol) was slowly added to the resulting yellow mixture, and the reaction mixture was stirred at RT for 4 h. The mixture was treated with ice-water (10 mL) and, after evolution of gas had ceased, with 2N HCl (10 mL). The aqueous phase was transferred to a separating funnel and extracted with Et_2O (3 × 20 mL); the organic phase was washed with water (20 mL), then brine (20 mL), dried ($MgSO_4$) and evaporated under reduced pressure to leave a pale yellow solid. The crude material was purified by FC,

eluting with PE containing 5% EtOAc to give 0.08 g (73%) of the desired product (**10**) as a vitreous semisolid material. ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.23 (s, 3H, $ArCH_3$), 3.19 (s, 3H, NCH_3), 3.37 (s, 3H, OCH_3), 7.03-7.31 ppm (m, 6H, $ArH_{3,4,6}$, $ArH_{3',4',5'}$); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 19.61 (s, $ArCH_3$), 39.75 (s, NCH_3), 51.06 (s, OCH_3), 115.6 (d, $J_{C\text{-}F}$ = 20.7 Hz, $C_{5'}$), 116.32 (s, C_3), 121.17 (d, $J_{C\text{-}F}$ = 1 Hz C_1), 126.2 (d, $J_{C\text{-}F}$ = 3.2 Hz, $C_{3' \text{ or } 2'}$), 127.3 (d, $J_{C\text{-}F}$ = 9.7 Hz, $C_{4'}$), 128.12 (s, C_5), 129.96 (s, C_4), 132.27 (s, C_6), 132.93 (d, $J_{C\text{-}F}$ = 3.4 Hz, $C_{2' \text{ or } 3'}$), 133.6 (d, $J_{C\text{-}F}$ = 13.2 Hz , $C_{1'}$), 144.37 (s, C_2), 159.34 (d, $J_{C\text{-}F}$ = 250 Hz, $C_{6'}$), 168.14 ppm (s, CO); MS (CI-isobutane) *m/z* 308-310 $[M+H]^+$.

2-[(2-chloro-6-fluorophenyl)methylamino]-5-methyl-benzenemethanol (11): In a flame-dried three-neck flask under nitrogen, $LiAlH_4$ (0.18 g; 4.97 mmol) was suspended in dry THF (30 mL). The suspension was cooled to 0 °C and a solution of **10** (0.51 g; 1.65 mmol) in dry THF (10 mL) was added dropwise. The reaction mixture was stirred at RT for 3 h. After reaction was complete, the mixture was cooled (0°C) and excess $LiAlH_4$ was destroyed by careful addition of water (2 mL), 10 % NaOH (1 mL) and water (2 mL). The precipitated salt was filtered off and washed with three portions of CH_2Cl_2; the organic layer was dried ($MgSO_4$) and evaporated under reduced pressure to give the crude product. The product was purified by FC, eluting with PE containing 10% EtOAc to give the desired product 0.36 g of **11** (78%) as a colourless oil. ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.26 (s, 3H, $ArCH_3$), 3.17 (s, 3H, NCH_3), 3.98 (d, J = 5.4 Hz, 2H, CH_2), 4.98 (t, J = 5.4 Hz, 1H, OH), 6.88-7.35 ppm (m, 6H, $ArH_{3,4,6}$, $ArH_{3',4',5'}$); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 20.56 (s, $ArCH_3$), 40.99 (s, NCH_3), 58.54 (s, CH_2), 115.7 (d, $J_{C\text{-}F}$ = 20.8 Hz, $C_{5'}$), 118.87 (s, C_3), 126.3 (d, $J_{C\text{-}F}$ = 3.2 Hz, $C_{3'}$), 126.8 (d, $J_{C\text{-}F}$ = 9.3 Hz, $C_{4'}$), 127.25 and 127.35 (2s, C_4, C_6), 130.61 (s, C_1), 132.5 (d, $J_{C\text{-}F}$ = 4.3 Hz, $C_{2'}$), 133.65 (s, C_5), 134.9 (d, $J_{C\text{-}F}$ = 12.6 Hz, $C_{1'}$), 143.65 (s, C_2), 159.8 ppm (d, $J_{C\text{-}F}$ = 249 Hz, $C_{6'}$; MS (CI-isobutane) *m/z* 280-282 $[M+H]^+$.

2-[(2-chloro-6-fluorophenyl)methylamino]-5-methylbenzeneacetonitrile (7): Pyridine (1.69 mL; 20 mmol) was added to a stirred solution of **11** (1 g; 3.7 mmol) in dry THF (25 mL) kept under nitrogen at 0 °C; this was followed by dropwise addition of a solution of $SOCl_2$ (1.69 mL; 23.4 mmol) in dry THF (25 mL); this latter addition was performed maintaining the temperature below 5 °C during 15 min. After addition was complete, the mixture was treated with ice-water (20 mL), then with 2N HCl (20 mL) and transferred to a separating funnel. The aqueous layer was extracted with EtOAc (3 × 25 mL), the combined organic layers were washed with 1N HCl (2 × 30 mL), water (2 × 20 mL) then with brine (30 mL), dried ($MgSO_4$) and evaporated under reduced pressure at RT to give an orange solid, which was used immediately in the next step. KCN (0.71 g; 1.09 mmol) was added to 0.44 g (1.5 mmol) of the resulting chloride in DMSO (20 mL), under nitrogen stream, and the reaction mixture was heated to 40°C for 1.5 h. The reaction mixture was treated with ice-water (30 mL) and extracted with EtOAc (3 × 30 mL). The organic phase was washed with 2N HCl (3 × 30 mL), then with water (2 × 20 mL) and brine (30 mL), dried ($MgSO_4$) and evaporated under reduced pressure to give crude cyano derivative as a yellow solid. The compound was purified by FC, eluting with PE containing 5 % EtOAc to afford 0.287 g (67%) of the desired product as a yellow oil. ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.27 (s, 3H, CH_3), 3.17 (s, NCH_3), 3.60 (s, CH_2), 7.12-7.46 ppm (m, 6H ArH); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 18.82 (s, CH_2), 20.11 (s, CH_3), 41.4 (d, $J_{C\text{-}F}$ = 3.4 Hz, NCH_3), 116.23 (d, $J_{C\text{-}F}$ = 20.8 Hz, $C_{5'}$), 118.24 (s, CN), 121.7 (d, $J_{C\text{-}F}$ = 1.9 Hz , C_3), 123.62 (s, C_1), 126.42 (d, $J_{C\text{-}F}$ = 3 Hz, $C_{3'}$), 127.3 (d, $J_{C\text{-}F}$ = 9.5 Hz, $C_{4'}$), 129.3 and 130.3 (2s, C_4, C_6), 132.34 (d, $J_{C\text{-}F}$ = 4.4 Hz, $C_{2'}$), 132.57 (s, C_5), 134.21 (d, $J_{C\text{-}F}$ = 12.2 Hz , $C_{1'}$), 145.23 (s, C_2), 159.9 ppm (d, $J_{C\text{-}F}$ = 249 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 289-291 $[M+H]^+$.

2-[(2-chloro-6-fluorophenyl)methylamino]-5-methyl-benzeneacetic acid (13): NaOH (1 g; 25 mmol) in H_2O (1 mL) was added to a stirred solution of **12** (0.08 g; 0.28 mmol) in EtOH (1 mL), and the mixture was refluxed for 24 h. The solvent was evaporated under reduced pressure, the residue taken up with water and extracted with Et_2O (20 mL), the aqueous layer was acidified with 1N HCl (pH 2-3) and extracted with EtOAc (2 × 20 mL). The organic phase was washed with water (15 mL), brine (20 mL), dried ($MgSO_4$) and evaporated to give the crude product as a light brown oil. The pure product was obtained by FC, eluting with PE containing 20% EtOAc to afford the title product (**13**) 0.05 g (58%) as a cream coloured solid. Mp: 75 °C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.22 (s, 3H, CH_3), 3.14 (s, 3H, NCH_3), 3.32 (s, 2H, CH_2), 6.93-7.38 (m, 6H ArH), 12.02 ppm (s, br, 1H, COOH); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 20.18 (s, CH_3), 36.09 (s, CH_2), 41.46 (d, $J_{C\text{-}F}$ = 3.6 Hz, NCH_3), 115.85 (d, $J_{C\text{-}F}$ = 20.8 Hz, $C_{5'}$), 120.95 (d, $J_{C\text{-}F}$ = 1.9 Hz , C_3), 126.15 (d, $J_{C\text{-}F}$ = 3.1 Hz, $C_{3'}$), 126.69 (d, $J_{C\text{-}F}$ = 9.6 Hz, $C_{4'}$), 127.65 (s, C_1), 127.91 (s, C_4 or C_6), 131.39 (s, $J_{C\text{-}F}$ = 3.9 Hz, C_5), 132.03 (s, C_6 or C_4), 132.42 (d, $J_{C\text{-}F}$ = 4.8 Hz, $C_{2'}$), 134.71 (d, $J_{C\text{-}F}$ = 12.2 Hz , $C_{1'}$), 145.64 (d, $J_{C\text{-}F}$ = 1.6 Hz, C_2), 159.93 (d, $J_{C\text{-}F}$ = 249 Hz, $C_{6'}$), 172.13 ppm (s, COOH); MS (CI-isobutane) *m/z* 308-310 $[M+H]^+$. Anal. calcd. for $C_{16}H_{15}ClFNO_2$, ¼ H_2O: C 61.54, H 5.00, N 4.48; found: C 61.75, H 4.87, N 4.45.

2-[(2-chloro-6-fluorophenyl)amino]-5-methyl-benzeneacetic acid, methyl ester (14): DMAP (0.13 g; 1.03 mmol) was added to a stirred solution of **4** (1.51 g; 5.14 mmol) in MeOH (37 mL). The reaction mixture was cooled to 0°C and a solution of DCC (1.27 g; 6.17 mmol) in dry CH_2Cl_2 (45 mL) was added dropwise. The reaction mixture was stirred at RT for 3 h. The solvent was evaporated under reduced pressure, the residue taken up with CH_2Cl_2 (20 mL), cooled to 0°C and the precipitate filtered. The filtrate was washed with 1N HCl (2 × 20 mL), H_2O (2 × 20 mL), dried (Na_2SO_4) and evaporated to leave 1.86 g of a cream coloured solid. The crude material was purified by FC, eluting with PE containing variable amounts of CH_2Cl_2 (from 10 to 20%) to give 1.17 g (74%) of the desired product (**14**) as a pink-shot white solid. Mp: 62 – 63°C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.22 (3H, s, CH_3), 3.65 (s, 3H, OCH_3), 3.78 (s, 2H, CH_2), 6.43 (dd, J = 8.1, 1.5 Hz, 1H, ArH_3), 6.91-7.38 ppm (m, 6H, $ArH_{4,6}$, $ArH_{3',4',5'}$,NH); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 20.97 (s, Ar*C*H_3), 37.86 (s, CH_2), 52.74 (s, OCH_3), 116.1 (d, $J_{C\text{-}F}$ = 20 Hz, $C_{5'}$), 117.78 (s, C_3), 124.46 (d, $J_{C\text{-}F}$ = 8.6 Hz, $C_{4'}$), 124.69 (s, C_1), 126.6 (d, $J_{C\text{-}F}$ = 3.2 Hz, $C_{3'}$), 128.7 (d, $J_{C\text{-}F}$ = 4.2 Hz, $C_{2'}$), 129 (s, C_4), 129.8 (d, $J_{C\text{-}F}$ = 13.8 Hz , $C_{1'}$), 130.88 (s, C_5), 132.23 (s, C_6), 140.94 (s, C_2), 156.73 (d, $J_{C\text{-}F}$ = 245 Hz, $C_{6'}$), 172.88 ppm (s CO); MS (CI-isobutane) *m/z* 308-310 $[M+H]^+$.

2-[(2-chloro-6-fluorophenyl)amino]-*N*-hydroxy-5-methyl-benzeneacetamide (15): Hydroxylamine 50% aqueous solution (0.53 mL, 18 mmol) and catalytic KCN (0.01 g) were added to a stirred solution of **14** (0.25 g; 0.81 mmol) in a THF/MeOH (1/1) mixture (4 mL). After 18 h of stirring at RT, the solvent was evaporated under reduced pressure, the residue taken up with CH_2Cl_2 (20 mL) and washed with water (2 × 20 mL), 1N HCl (2 × 25 mL) and brine (20 mL). The organic phase was dried ($MgSO_4$) and

evaporated to afford a crude product which was purified by FC, eluting with CH_2Cl_2 containing 5 % MeOH, to afford 0.18 g of a white solid (**15**) (74%). An analytical sample was obtained by recrystallisation from hexane/iPrOH. Mp: 146 - 147°C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.21 (3H, s, CH_3), 3.41 (s, 2H, ArCH_2-), 6.43 (dd, J = 8.1 Hz, 3.3 Hz, 1H, ArH_3), 6.90 (d, J = 8.1 Hz, 1H, ArH_4), 6.97 (s, 1H, ArH_6), 7.02-7.09 (m, 1H, Ar$H_{4'}$), 7.19-7.26 (m, 1H, Ar-$H_{5'}$), 7.33 (d, J = 8.1 Hz, 1H, Ar$H_{3'}$), 8.42 (s, 1H, ArNH), 9.05, (s, 1H, CONH), 10.94 ppm (s, 1H OH); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 20.06 (CH_3), 36.43 (CH_2), 115.2 (d, $J_{C\text{-}F}$ = 20 Hz, $C_{5'}$), 116.2 (d, $J_{C\text{-}F}$ = 2.9 Hz, C_3), 122.6 (d, $J_{C\text{-}F}$ = 8.6 Hz, $C_{4'}$), 125.1 (s, C_1), 125.7 (d, $J_{C\text{-}F}$ = 3.3 Hz, $C_{3'}$), 126.7 (d, $J_{C\text{-}F}$ = 4.5 Hz, $C_{2'}$), 127.6 (s, C_4), 128.8 (d, $J_{C\text{-}F}$ = 13.2 Hz , $C_{1'}$), 129.6 (s, C_5), 130.6 (s, C_6), 140.1 (d, J_{CF} = 1 Hz, C_2), 155.1 (d, $J_{C\text{-}F}$ = 246 Hz, $C_{6'}$), 168.13 ppm (s, CO); MS (CI-isobutane) *m/z* 309-311 $[M+H]^+$. Anal. calcd. for $C_{15}H_{14}ClFN_2O_2$: C 58.36, H 4.57, N 9.07; found: C 58.43, H 4.55, N 9.01.

5-[[2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl]methyl]1,3,4-oxadiazol-2(3*H*)-one (16): To a stirred solution of **14** (0.05 g; 0.16 mmol) in absolute EtOH, hydrazine hydrate (0.78 mL; 16.2 mmol) was added and the mixture was refluxed for 1.5 h. Ethanol was evaporated under reduced pressure at RT, the remaining residue was dissolved in water and the product was extracted with EtOAc (3 × 20 mL). The organic phase was washed with water (20 mL), brine (20 mL), dried ($MgSO_4$) and evaporated under reduced pressure to give a white solid. The solid was purified by FC, eluting with CH_2Cl_2 containing 2.5% MeOH to afford the intermediate hydrazide as a white solid. The hydrazide (0.05 g; 0.16 mmol) was added under nitrogen to a stirred solution of 1,1-carbonyldiimidazole (0.029g; 0.18 mmol) in dry THF (3 mL) and the reaction mixture was stirred at RT for 5 h. The solvent was evaporated under reduced pressure, the residue was taken up with water (15 mL) and extracted with EtOAc (3 × 15 mL). The organic phase was washed with water (15 mL) and brine (15 mL), dried ($MgSO_4$) and evaporated under reduced pressure to leave an oil which solidified upon trituration with hexane. The solid was purified by FC, eluting with PE containing 30% EtOAc to give 0.04 g (74%) of the title product (**16**) as a white solid. Mp: 133 - 134 °C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.21 (3H, s, CH_3), 4.01 (s, 2H, CH_2), 6.36 (dd, J = 8.1, 1.5 Hz, 1H, ArH_3), 6.89-7.35 (m, 6H, $ArH_{4,6}$, $ArH_{3',4',5'}$, ArNH), 12.09 ppm (s, 1H, CONH); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 20.04 (s, ArCH_3), 28.30 (s, CH_2), 115.09 (d, $J_{C\text{-}F}$ = 20.2 Hz, $C_{5'}$), 117.05 (d, $J_{C\text{-}F}$ = 1.5 Hz, C_3), 122.96 (s, C_1), 124 (d, $J_{C\text{-}F}$ = 8.8 Hz, $C_{4'}$), 125.74 (d, $J_{C\text{-}F}$ = 3.3 Hz, $C_{3'}$), 128.4 (s, C_4), 128.58 (d, $J_{C\text{-}F}$ = 3.7 Hz, $C_{2'}$), 129.05 (d, $J_{C\text{-}F}$ = 14.3 Hz, $C_{1'}$), 129.91 (s, C_5), 130.87 (s, C_6), 140.28 (d, $J_{C\text{-}F}$ = 1 Hz C_2), 156.41 (d, $J_{C\text{-}F}$ = 245 Hz, $C_{6'}$), 154.97 and 155.5 ppm (2s, 2C, O(C)*C*=N), *C*O); MS (CI-isobutane) *m/z* 334-336 $[M+H]^+$. Anal. calcd. for $C_{16}H_{13}ClFN_3O_2$: C 57.58, H 3.93, N 12.59; found: C 57.65, H 4.01, N 12.40.

***N*-[2-[2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl]ethyl]1,1,1-trifluoro-methansulfonamide (17):** A solution of **9** (0.50 g; 1.90 mmol) in dry THF (8 mL) was added to a solution of BH_3 1M in THF (6.66 mL; 6.66 mmol) kept under nitrogen. The reaction mixture was refluxed for 3 h, cooled to 0 °C, and 5N HCl was cautiously added. After evolution of gas had ceased, the mixture was transferred into a separating funnel and extracted with Et_2O (4 × 30 mL). The aqueous layer was basified with NaOH and extracted with Et_2O (3 × 30 mL), the organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to give 0.3 g of a pale yellow oil, consisting of the intermediate amine. The product was checked by MS and used directly in the next step. MS (CI-isobutane) *m/z* 279-281 $[M+H]^+$. A solution of trifluoromethansulfonic anhydride (0.36 g; 1.28 mmol) in dry CH_2Cl_2 (5 mL) was added dropwise to a stirred solution of the intermediate amine (0.3 g; 1.08 mmol) and triethylamine (0.30 mL; 2.15 mmol) in dry CH_2Cl_2 (12 mL), kept under nitrogen at 0 °C. After 1 h of stirring at RT, the mixture was diluted with CH_2Cl_2 (15 mL), washed with 2N HCl (2 × 20 mL) then brine (2 × 20 mL) and the organic phase dried ($MgSO_4$) and evaporated under reduced pressure to leave a brown oil. The crude residue was purified by FC, eluting with PE with EtOAc ranging from 5% to 10%, to afford 0.30 g (68%) of **17** as a pale yellow oil, which solidified upon standing. An analytical sample was recrystallised from hexane. Off-white solid. Mp: 65 – 66°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.30= (s, 3H, CH_3), 3.03 (t, J = 6.6 Hz, 2H, ArCH_2), 3.67 (m, 2H, CH_2NH), 5.13 (s, br, 1H $NHSO_2$), 6.61 (dd, J = 8.1 2.1 Hz, 1H, Ar-H_3), 6.93-7.26 ppm (m, 6H, Ar$H_{4,6}$, Ar$H_{3',4',5'}$, ArNH); ^{13}C NMR (75 MHz, $CDCl_3$): δ= 20.67 (CH_3), 32.52 (CH_2), 44.09 (CH_2), 115 (d, $J_{C\text{-}F}$ = 20 Hz, $C_{5'}$), 119.1 (d, $J_{C\text{-}F}$ = 2.2 Hz, C_3), 119.7 (q, $J_{C\text{-}F}$ = 319 Hz, CF_3), 123 (d, $J_{C\text{-}F}$ = 8.7 Hz, $C_{4'}$), 125.5 (d, $J_{C\text{-}F}$ = 3.3 Hz, $C_{3'}$), 127.3 (s, C_1), 127.7 (d, $J_{C\text{-}F}$ = 3.9 Hz, $C_{2'}$), 128.7 (s, C_4), 129.3 (d, $J_{C\text{-}F}$ = 13 Hz, $C_{1'}$), 131.3 (s, C_6), 133 (s, C_5), 139.2 (d, J_{CF} = 1 Hz, C_2), 155.7 ppm (d, $J_{C\text{-}F}$ = 246 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 411-413 $[M+H]^+$. Anal. calcd. for $C_{16}H_{15}ClF_4N_2O_2S$: C 46.78, H 3.68, N 6.82; found: C 46.65, H 3.61, N 6.73.

***N*-(2-chloro-6-fluorophenyl)-4-methyl-2-(1*H*-tetrazol-5-ylmethyl)benzenamine (18):** Sodium azide (0.48 g, 7.6 mmol) and NH_4Cl (0.4 g; 7.6 mmol) were added to a stirred solution of **9** (0.2 g; 0.76 mmol) in dry DMF (8 mL), and the reaction mixture was heated to 120°C for 18 h. After cooling, the mixture was treated with 0.5N HCl (10 ml) and extracted with EtOAc (3 × 20 mL). The organic layers were dried ($MgSO_4$) and evaporated under reduced pressure to leave a brown oil, which was purified by FC, eluting first with PE containing variable amounts of EtOAc (from 40-60%), then with CH_2Cl_2 containing variable amounts of MeOH (from 0-10%) to give **18** as a pale brown solid (0.24 g; quant.) The compound was recrystallized from EtOH/H_2O to afford a cream coloured solid. Mp: 163 – 164°C (dec); ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.19 (s, 3H, CH_3), 4.34 (s, 2H, CH_2), 6.40 (dd, J = 8.7 Hz, 1.8 Hz, 1H, ArH_3), 6.90-6.93 (m, 2H, ArH_4, ArNH), 7.07-7.14 (m, 2H, ArH_6, Ar$H_{4'}$), 7.21-7.32 (m, 1H, ArH_5), 7.33 (m, 1H, Ar$H_{3'}$), 15.9 ppm (s, br, 1H, Tetrazole-NH); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 20.1 (CH_3), 25.41 (CH_2), 115.1 (d, $J_{C\text{-}F}$ = 20 Hz, $C_{5'}$), 117.25 (d, $J_{C\text{-}F}$ = 1.7 Hz, C_3), 123.75 (d, $J_{C\text{-}F}$ = 8.8 Hz, $C_{4'}$), 125.28 (s, C_1), 125.73 (d, $J_{C\text{-}F}$ = 3.2 Hz, $C_{3'}$), 128.05 (d, $J_{C\text{-}F}$ = 4 Hz, $C_{2'}$), 128.19 (s, C_4), 129.13 (d, $J_{C\text{-}F}$ = 14 Hz, $C_{1'}$), 130.17 (s, C_5), 130.28 (s, C_6), 139.91 (d, $J_{C\text{-}F}$ = 1 Hz, C_2), 154.88 (s, C-tetrazole), 155.1 ppm (d, $J_{C\text{-}F}$ = 245 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 318-320 $[M+H]^+$. Anal. calcd. for$C_{15}H_{13}ClFN_5$ ⅓ H_2O: C 55.66, H 4.25, N 21.63; found: C 55.71, H 4.30, N 21.30.

2-[(2-chloro-6-fluorophenyl)amino]-5-methyl-benzamide (19): Procedure A: To a stirred solution of **7** (1.1 g; 3.9 mmol), thionyl chloride (2.85 mL; 39 mmol) was added and the reaction mixture heated to 60°C for 30 min. Thionyl chloride was evaporated under reduced pressure, the residue treated with benzene and evaporated again. The flask containing the oily residue was cooled (0°C) under nitrogen and concentrated aqueous NH_3 was slowly added. The reaction mixture was stirred at RT for 4 h, transferred to a separating funnel and extracted with EtOAc (2 × 20 mL). The organic layer was washed with water

(20 mL) then with brine (20 mL), dried ($MgSO_4$) and evaporated under reduced pressure to afford a yellowish solid. The solid was purified by FC, eluting with PE containing 30% EtOAc to give 0.9 g (83%) of the title product (**19**) as a yellow solid. Mp: 182 - 183°C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.3 (s, 3H, CH_3), 6.35 (dd, J = 8.4 Hz, 4.5 Hz, 1H, ArH_3), 7.10 (dd, J = 8.4, 1.5 Hz, 1H, ArH_4), 7.19-7.41 (m, 3H, $ArH_{3',4',5'}$), 7.45 (s, br 1H, CON*H*H), 7.57 (d, J = 1.5 Hz, 1H, ArH_6), 8.08 (s, br, 1H, CONH*H*), 9.83 ppm (s, 1H, ArNH); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 19.95 (s, CH_3), 113.6 (d, $J_{C\text{-}F}$ = 3.5 Hz, C_3), 115.38 (d, $J_{C\text{-}F}$ = 20 Hz, $C_{5'}$), 116.34 (s, C_1), 125.55 (d, $J_{C\text{-}F}$ = 9 Hz, $C_{4'}$), 125.73 (d, $J_{C\text{-}F}$ = 3.2 Hz, $C_{3'}$), 126.37 (s, C_5), 126.59 (d, $J_{C\text{-}F}$ = 14.3 Hz , $C_{1'}$), 128.95 (s, C_4), 129.99 (d, $J_{C\text{-}F}$ = 3.9 Hz, $C_{2'}$), 132.56 (s, C_6), 142.7 (d, $J_{C\text{-}F}$ = 1 Hz, C_2), 157.02 (d, $J_{C\text{-}F}$ = 247 Hz, $C_{6'}$), 171.31 ppm (s, $CONH_2$); MS (CI-isobutane) *m/z* 279-281 $[M+H]^+$.

Procedure B: DBU (1.51 g; 9.93 mmol), $Cu(OAc)_2$ monohydrate (0.66 g; 3.31 mmol) and 2-chloro-6-fluorophenylboronic acid (0.58 g; 3.31 mmol) were added to a stirred solution of **21** (0.50 g; 3.31 mmol) in distilled dioxane (25 mL). After 2 h of stirring at RT, a further quantity of 2-chloro-6-fluorophenylboronic acid (0.15 g; 0.89 mmol) was added and, after 30', the mixture was treated with pH 4.5 NaOAc/AcOH buffer (50 mL) and 0.3M EDTA tetrasodium (15 mL), then extracted with EtOAc (3 × 50 mL). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to leave a yellowish oil. The crude product was purified by FC, eluting with PE containing 30% EtOAc to give 0.31 g (34%) of the title product (**19**) as a yellow solid. M.p. and spectral data are identical to those of a sample obtained by procedure A.

***N*-[[2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl]methyl]1,1,1-trifluoro-methansulfonamide (20):** $AlCl_3$ (0.50 g; 3.7 mmol) was added to a cooled (0°C) suspension of $LiAlH_4$ (0.16 g; 4.3 mmol) in dry THF (40 mL), in a flame-dried reaction flask under a nitrogen atmosphere. To the resulting solution, **19** (0.3 g; 1.0 mmol), dissolved in dry THF (10 mL), was added dropwise. The reaction mixture was refluxed for 8 h. The reaction mixture was cooled (0 °C) and treated with water (1 mL), 10 % aqueous NaOH (1 mL) then again with water (1mL). The aluminium salts were filtered off, and the organic phase was dried ($MgSO_4$) and evaporated under reduced pressure to give a yellowish semisolid. This compound was purified by FC, eluting with CH_2Cl_2 containing variable amounts of MeOH (from 1 to 10%) to afford 0.21 g (74%) of the expected amine as a yellow solid. This product was checked by NMR and RP-HPLC (column: Nucleosil Nautilus (100-SC18, 250 × 4.6, Macherey-Nagel); eluent: acetonitrile/water 45/55 + 0.1% TFA 1.2 mL/min; λ = 226, 254 nm) to verify the absence of defluorinated by-product and used immediately in the next step. ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.21 (s, 3H, CH_3), 3.80 (s, 2H, CH_2), 4.42 (s, br, 2H, NH_2), 6.37 (dd, J = 8.1 Hz, 2.7 Hz, 1H, ArH_3), 6.92 (d, J = 8.1 Hz, 1H, ArH_4), 7.05-7.34 (m, 4H, $ArH_{3',4',5'}$, ArH_6), 8.17 ppm (s, br, 1H, ArNH); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 20.49 (s, CH_3), 42.15 (s, CH_2), 115.47 (d, $J_{C\text{-}F}$ = 20.2 Hz, $C_{5'}$), 117.16 (d, $J_{C\text{-}F}$ = 2.5 Hz, C_3), 123.81 (d, $J_{C\text{-}F}$ = 8.8 Hz, $C_{4'}$), 126.65 (d, $J_{C\text{-}F}$ = 3.2 Hz, $C_{3'}$), 126.93 (s, C_5), 128.22 (d, $J_{C\text{-}F}$ = 4.3 Hz, $C_{2'}$), 128.74 (s, C_4), 128.84 (d, $J_{C\text{-}F}$ = 14 Hz , $C_{1'}$), 129.22 (s, C_1), 130.44 (s, C_6), 141 (d, $J_{C\text{-}F}$ = 1 Hz, C_2), 156.28 ppm (d, $J_{C\text{-}F}$ = 245 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 265-267 $[M+H]^+$. Triethylamine (0.61 mL; 4.4 mmol) was added under stirring to a solution of the resulting amine (0.39 g; 1.47 mmol) in dry CH_2Cl_2 (10 mL) kept under nitrogen. The reaction mixture was cooled (0°C) and trifluoromethansulfonyl anhydride (0.37 mL; 2.2 mmol) was added dropwise. The reaction mixture was stirred at RT for 5 h. The mixture was diluted with CH_2Cl_2 (20 mL), transferred to a separating funnel and washed with 2N HCl (2 × 20 mL), water (20 mL) and brine (20 mL), then dried ($MgSO_4$) and evaporated under reduced pressure to give the crude product as an orange liquid. The product was purified by FC, using PE containing 25% CH_2Cl_2 to afford 0.2 g (34%) of the desired product (**20**) as a hygroscopic semisolid material. ^{1}H NMR (300 MHz, [D_6]DMSO): δ= 2.25 (s, 3H, CH_3), 4.49 (s, 2H, CH_2), 6.40 (d, J = 8.1 Hz, 1H, ArH_3), 6.82 (s, 1H, ArH_6), 6.95 (d, J = 8.1 Hz, 1H, ArH_4), 7.03-7.39 (m, 4H, $ArH_{3',4',5'}$, ArNH), 9.85 ppm (s, br, 1H, $NHSO_2CF_3$); ^{13}C NMR (75 MHz, [D_6]DMSO): δ= 20.39 (s, CH_3), 43.48 (s, CH_2), 115.24 (d, $J_{C\text{-}F}$ = 20.2 Hz, $C_{5'}$), 117.65 (d, $J_{C\text{-}F}$ = 1.5 Hz, C_3), 119.73 (q, $J_{C\text{-}F}$ = 321 Hz, CF_3), 124.14 (d, $J_{C\text{-}F}$ = 9 Hz, $C_{4'}$), 125.91 (d, $J_{C\text{-}F}$ = 3.7 Hz, $C_{3'}$), 126.16 (s, C_5), 128.22 (d, $J_{C\text{-}F}$ = 3.8 Hz, $C_{2'}$), 128.54 (s, C_4), 128.73 (s, C_6), 129.64 (d, $J_{C\text{-}F}$ = 14.2 Hz , $C_{1'}$), 130.23 (s, C_1), 139.59 (d, $J_{C\text{-}F}$ = 1.5 Hz, C_2), 156.48 ppm (d, $J_{C\text{-}F}$ = 245 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 397399 $[M+H]^+$. Anal. calcd. for$C_{15}H_{13}ClFN_4O_2S$ ¼ H_2O: C 44.89, H 3.39, N 6.98; found: C 44.83, H 3.20, N 6.81.

***N*-[(2-amino-5-methylphenyl)amino]-methansulfonamide (23):** In a flame-dried flask, BH_3 1M in THF (29 mL; 29 mmol) was added to a stirred solution of **21** (1.45 g; 9.65 mmol) in dry THF (70 mL) kept at 0°C under nitrogen. The ice-bath was removed and the mixture refluxed for 2 h. The mixture was cooled to 0°C and excess borane destroyed by cautious addition of ice; gentle stirring of the mixture was continued until evolution of gas ceased. The reaction mixture was treated with brine (70 mL) and transferred to a separating funnel. The organic solvent was separated and the aqueous layer extracted with EtOAc (3 × 50 mL). The combined organic layers were dried (K_2CO_3) and evaporated, to leave 1.49 g of a pale yellow solid. This intermediate was suspended in CH_2Cl_2 (40 mL) and treated with triethylamine (2.93 g; 29 mmol), methansulfonyl chloride (1.11 g; 9.65 mmol) and stirred at RT for 1.5 h. The reaction mixture was diluted with water, the organic phase separated and the aqueous layer extracted with CH_2Cl_2 (2 × 40 mL). The combined organic layers were dried (Na_2SO_4) and evaporated, to leave 2.39 g of a pale yellow oil. The crude material was purified by FC, eluting with CH_2Cl_2 containing variable amounts of MeOH (1% to 2%) to afford 1.43 g (68%) of the desired product as a colourless oil. MS (CI-isobutane) *m/z* 215 $[M+H]^+$. The product was not characterized further, and was used directly in the next step.

***N*-[(2-amino-5-methylphenyl)amino]-4-chloro-benzenesulfonamide (24):** In a flame dried flask, BH_3 1M in THF (29 mL; 29 mmol) was added to a stirred solution of **21** (1.45 g; 9.65 mmol) in dry THF (70 mL) kept at 0°C under nitrogen. The ice-bath was removed and the mixture was refluxed for 2 h. The mixture was cooled to 0°C and excess borane destroyed by cautious addition of ice; gentle stirring of the mixture was continued until evolution of gas had ceased. The reaction mixture was treated with brine (70 mL) and transferred to a separating funnel. The organic solvent was separated and the aqueous layer extracted with EtOAc (3 × 50 mL). The combined organic layers were dried (K_2CO_3) and evaporated to leave 1.38 g of a pale yellow oil. This intermediate was suspended in CH_2Cl_2 (55 mL) and treated with triethylamine (2.61 g; 26.8 mmol), *p*chlorobenzensulfonyl chloride (0.94 g; 4.48 mmol) and stirred at RT for 1.5 h. The reaction mixture was diluted with water, the organic phase separated and the aqueous layer extracted with CH_2Cl_2 (2 × 40 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to

leave 3.14 g of a pale yellow oil. The crude material was purified by FC, eluting with CH_2Cl_2 containing variable amounts of EtOAc (10% to 25%) to afford 2.40 g (80%) of the desired product as a colourless oil, which solidified on standing. ^{1}H NMR (300 MHz, $CDCl_3$): δ= 2.14 (s, 3H, ArC*H*$_3$-), 3.50 (s, br, 2H, NH_2), 3.96 (s, 2H, CH_2), 5.18 (s, br, 1H, NH), 6.53 (d, J = 8.1 Hz, 1H, ArH_3), 6.65 (d, 1H, J = 1.7 Hz, ArH_6), 6.89 (dd, J = 8.1, 1.7 Hz, 1H, ArH_4), 7.44 (d, J = 6.9 Hz, 2H, Ar*H*$_{3'\text{-}5'}$), 7.75 ppm (d, J = 6.9 Hz, 2H, Ar*H*$_{2'\text{-}6'}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ= 20.19 (CH_3), 45.22 (CH_2), 116.71 (C_5), 119.09 (C_3), 127.89 (C_1), 128.58/129.40 (two peaks, 4C, $C_{2',3',5',6'}$), 130.14 (C_6*), 130.73 (C_4*), 137.80 ($C_{4'}$§), 139.26 ($C_{1'}$§), 142.52 ppm (C_2); MS (CI-isobutane) *m/z* 310-312 $[M+H]^+$.
* Assignments might be reversed. § Assignments might be reversed.

***N*-[(2-amino-5-iodophenyl)amino]-4-chloro-benzenesulfonamide (25):** In a flame dried flask, BH_3 1M in THF (38 mL; 38 mmol) was added to a stirred solution of 2-amino-5-iodo benzencarboxamide (**22**) (2.00 g; 7.63 mmol) in dry THF (100 mL) kept at 0°C under nitrogen. The ice-bath was removed and the mixture was refluxed for 30 min. The mixture was cooled to 0°C and excess borane destroyed by cautious addition of ice; gentle stirring of the mixture was continued until evolution of gas had ceased. The reaction mixture was treated with brine (100 mL) and transferred to a separating funnel. The organic solvent was separated and the aqueous layer extracted with EtOAc (2 × 50 mL). The combined organic layers were dried (K_2CO_3) and evaporated to leave 2.32 g of a pale yellow solid. This intermediate was suspended in CH_2Cl_2 (60 mL) and treated with triethylamine (2.31 g; 22.9 mmol), *p*-chlorobenzensulfonyl chloride (1.61 g; 7.63 mmol) and stirred at RT for 1.5 h. The reaction mixture was diluted with water, the organic phase separated and the aqueous layer extracted with CH_2Cl_2 (2 × 40 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to leave 3.81 g of a pale yellow oil. The crude material was purified by FC, eluting with CH_2Cl_2 containing variable amounts of EtOAc (1.5% to 5%) to afford 2.83 g (88%) of the desired product as a white solid. Mp: 117 - 11°C; ^{1}HNMR (300 MHz, CD_3OD): δ= 3.91 (s, 2H, ArC*H*$_2$-), 6.46 (d, J = 8.4 Hz, 1H, ArH_3), 7.16 (d, J = 2.1 Hz, 1H, ArH_6), 7.24 (dd, J = 8.4, 2.1 Hz, 1H, ArH_4), 7.54 (d, J = 6.9 Hz, 2H, Ar*H*$_{3'\text{-}5'}$), 7.78 ppm (d, J = 6.9 Hz, 2H, Ar*H*$_{2'\text{-}6'}$); ^{13}C NMR (75 MHz, CD_3OD): δ= 44.61 (CH_2), 78.42 (C_5), 119.09 (C_3), 124.43 (C_1), 129.7/129.6 (two peaks, 4C, $C_{2',3',5',6'}$), 138.45 (C_6*), 139.03 (C_4*), 139.85 ($C_{4'}$§), 140.45 ($C_{1'}$§), 147.1 ppm (C_2); MS (CI-isobutane) *m/z* 423-425 $[M+H]^+$. * Assignments might be reversed. § Assignments might be reversed.

4-chloro-*N*-[[2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl]methyl]-methansulfonamide (26): To a stirred solution of **23** (0.76 g; 3.55 mmol) in distilled dioxane (30 mL), DBU (1.59 g; 10.6 mmol), $Cu(OAc)_2$ monohydrate (0.78 g; 3.90 mmol) and 2-chloro-6fluorophenylboronic acid (0.68 g; 3.90 mmol) were added. After 2 h of stirring at RT, a further quantity of 2-chloro-6-fluorophenylboronic acid (0.15 g; 0.89 mmol) was added and, after 30', the mixture was treated with pH 4.5 NaOAc/AcOH buffer (70 mL), with 0.3M EDTA tetrasodium (20 mL) then extracted with EtOAc (3 × 50 mL). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to leave 1.25 g of a brown oil. The crude product was purified by FC, eluting with CH_2Cl_2 to yield 0.38 g (31%) of the desired product as a yellow semisolid foam. An analytical sample was recrystallized from iPrOH to afford **26** as yellow needles. Mp: 137 – 138°C. ^{1}H NMR (300 MHz, $[D_6]$DMSO): δ= 2.23 (s, 3H, CH_3), 2.94 (s, 3H, SO_2CH_3), 4.23 (s, 2H, CH_2), 6.35 (dd, J = 8.1, 2.7 Hz, 1H, ArH_3), 6.83 (s, 1H, ArH_6), 6.95 (d, J = 8.1 Hz, 1H, ArH_4), 7.11-7.39 (m, 4H, $ArH_{3',4',5'}$, ArNH), 7.51 ppm (t, J = 6 Hz, 1H, $NHSO_2$); ^{13}C NMR (75 MHz, $[D_6]$DMSO): δ= 20.14 (s, CH_3), 39.30 (s, CH3), 43.43 (s, CH_2), 115.20 (d, $J_{C\text{-}F}$ = 20.3 Hz, $C_{5'}$), 115.65 (d, $J_{C\text{-}F}$ = 2.1 Hz, C_3), 124.26 (d, $J_{C\text{-}F}$ =4.5 Hz, $C_{4'}$), 125.74 (d, $J_{C\text{-}F}$ = 4.4 Hz, $C_{3'}$), 125.74 (s, C_5), 128.36 (s, C_4), 128.47 (d, $J_{C\text{-}F}$ = 5 Hz, $C_{2'}$), 128.59 (d, $J_{C\text{-}F}$ = 5.6 Hz, $C_{4'}$), 129.20 (s, C_1), 129.64 (s, C_6), 139.57 (d, $J_{C\text{-}F}$ = 1.0 Hz, C_2), 156.28 ppm (d, $J_{C\text{-}F}$ = 246 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 397-399 $[M+H]^+$.MS (CI-isobutane) *m/z* 343/345 $[M+H]^+$. Anal. calcd. for$C_{15}H_{16}ClFN_2O_2S$: C 52.55, H 4.70, N 8.17; found: C 52.85, H 4.86, N 8.30.

4-chloro-*N*-[[2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl]methyl]-benzenesulfonamide (27): DBU (0.13 g; 0.86 mmol), $Cu(OAc)_2$ monohydrate (0.12 g; 0.58 mmol) and 2-chloro-6fluorophenylboronic acid (0.086 g; 0.43 mmol) were added to a stirred solution of **24** (0.09 g; 0.29 mmol) in distilled dioxane (4 mL). After 5 h of stirring at RT, the mixture was treated with pH 4.5 NaOAc/AcOH buffer (70 mL) then with 0.3M EDTA tetrasodium (20 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to leave a brown oil. The crude product was purified by FC, eluting with PE containing 10% EtOAc to yield 0.05 g (39%) of the desired product as a white solid. An analytical sample was obtained by recrystallisation from EtOH. M.p.: 147 – 148°C; ^{1}H NMR (300 MHz, $[D_6]$DMSO): δ= 2.17 (s, 3H, CH_3), 4.09 (m, 2H, CH_2), 6.34 (d, J = 7.8 Hz, 1H, ArH_3), 6.78–7.37 (m, 6H, $ArH_{4,6}$, $ArH_{3',4',5'}$, ArNH), 7.66 (d, J = 9 Hz, 2H, $ArH_{3'',5''}$), 7.86 (d, J = 9 Hz, 2H, $ArH_{2'',6''}$), 8.26 ppm (m, br, 1H, $NHSO_2$); ^{13}C NMR (75 MHz, $[D_6]$DMSO): δ= 20.09 (s, CH_3), 43.38 (s, CH_2), 115.17 (d, $J_{C\text{-}F}$ = 20.1 Hz, $C_{5'}$), 116.24 (s, C_3), 124.09 (d, $J_{C\text{-}F}$ = 8.9 Hz, $C_{4'}$), 125.33 (s, C_1), 125.74 (d, $J_{C\text{-}F}$ = 3.1 Hz, $C_{3'}$), 128.32 (s, C_4), 128.33 (d, $J_{C\text{-}F}$ = 3.8 Hz, $C_{2'}$), 128.38 (s, C_5), 128.49/129.24 (2s, $C_{3'',5''}$/$C_{2'',6''}$), 128.76 (d, $J_{C\text{-}F}$ = 14.2 Hz, $C_{1'}$), 129.42 (s, C_6), 137.33 (s, $C_{4''}$), 139.05 (s, $C_{1''}$), 139.51 (s, C_2), 156.48 ppm (d, $J_{C\text{-}F}$ = 245 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 439-441 $[M+H]^+$. Anal. calcd. for $C_{20}H_{17}Cl_2FN_2O_2S$: C 54.68, H 3.90, N 6.38; found: C 54.33, H 4.19, N 6.19.

4-chloro-*N*-[[2-[(2-chloro-6-fluorophenyl)amino]-5-iodophenyl]methyl]-benzenesulfonamide (28): DBU (1.08 g; 7.1 mmol), $Cu(OAc)_2$ monohydrate (0.52 g; 2.60 mmol) and 2-chloro-6fluorophenylboronic acid (0.45 g; 2.60 mmol) were added to a stirred solution of **25** (1.00 g; 2.37 mmol) in distilled dioxane (40 mL). After 2 h stirring at RT, a further quantity of 2-chloro-6-fluorophenylboronic acid (0.11 g; 0.59 mmol) was added and, after 30', the mixture was treated with pH 4.5 NaOAc/AcOH buffer (70 mL), with 0.3M EDTA tetrasodium (20 mL) then extracted with EtOAc (3 × 50 mL). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to leave 1.82 g of a brown oil. The crude product was purified by FC, eluting with CH_2Cl_2/PE 60/40 to yield 0.61 g (47%) of the desired product as a yellow semisolid foam. ^{1}H NMR (300 MHz, CD_3OD): δ= 4.12 (s, 2H, C*H*$_2$), 6.22 (d, J = 8.4 Hz, 3 Hz, 1H, ArH_3), 7.11-7.38 (m, 5H, $ArH_{3',4',5'}$, ArH_4, ArH_6), 7.54 (d, J = 6.9 Hz, 2H, Ar*H*$_{3''4''}$), 7.78 ppm (d, J = 6.9 Hz, 2H, Ar*H*$_{2''\text{-}6''}$); ^{13}C NMR (75 MHz, CD_3OD): δ= 44.87 (CH_2), 82.88 (C_5), 116.1 (d, $J_{C\text{-}F}$ = 20.7 Hz, $C_{5'}$), 118.8 (d, J_{CF} = 2.3 Hz, C_3), 126.1 (d, $J_{C\text{-}F}$ = 8.8 Hz, $C_{4'}$), 126.9 (d, $J_{C\text{-}F}$ = 3.3 Hz, $C_{3'}$), 127.9 (C_1), 129.1 (d, $J_{C\text{-}F}$ = 14.2 Hz, $C_{1'}$), 129.8/130.5 (two peaks, 4C, $C_{2'',3'',5'',6''}$), 131.1 (d, $J_{C\text{-}F}$ = 3.4 Hz, $C_{2'}$), 138.9 (C_6*), 139.4 (C_4*), 140 ($C_{4''}$§), 140.3 ($C_{1''}$§), 143.8 (d, $J_{C\text{-}F}$ = 1.3 Hz, C_2), 158.5 ppm (d, $J_{C\text{-}F}$ = 247 Hz, $C_{6'}$); MS (CI-isobutane) *m/z* 551-553 $[M+H]^+$. * Assignments might be reversed. § Assignments might be reversed.

(2*E*)-3-[4-[(2-chloro-6-fluorophenyl)amino]-3-[[[(4-chlorophenyl)sulfonyl]amino]methyl]phenyl-2-propenoic acid, ethyl ester (29): Ethylacrylate (0.60 mL; 5.57 mmol), triethylamine (0.45 mL; 3.21 mmol), triphenylphosphine (0.07 g; 0.27 mmol) and $Pd(dba)_2$ (0.071 g; 0.13 mmol) were added to a stirred solution of **28** (0.74 g; 1.34 mmol) in dry DMF (15 mL) and the reaction mixture was heated under nitrogen to 100°C for 20 h. The reaction mixture was cooled, diluted with water (25 mL) and extracted with EtOAc (3 × 30 mL). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to afford 1.04 g of crude product as a brown oil. The crude product was purified by FC, eluting with CH_2Cl_2 containing 2% EtOAc, affording 0.1 g of unreacted **28** and 0.41 g (58%) of the desired product (**29**) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ= 1.30 (t, J = 6.9 Hz, 3H, CH_3), 4.21 (m, 4H, CH_2NH, CH_2O), 5.06 (t, J = 5.8 Hz, 1H, NHSO$_2$),6.20 (d, J = 15.7 Hz, 1H, O(O)CCH=), 6.49-6.54 (m, 2H, ArH$_3$, ArH$_4$), 7.04-7.32 (m, 5H, ArH$_{3',4',5'}$, ArNH, ArH$_6$), 7.46-7.53 (m, 3H, Ar$H_{3''\text{-}4''}$, CH=CHAr), 7.81 ppm (d, J = 8.4 Hz, 2H, Ar$H_{2''\text{-}6''}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ= 14.32 (CH_3), 45.68 (ArCH_2), 60.43 (OCH_2), 115 (d, $J_{C\text{-}F}$ = 20 Hz, $C_{5'}$), 115.5 (O(O)CCH=), 121.9 (d, $J_{C\text{-}F}$ = 1.8 Hz, C_3), 125.1 (d, $J_{C\text{-}F}$ = 8.8 Hz, $C_{3'}$), 125.7 (d, $J_{C\text{-}F}$ = 2.9 Hz, $C_{4'}$), 126.6 (C_5), 126.6 (C_1), 126.8 (d, $J_{C\text{-}F}$ = 13.8 Hz, $C_{1'}$), 128.7 (C_6), 128.73 ($C_{3'',5''}$), 129.51 (C_4), 129.55 ($C_{2'',6''}$), 130.2 (d, $J_{C\text{-}F}$ = 10.6 Hz, $C_{2'}$), 130.5 (C_3), 137.4 (d, $J_{C\text{-}F}$ = 2.4 Hz, $C_{4''}$), 139.67 (d, $J_{C\text{-}F}$ = 2 Hz, $C_{2''}$), 143.8 (CH=CH), 144.54 (C_2), 156.9 (d, $J_{C\text{-}F}$ = 249 Hz, $C_{6'}$), 167.38 ppm (CO); MS (CI-isobutane) *m/z* 523-525-527 $[M+H]^+$.

4-[(2-chloro-6-fluorophenyl)amino]-3-[[[(4-chlorophenyl)sulfonyl]amino]methyl]-benzenepropanoic acid, ethyl ester (30): To a stirred solution of **29** (0.32 g; 6.61 mmol) in EtOH (14 mL), 10% Pd/C (0.13 g) was added. The mixture was hydrogenated at 1 bar pressure for 2 h. The catalyst was removed by filtration through celite, the filtrate evaporated under reduced pressure and purified by FC, eluting with CH_2Cl_2 containing 1 % EtOAc to give 0.25 g (78%) of the title product as a pale yellow semisolid. ^{1}H NMR (300 MHz, $CDCl_3$): δ= 1.2 (t, J = 7.1 Hz, 3H, CH_3), 2.5 (t, J = 7.7 Hz, 2H, ArCH_2), 2.78 (t, J = 7.7 Hz, 2H, CH_2CO), 4.08 (q, J = 7.1 Hz, 2H, CH_2O), 4.18 (d, J = 5.7 Hz, 2H, CH_2NH), 5.19 (t, J = 5.7 Hz, 1H, NHSO$_2$), 6.09 (s, 1H, NH), 6.5 (m, 1H, ArH$_3$), 6.88 (s, 1H, ArH$_6$), 6.94-7.02 (m, 3H, ArH$_{3',4',5'}$), 7.23 (m, 1H, ArH$_4$), 7.41 (d, J = 8.4 Hz, 2H, Ar$H_{3''\text{-}5''}$), 7.77 ppm (d, J = 8.4 Hz, 2H, ArH$_{2''\text{-}6''}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ= 14.18 (CH_3), 29.93 (ArCH_2), 35.88 (COCH_2), 45.57 (CH_2NH), 60.49 (OCH_2), 114.9 (d, $J_{C\text{-}F}$ = 20.1 Hz, $C_{5'}$), 116.9 (d, $J_{C\text{-}F}$ = 2.9 Hz, C_3), 123.5 (C_1), 123.6 (d, $J_{C\text{-}F}$ = 8.8 Hz, $C_{3'}$), 125.5 (d, $J_{C\text{-}F}$ = 3.3 Hz, $C_{4'}$), 128.3 (d, $J_{C\text{-}F}$ = 13.5 Hz, $C_{1'}$), 128.6 (C_5), 128.7 ($C_{3'',5''}$), 129 (C_6), 129.4 ($C_{2'',6''}$), 130.7 (C_4), 133.5 (C_2), 137.8 ($C_{4''}$), 139.3 ($C_{1''}$), 140.6 (C_2), 157.7 (d, $J_{C\text{-}F}$ = 248 Hz, $C_{6'}$), 173 ppm (CO); MS (CI-isobutane) *m/z* 525-527-529 $[M+H]^+$.

4-[(2-chloro-6-fluorophenyl)amino]-3-[[[(4-chlorophenyl)sulfonyl]amino]methyl]-benzenepropanoic acid (31): Compound **30** (0.13 g; 0.25 mmol) was dissolved in EtOH (5 mL) then NaOH 10% (w/V) (5 mL) was added and the mixture was heated to 60 °C for 1h. The solvent was evaporated and the aqueous residue acidified with 1N HCl, extracted with CH_2Cl_2 (3 × 15 mL), the organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to afford 0.13 g of crude material. Purification by FC, eluting with CH_2Cl_2 containing 5% MeOH, afforded 0.12 g (quant.) of **31** as a pale yellow oil, which solidified upon standing to give a pale yellow solid. Mp. 70 – 71°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ= 2.58 (t, J = 7.5 Hz, 2H, CH_2CO), 2.81 (t, J = 7.5 Hz, 2H, ArCH_2), 4.19 (q, J = 5.4 Hz, 2H, CH_2NH), 5.01 (m, br, 1H, NHSO$_2$), 6.05 (s, 1H, NH), 6.49-6.54 (m, 1H, ArH$_3$), 6.90 (s, 1H, ArH$_6$), 6.96-7.06 (m, 4H, ArH$_{3',4',5'}$), 7.22-7.25 (m, 1H, ArH$_4$), 7.43 (d, J = 8.4 Hz, 2H, Ar$H_{3''\text{-}5''}$), 7.78 ppm (d, J = 8.4 Hz, 2H, ArH$_{2''\text{-}6''}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ= 29.63 (ArCH_2), 35.49 (COCH_2), 45.37 (CH_2NH), 114.9 (d, $J_{C\text{-}F}$ = 20.6 Hz, $C_{5'}$), 117 (C_3), 123.57 (C_1), 123.72 ($C_{3'}$), 125.6 (d, $J_{C\text{-}F}$ = 3.4 Hz, $C_{4'}$), 128.3 (d, $J_{C\text{-}F}$ = 14.1 Hz, $C_{1'}$), 128.6 (C_5), 128.7 ($C_{3'',5''}$), 129 (C_6), 129.42 ($C_{2'',6''}$), 130.18 (C_4), 133.16 ($C_{2'}$), 137.72 ($C_{4''}$), 139.4 ($C_{1''}$), 140.68 (C_2), 157.72 (d, $J_{C\text{-}F}$ = 248 Hz, $C_{6'}$), 184.03 ppm (COOH); MS (CI-isobutane) *m/z* 497-499-501 $[M+H]^+$. Anal. calcd. for$C_{22}H_{19}Cl_2FN_2O_4S$: C 53.13, H 3.85, N 5.63; found: C 53.02, H 3.78, N 5.29.

Determination of ionisation constants The ionisation constants of compounds were determined by potentiometric titration with the GLp*K*a apparatus (Sirius Analytical Instruments Ltd, Forest Row, East Sussex, UK). Because of the low aqueous solubility, lumiracoxib **4** and compounds **13**, **15**-**18**, **20**, **26**-**27** and **31** required titrations in the presence of methanol as cosolvent: at least five different hydroorganic solutions (ionic strength adjusted to 0.15 M with KCl) of the compounds (20 mL, about 1 mM in 20–60 wt% methanol) were initially acidified to pH 1.8 with 0.5 N HCl; the solutions were then titrated with standardized 0.5N KOH to pH 12.2 at 25 °C under nitrogen. The apparent ionization constants in the water–methanol mixtures (p_sK_a) were obtained and aqueous pK_a values were calculated by extrapolation to zero content of the cosolvent, following the Yasuda-Shedlovsky procedure.[32]

Biological Data

Isolation of human platelets and analysis of platelet aggregation. Human blood was taken from the antecubital vein of healthy volunteers of both genders who had not taken medications for at least 72 h and had no history of cardiovascular disease; age range was 18 to 60 years. Volunteers gave their informed and signed consent to the use of blood samples for research purposes. Blood was anticoagulated with anticoagulant citrate dextrose solution (ACD) (84 mM sodium citrate, 41 mM citric acid and 136 mM glucose; 1:7, v:v) and treated with 100µM acetylsalicylic acid. Platelet-rich plasma was obtained by centrifugation at 180 g for 15 min at room temperature, and further centrifugation at 650 g for 10 min at room temperature, to obtain a platelet pellet that was resuspended in HBSS. Washed platelet suspension was adjusted to 2 x10^8 cell ml^{-1}. and each sample was prewarmed (37°C) before drug or vehicle incubation. Agonistinduced platelet aggregation was determined using the Born turbidimetric assay[40] in a 0.5-mL sample of washed platelets at 37°C, using a Chrono-Log aggregometer (Mascia Brunelli, Milano, Italy). The baseline was set using HBSS solution as blank (100% light transmission vs. platelet suspension). The platelet samples were incubated with drug or vehicle (DMSO, maximum 0.2%, v:v) for 5 min at 37°C, challenged with the TP agonist U-46619 (0.1-0.5 µM) under stirring, and aggregation monitored for 6 min. In a few selected experiments, platelet aggregation was induced with thrombin (0.1U mL^{-1} or the calcium ionophore A-23187 (3 µM). The use of DMSO did not affect either thrombin or U-46619-

induced aggregation. Experiments were repeated in triplicate using platelets from different subjects (n= 3–5). Given the significant inter-subject variability of the platelet response to agonist challenge, the anti-aggregating activity of different compounds was compared with the appropriate control aggregation, recorded immediately before and after drug testing.

*COX-2 inhibition (*buffy coat from human *whole blood).* A whole blood assay[41] was performed to evaluate the ability of synthesised compounds to inhibit COX-2. The test compound was dissolved in DMSO and 1µL aliquots were placed in plastic tubes containing 1mL blood sample (Buffy coat diluted 1:1) treated with 10µg mL^{-1} ASA. LPS challenge (10µg mL^{-1}, overnight, 37°C), was given to promote COX-2 expression. Samples were centrifuged (5000×g, 5 min) to obtain plasma. COX-2 activity was detected by PGE_2 production, evaluated by enzyme immunoassay (PGE_2 EIA kit, *Cayman Chemical*). Standard curves with known concentrations of PGE_2 were used to determine prostanoid concentrations in the sample wells, and % inhibition by compounds was calculated versus control samples. The concentration of each test compound that caused 50% inhibition (IC_{50}) was then calculated.

COX-2 inhibition (lympho-monocytes). COX-2 activity was also detected in aqueous buffer (HBSS) lympho-monocytes suspension, in order to avoid compound binding to plasma-protein. Cells were obtained from diluted buffy coat layered on Ficoll Paque, centrifuged at 400×g 30 min to separate lympho-monocytes from the pelletted PMNL and red cells. Lympho-monocytes were collected and suspended in HBSS. Samples were processed as described above for COX-2 expression/activity in whole blood.

COX-1 inhibition. A whole blood assay was performed to evaluate the ability of synthesised compounds to inhibit COX-1. Methanolic solutions of test compounds at different concentrations were prepared, 10 µL aliquots were placed in incubation tubes and the solvent was evaporated. The residues were dissolved by vortexing in 1 mL untreated blood to test COX-1 inhibition. The final concentrations of the test compounds were therefore diluted 100 times in the incubation tubes. The COX-1 aliquots were incubated in glass tubes for 1 h at 37°C, which is sufficient to complete coagulation, then centrifuged at 2000×*g* for 10 min, after which the serum was ready to be tested for platelet TXB_2 production. Percentage inhibition in samples treated with the test compounds was evaluated versus control samples with basal TXB_2 production. Prostanoid production was evaluated by the enzyme immunoassay, following the specific instructions provided by *Cayman Chemical*, based on a competitive reaction between TXB_2 and a TXB_2-acetylcholinesterase conjugate (TXB_2 tracer) for a specific TXB_2 antiserum. Standard curves with known concentrations of TXB_2 were used to determine prostanoid concentrations in the sample wells. Percent inhibition in compound-treated samples was calculated versus untreated controls. The concentration of the test compounds causing 50% inhibition (IC_{50}) was calculated from the concentration-inhibition response curve (5-6 experiments).

Culture and transfection of COS-7 and HEK293 cells. COS-7 and HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 20 mM HEPES buffer, pH 7.4, at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were plated out into 24-well dishes previously coated with poly-D-lysine, following a standard seeding protocol to obtain a 50–60% confluence at the time of transfection. This was performed using Lipofectamine2000 according to the manufacturer's instructions. In brief, Lipofectamine2000/DNA transfection mix was prepared in Opti-MEM I Medium at an optimized 2:1 ratio. Transfection mix was added to cells after 20-min incubation at room temperature to allow the complexes to form. Equal protein content was ensured at the end of each assay by the Lowry dye-binding procedure.

Radioligand binding assays. Receptor expression was monitored 48 h after transfection. Equilibrium mixed-type binding curve[42] of [^{3}H]SQ29,548 (Perkin-Elmer, Boston, MA, USA) together with heterologous competition curves of the specified ligands were generated as described elsewhere.[36] Briefly, confluent adherent cells in 250 mL of serum-free Dulbecco's modified Eagle's medium, containing 0.2% (w:v) bovine serum albumin, were assayed in the presence of 0.1–1 nM of the specific receptor antagonist [^{3}H]SQ29548 (48 Ci $mmol^{-1}$), 3 nM–10 µM of the homologous cold ligand, or 1–300 µM of the heterologous cold ligands. All samples contained 0.2% ethanol (v:v) as vehicle for SQ29548, and 0.3% DMSO (v:v) as the drug vehicle. After 30 min incubation at room temperature, cells were lysed in 0.5 N NaOH and radioactivity measured by liquid scintillation counting (Ultima Gold; Packard Instruments, Meriden, CT, USA).

Rat aorta preparation. Male Sprague–Dawley rats (n = 3) weighing 180–220 g were used. The animals were killed by inhalation of high concentrations of CO_2 in air. The middle part of aorta was quickly removed and placed in ice-cold Tyrode's solution. The aorta was dissected from surrounding tissue and prepared as rings. The aortic rings were placed in 5ml organ baths filled with Tyrode's solution (composition in mM: NaCl, 142.9; KCl, 2.7; $NaHCO_3$, 11.9; glucose,
5.5; $CaCl_2$, 1.8; $MgCl_2$ $6H_2O$, 0.5; NaH_2PO_4, 0.4). The pH was kept at 7.4 by gassing with 6.5% CO_2 in O_2 and the temperature was kept constant at 37 °C. The aortic rings were mounted on lower and upper organ hooks, connected to the isometric force-displacement transducers. Changes in smooth-muscle tension in the preparations, that is vascular smooth-muscle contractions and relaxations, were recorded and displayed by a computerized data acquisition system connected to a "Power MacLab" Bridge Amplifier. The rat aortic rings were allowed to equilibrate for 60 min; the baseline resting tension was set at 10 mN with a load of 1 g and the preparations were treated for 20 min with 10 µM indomethacin. The capacity of the aortic rings to contract and to relax was checked by challenges with 10 µM noradrenaline and 0.1–10 µM acetylcholine, respectively. After another equilibration period of 60 min and the pretreatment period of 20 min with 10 µM indomethacin, cumulative concentration–response curves for U-46619 were established in control or in the presence of each compound, added to the organ bath fluid 20 min before the concentration–response curves for U-46619 were determined. All responses were expressed as percent of the maximal contraction induced by U-46619.

Statistical analysis. Data from radioligand binding were evaluated by a nonlinear, least-squares curve fitting procedure using GraphPad Prism version 4 software package, implemented with the n-ligand mbinding site model, as described in the LIGAND software.[43] Parameter errors are in all cases expressed in percentage coefficient of variation (%CV) and calculated by simultaneous analysis of at least two different independent experiments performed in duplicate or triplicate. A statistical level of significance of $P<0.05$ was set.

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