



In vitro pharmacological evaluation of multitarget agents for thromboxane prostanoid receptor antagonism and COX-2 inhibition



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ABSTRACT

Purpose: Patients with high cardiovascular risk due to ageing and/or comorbidity (diabetes, atherosclerosis) that require effective management of chronic pain may take advantage from new non-steroidal anti-inflammatory drugs (NSAIDs) that at clinical dosages may integrate the anti-inflammatory activity and reduced gastrointestinal side effects of selective cyclooxygenase-2 (COX-2) inhibitor (coxib) with a cardioprotective component involving antagonism of thromboxane A₂ prostanoid (TP) receptor.

Methods: New compounds were obtained modulating the structure of the most potent coxib, lumiracoxib, to obtain novel multitarget NSAIDs endowed with balanced coxib and TP receptor antagonist properties. Antagonist activity at TP receptor (pA₂) was evaluated for all compounds in human platelets and in an heterologous expression system by measuring prevention of aggregation and Gq-dependent production of intracellular inositol phosphate induced by the stable thromboxane A₂ (TXA₂) agonist U46619. COX-1 and COX-2 inhibitory activities were assessed in human washed platelets and lympho-monocytes suspension, respectively. COX selectivity was determined from dose–response curves by calculating a ratio (COX-2/COX-1) of IC₅₀ values.

Results: The tetrazole derivative **18** and the trifluoromethan sulfonamido-isoster **20** were the more active antagonists at TP receptor, preventing human platelet aggregation and intracellular signalling, with pA₂ values statistically higher from that of lumiracoxib. Comparative data regarding COX-2/COX-1 selectivity showed that while compounds **18** and **7** were rather potent and selective COX-2 inhibitor, compound **20** was somehow less potent and selective for COX-2.

Conclusion: These results indicate that compounds **18** and **20** are two novel combined TP receptor antagonists and COX-2 inhibitors characterized by a fairly balanced COX-2 inhibitor activity and TP receptor antagonism and that they may represent a first optimization of the original structure to improve their multitarget activity.

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1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) provide analgesic and anti-inflammatory properties by virtue of cyclooxygenase

Abbreviations: AA, arachidonic acid; CV, cardiovascular; COX, cyclooxygenase coxibs; COX-2, selective inhibitors; DMEM, Dulbecco's modified eagle's medium; EIA, enzyme immunoassay; IP, inositol phosphate; HEK293, human embryonic kidney 293; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; TXA₂, thromboxane A₂; TP, thromboxane prostanoid receptor.

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(COX) inhibition. COX is responsible for prostanoid production from arachidonic acid (AA) and can be inhibited reversibly by non-aspirin NSAIDs and irreversibly by aspirin. COX exists in two isoforms, the housekeeping enzyme COX-1 responsible for the gastric cytoprotection and haemostatic integrity, and the inducible isoform COX-2, mostly expressed in response to inflammatory stimuli and constitutively present in some specific tissue such as endothelial cells, brain and kidney [1,2].

Severe gastric problems such as bleeding, gastric erosion and ulcers are the main side effect of chronic use of conventional NSAIDs and aspirin, mainly due to the inhibition of the COX-1-derived gastroprotective prostaglandin (PG) E₂ production [3]. Celecoxib (Celebrex) and rofecoxib (Vioxx) were the first COX-2

selective inhibitors (coxibs) to enter the market as second generation NSAIDs to be used in symptomatic treatments of patients with osteoarthritis and rheumatoid arthritis with the promise of being antiinflammatory while minimizing gastrointestinal (GI) toxicity [4]. However, in 2004 Vioxx was withdrawn from the market and concern over potential cardiovascular (CV) toxicity and risk of myocardial infarction and stroke associated with the extended use of coxibs [5,6] and traditional NSAIDs in general [7–9] widespread rapidly, leading the official medicine agencies to issue CV safety warnings for coxibs still on the market and successively also for non-selective NSAIDs.

Early explanation for the potential thrombotic risk included an imbalance in the biosynthesis of thromboxane A_2 (TXA_2 , a potent platelet aggregator and vasoconstrictor) and prostacyclin (PGI_2 , which has opposing actions) [10] as a result of the observation that urinary excretion of the principal PGI_2 metabolite, 2,3-dinor 6-keto $PGF_{1\alpha}$, was reduced in patients treated with celecoxib and rofecoxib, while TXB_2 , urinary metabolite of TXA_2 , was unaltered [11,12]. Indeed, PGI_2 is the major end product of COX-2 in vascular endothelium and reduced prostacyclin receptor signalling has been suggested to contribute to the adverse CV outcomes observed with coxibs [13]. Other explanations have been also proposed to clarify the effect of coxibs (and conventional NSAIDs) that do not involve the isoform of COX present on endothelial cells. For example, it was hypothesized that the hazard could depend upon differences in the levels of lipid peroxides or in the supply of AA substrate between platelets and endothelial cells, such that PGI_2 synthesis is inhibited by NSAIDs more readily than platelet-derived TXA_2 [14]. Another report suggests that the CV toxicity of rofecoxib could be due to its intrinsic physico-chemical properties and primary metabolism that increase Low Density Lipoproteins and membrane lipids oxidation thus promoting formation of isoprostanes, a characteristic feature of atherogenesis [15]. The involvement of isoprostanes is of particular interest considering that they are nonenzymatic products of fatty acid oxidation, therefore insensitive to the action of aspirin and NSAIDs, they are chemically stable and are produced *in vivo* in quantities exceeding those of TXA_2 and, finally act through the TXA_2 prostanoid (TP) receptor [16].

For these reasons, we consider that the addition of a TP antagonist component to a coxib may provide protection against all the harmful activities mediated through the activation of the TP receptor by mediators sensitive and insensitive to aspirin/NSAIDs such as the unopposed platelet-derived TXA_2 and the nonenzymatic product isoprostanes.

Recently, an unexpected mechanism of action for diclofenac, a traditional NSAID with a non-selective profile of COX

inhibition, and its derivative lumiracoxib was described: the competitive antagonism at the TP receptor [17]. While it is true that increase in CV risk has been reported not only for selective coxibs, but also for conventional NSAIDs [18–20] including diclofenac [21], its potency as TP receptor antagonist is certainly not sufficient to have an impact in therapy at the prescribed clinical doses [17]. In addition, despite it has been withdrawn from the market due to hepatotoxicity problems (Novartis News (2007) Prexige® receives “not approvable” letter in the US despite being one of the most studied COX-2 inhibitors), a recent meta-analysis of eighteen clinical trials in patients with osteoarthritis taking lumiracoxib concluded that no significant differences in CV outcomes was evident between lumiracoxib and placebo or between lumiracoxib and other NSAIDs [22]. Thus, these findings seems to corroborate our choice to use lumiracoxib as a starting structure for chemical modification and to reinforce the hypothesis that cardiotoxicity associated to the different NSAIDs and coxibs might not depend on COX selectivity *per se*, but rather on distinctive characteristics of each single molecule, including its pharmacokinetic, that might affect differently the intricate inter-eicosanoid network of biosynthetic and signaling pathways leading to multiple events that may synergize or be functionally opposed, as it is the case for platelet function [23].

In the present study we report the physico-chemical profile and the full pharmacological characterization of four different compounds **18**, **20**, **7** and **32** (Fig. 1) endowed with dual COX-2 inhibitor activity and TP receptor antagonism out of a large series of compounds previously reported [24]. TP antagonism of these newly synthesized compounds has been evaluated calculating their pA_2 for anti-aggregating activity in human platelets as well as for their activity in inhibiting phospholipase C induced inositol phosphate production in Human Embryonic Kidney 293 (HEK293) cells transiently transfected with the $TP\alpha$ receptor in response to the TXA_2 stable analogue U46619. Moreover, we determined their COX-1 and COX-2 activity and selectivity in washed platelets and isolated human monocytes, respectively. The same was performed with reference molecules, namely the traditional NSAIDs naproxen, the coxib lumiracoxib, as well as the potent and selective TP antagonist terutroban [25].

2. Material and methods

2.1. Reagents

Animal serum, antibiotics, other supplements, Lipofectamine 2000, Opti-MEM I and molecular biology reagents were purchased

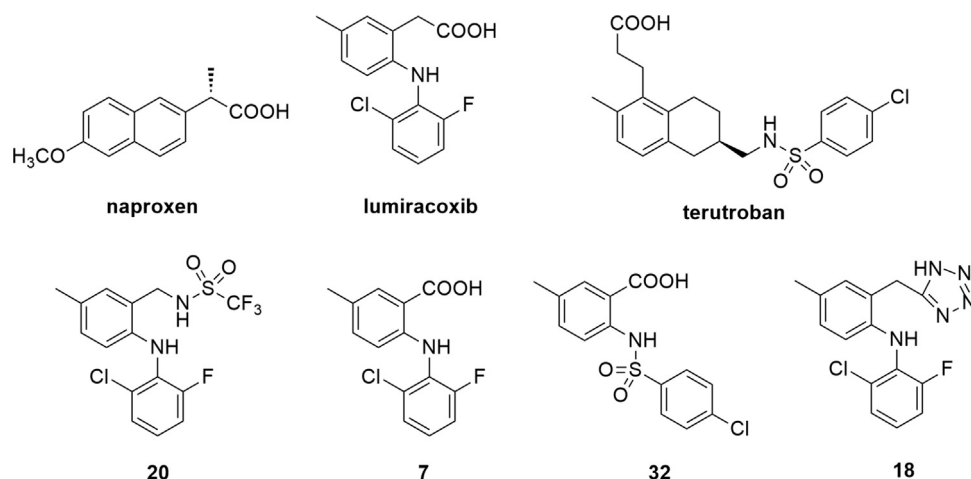


Fig. 1. Chemical structures of reference compounds naproxen, lumiracoxib and terutroban, as well as of the newly synthesized compounds **7**, **18**, **20**, and **32**.

from Invitrogen (Carlsbad, CA). Inositol-free Dulbecco's modified Eagle's medium (DMEM) was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Ultima Gold was from PerkinElmer Life and Analytical Sciences (Boston, MA), as were myo-[2-³H]inositol. U46619 ([1R-[1 α β (Z) α (1E, 3S*)][1 α ,4 α ,5 β (Z), 6 α (1E, 3S*)][1 α ,5 β (Z), 6 α (1E, 3S*)][1 α ,4 α ,5 β (Z), 6 α (1E, 3S*)][1 α β (Z), 6 α (1E, 3S*)][1 α ,4 α ,5 β (Z), 6 α (1E, 3S*)][1 α ,5 β (Z), 6 α (1E, 3S*)][1 α ,4 α ,5 β (Z), 6 α (1E, 3S*)]-7-[6-(3-hydroxy-1-octenyl)-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid), SQ29,548 ([1S-[1 α (Z) α ,4 α][1 α ,2 α (Z), 3 α ,4 α][1 α α (Z), 3 α ,4 α][1 α ,2 α (Z), 3 α ,4 α]-7-[3-[[2-[(phenylamino) carbonyl]-hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid), deuterated standards ([d₄]PGE₂ and [d₄]TXB₂) and PGE₂ enzyme immunoassay- (EIA) kit were from Cayman Chemical (Ann Arbor, MI). Anion exchange resin AG 1X-8 (formate form, 200–400 mesh) and Lowry dye-binding protein reagents were from Bio-Rad (Hercules, CA). All other reagents of the highest purity were available from Sigma–Aldrich (St. Louis, MO).

2.2. Isolation of human platelets and analysis of platelet aggregation

CPD-anticoagulated human blood (Citrate Phosphate Dextrose solution: sodium citrate, dihydrate, 26.3 g/L; dextrose, monohydrate, 25.5 g/L; citric acid, anhydrous 3.27 g/L; monobasic sodium phosphate, monohydrate, 2.22 g/L) was sampled following informed consent from healthy volunteers of both genders aged from 18 to 60 years that had no history of CV disease. Blood was treated with 100 μ M acetylsalicylic acid and 25 mL buffy coat was centrifuged at 280 $\times$ g for 15 min at room temperature to obtain platelet-rich plasma (PRP), which was further centrifuged at 650 $\times$ g for 10 min at room temperature. The pelleted platelets were suspended in 8 mL washing buffer (mM composition: citric acid monohydrate 39, glucose monohydrate 5, KCl 5, CaCl₂ 2, MgCl₂ $\times$ 6H₂O 1, NaCl 103, pH 6.5), recentrifuged at 650 $\times$ g for 15 min at room temperature, and finally resuspended in 15 mL of Hank's Balance Salt Solution (HBSS): CaCl₂ $\cdot$ 2H₂O 0.185 g/L; KCl 0.40 g/L; KH₂PO₄ 0.06 g/L; MgCl₂ $\cdot$ 6H₂O 0.10 g/L; MgSO₄ $\cdot$ 7H₂O 0.10 g/L; NaCl 8.00 g/L; NaHCO₃ 0.35 g/L; Na₂HPO₄ 0.048 g/L; D-glucose 1.00 g/L). The concentration was adjusted at approximately 2 $\times$ 10⁸ cell/mL and platelet aggregation was assessed with a Chrono-Log aggregometer (Mascia Brunelli, Milano, Italy), using the Born turbidimetric assay at 37 °C in a 0.5 mL sample. After incubation with drug or vehicle dimethyl sulfoxide (DMSO, maximum 0.2%, v/v) for 5 min at 37 °C, platelet aggregation was induced by U46619 (0.1 μ M) under continuous stirring and monitored for 6 min. Experiments were repeated at least in triplicate using platelets from different subjects. The anti-aggregating activity of each compound was compared with its corresponding control aggregation, recorded immediately before and after drug testing due to the inter-subject variability of the platelet response to the agonist challenge.

2.3. COX-2 inhibitory activity (lympho-monocytes)

The study of COX-2 activity was carried out in a lymphomonocytes suspension, in order to avoid eventual compound binding to plasma-protein. Lymphomonocytes were isolated from buffy coat (diluted in NaCl 0.9% 1:1) Ficoll–Paque gradient density centrifugation (400 $\times$ g for 30 min at 10 °C); enriched cell ring was collected and twice saline washing (280 $\times$ g for 15 min at 10 °C) performed to remove the remaining suspended platelets. Soon after, a lysis buffer (NaCl 0.2% weight/volume, w/v) was added to remove the remaining erythrocytes, immediately balanced with an equal volume of equilibrating solution (NaCl 1.6% + saccharose 0.2%, w/v). Lymphomonocytes were then resuspended in HBSS and

COX-2 inhibition was evaluated quantifying PGE₂ production in 24 h lipopolysaccharide (LPS) challenged preparations pretreated (30 min, 37 °C) with increasing concentration of the tested compound. PGE₂ determination was carried out by EIA, according to the manufacturer's instructions, or mass spectrometry as described below.

2.4. COX-1 inhibitory activity (human platelets)

Human platelets were recovered from PRP after centrifugation at 650 $\times$ g for 15 min at room temperature. Their concentration was adjusted at 2 $\times$ 10⁸ cells/mL. Platelets were treated with increasing concentration of the tested compounds, and incubated at 37 °C in a Dubnoff bath for 30 min. In order to stimulate the TXB₂ production by platelet degranulation, 2 μ M calcium ionophore A23187 was added to each test tube sample for 10 min at 37 °C. Following centrifugation at 1500 $\times$ g for 5 min, TXB₂ production was evaluated in the supernatant by mass spectrometry as described below.

2.5. Mass spectrometry determination of eicosanoids

PGE₂ and TXB₂ concentrations were evaluated by liquid chromatography–tandem mass spectrometry using the isotopic dilution of the deuterated internal standards [d₄]PGE₂ and [d₄]TXB₂. Briefly, samples were spiked with internal standards and an aliquot injected into a liquid chromatograph Agilent 1100 (Agilent Technologies, Santa Clara, CA). Chromatography was carried out using a reverse phase column (Synergi 4 μ m Hydro-RP, 150 $\times$ 2 mm; Phenomenex, Torrance, CA). The column was eluted with a linear gradient from 25 to 100% solvent B (Methanol/Acetonitrile, 65/35) over 10 min (Solvent A: 0.05% acetic acid pH 6 with ammonia). The effluent from the high-performance liquid chromatography (HPLC) column was directly infused into an API4000 triple quadrupole operated in negative ion mode, monitoring the following specific transitions: *m/z* 351 > 271 for PGE₂, *m/z* 355 > 275 for [d₄]PGE₂, *m/z* 369 > 169 TXB₂ and *m/z* 373 > 173 for [d₄]TXB₂. Quantitation was carried out using standard curves obtained with synthetic standards (Cayman Chemical, Ann Arbor, MI).

2.6. Culture and transfection of HEK293 cells

HEK293 cells (ATCC, Manassas, VA) were transiently transfected as previously reported [17,26–30]. Briefly, cells were cultured in DMEM and supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/mL penicillin, 100 μ g/mL streptomycin, and 20 mM HEPES buffer, pH 7.4, at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells were plated onto 12-well dishes previously coated with poly-D-lysine, in order to obtain a 50–60% confluence at the time of transfection. DNA constructs of TP α wild type receptor were previously obtained in our laboratory [26]. Ultrapure plasmids for cell transfection were obtained using the QIAfilter Plasmid Kits by Qiagen (Hilden, Germany). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as a transfectant agent and the transfection mix Lipofectamine 2000/DNA was prepared in Opti-MEM I Medium, which was optimized at 2:1 ratio and later added to the cells.

2.7. Total inositol phosphate determination in HEK293 cells

The functional activity of the TP α receptor was assessed 48 h after transfection by measuring the accumulation of total labelled IPs through a column ion exchange chromatography as previously described [26]. Briefly, the day before the assay, transfected HEK293 cells were labelled with 0.5–1 μ Ci/mL of myo-[2-³H]inositol for 24 h in DMEM, free of inositol and serum, containing 20 mM HEPES

Table 1

Topological polar surface area (tPSA), solubility in simulated gastric fluid (SGF) and phosphate buffered solution (PBS), dissociation constants and lipophilicity descriptors of compounds under study.

Compound	tPSA ^a (Å ²)	Solubility (μM) ± SD		pK _a ± S.D. ^b	clog P ^c	log D 7.4 ± S.D. ^d
		SGF	PBS			
Lumiracoxib	49.3	23.4 ± 0.7	442.1 ± 21.4	4.15 ± 0.03 ^e	4.66	1.19 ± 0.05
7	49.3	8.7 ± 0.1	440.2 ± 3.9	4.31 ± 0.05	5.84	1.86 ± 0.03
18	61.1	54.1 ± 5.4	533.9 ± 16.2	4.85 ± 0.01 ^e	4.85	1.60 ± 0.03
20	58.2	13.9 ± 1.6	60.7 ± 2.3	6.70 ± 0.01 ^e	6.16	>3.5
32	83.5	19.3 ± 4.9	553.1 ± 15.0	4.33 ± 0.01	4.54	0.87 ± 0.04

^a Topological polar surface area values calculated using ChemBioDraw v.12 CambridgeSoft.

^b Data obtained by potentiometric titration (SIRIUS GlpK_a).

^c Values calculated by using Bio-Loom for Windows v.1.5 BioByte Corp.

^d Data obtained by shake-flask method.

^e According to [24].

buffer pH 7.4, 0.5% (w/v) Albumax, 2 mM glutamine, and 100 U/mL penicillin streptomycin. On the day of the assay, cells were incubated with 25 mM LiCl for 10 min. Soon after they were pretreated with antagonists (diclofenac, lumiracoxib, and tested compounds) for 30 min at 37 °C, and stimulated with either vehicle or 0.1 μM U46619. The reaction was stopped by lysing the cells with 10 mM formic acid for 30 min at 4 °C, and acidic phase transferred in 5 mM NH₄OH pH 8–9. Finally, total IPs were extracted by chromatography AG 1X-8 columns formate form, 200–400 mesh size.

2.8. Determination of dissociation constants

The ionization constants of compounds were determined by potentiometric titration with the GLpK_a apparatus (Sirius Analytical Instruments Ltd., Forest Row, East Sussex, UK) as previously described [24]. Briefly, because of the low aqueous solubility, compounds required titrations in the presence of MeOH as co-solvent: at least five different hydro-organic solutions (ionic strength adjusted to 0.15 M with KCl) of the compounds (20 mL, ca. 0.5 mM in 20–60 w% MeOH) were initially acidified to pH 1.8 with 0.5N HCl; the solutions were then titrated with standardized 0.5 N KOH to pH 12.2 at 25 °C under N₂.

The apparent ionization constants in the H₂O–MeOH mixtures (pK_a) were obtained and aqueous pK_a values were calculated by extrapolation to zero content of the co-solvent, following the Yasuda–Shedlovsky procedure [31].

2.9. Determination of lipophilicity descriptors

Calculated partition coefficients of compounds in neutral form (clog P) were obtained by using Bio-Loom for Windows v.1.5 (BioByte Corp. Claremont, CA, USA).

The distribution coefficient at pH 7.4 (log D^{7.4}) of the compounds between *n*-octanol and water was experimentally obtained by shake-flask technique at room temperature. In the shake-flask experiments phosphate 50 mM buffer with ionic strength adjusted to 0.15 M with KCl, was used as aqueous phase; the organic (*n*-octanol) and aqueous phase were mutually saturated by shaking for 4 h. The compounds were solubilised in the buffered aqueous phase at a concentration of about 0.1 mM and an appropriate amount of *n*-octanol was added. The two phases were shaken for about 20 min, by which time the partitioning equilibrium of solutes is reached, and then centrifuged (1100 × g, 10 min). The concentration of the solutes was measured in the aqueous phase by HPLC. Each log D value is an average of at least six measurements. All the experiments were performed avoiding exposure to light.

2.10. Solubility assessment in phosphate buffered saline (PBS) and simulated gastric fluid (SGF)

The solubility of compounds **7**, **18**, **20**, **32** and lumiracoxib was studied in simulated gastric fluid (SGF-without pepsin) and phosphate buffered saline (PBS) 0.05 M to evaluate the solubility of compounds in acid (pH 1.5 for SGF) and neutral conditions (pH 7.4 for PBS) to simulate the gastric and the body fluid environment respectively (Table 1) [32].

Stock solutions of compounds lumiracoxib, **7**, **18**, **20** and **32** (10 mM) were prepared in DMSO. Eight point calibration standards (1, 5, 10, 20, 50, 100, 200 and 500 μM) were prepared from each 10 mM solution stock by dilution in HPLC mobile phase and analyzed by HPLC. 100 μL of each test compound (stock solution 10 mM in DMSO) were added to 1900 μL of PBS and SGF in glass tubes in triplicate and shaken for 90 min at 100 rpm at room temperature. The samples were filtered using 0.45 μL PTFE filters and analysed by HPLC.

HPLC analysis were performed with a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1379A), a diode-array detector (DAD) (model G1315B) integrated in the HP1100 system. Data analysis were processed using a HP ChemStation system (Agilent Technologies). The analytical column was a Tracer Excel C18 (250 × 4.6 mm, 5 μm; Teknokroma). The mobile phase consisting of acetonitrile/HCOOH 0.1% 70/30 (v/v) at flow-rate = 1.0 mL/min. The injection volume was 20 mL (Rheodyne, Cotati, CA). The column effluent was monitored at 254 nm and 280 nm referenced against a 800 nm wavelength. Quantitation of compounds was done using calibration curves of compounds; the linearity of the calibration curves was determined in a concentration range of 1–500 μM (*r*² > 0.99).

2.11. Stability in human serum

A solution of each compound (10 mM) in DMSO was added to human serum (from human male AB plasma, USA origin, sterile-filtered, Sigma–Aldrich) preheated at 37 °C; the final concentration of the compound was 100 μM. Resulting solution were incubated at 37 ± 0.5 °C and at appropriate time intervals (2, 6, 24 h) 300 μL of the reaction mixture was withdrawn and added to 300 μL of acetonitrile containing 0.1% trifluoroacetic acid in order to deproteinize the proteins. Sample was sonicated, vortexed and then centrifuged for 10 min at 2150 × g. The clear supernatant was filtered by 0.45 μm PTFE filters (Alltech) and analyzed by RP-HPLC with method previously described for solubility assessment.

Table 2
TP receptor antagonism at human washed platelet aggregation and total IP production in transfected HEK 293 cells. pA_2 values were determined by measuring inhibition of aggregation response to the stable agonist U46619.

Compound	$pA_2 \pm \%CV$	
	Human washed platelet aggregation	Total IP production in HEK293 cells
Lumiracoxib	5.0 ± 2.5	4.6 ± 5.2
Diclofenac	5.4 ± 4.9	5.3 ± 4.7
Naproxen	4.1 ± 2.5	3.9 ± 16.3
Terutroban	9.4 ± 4.1	9.3 ± 2.8
18	5.6 ± 3.5	$5.5^a \pm 2.2$
20	$5.9^a \pm 4.1$	$5.7^a \pm 2.3$
7	5.0 ± 1.8	4.9 ± 5.1
32	4.8 ± 2.4	4.8 ± 2.5

^a95% CI vs lumiracoxib, see results.

2.12. Statistical analysis

pA_2 were calculated accordingly to the following set of equations as described in Prism 5 (GraphPad Software Inc., San Diego, CA):

(1) Agonist response = Bottom + (Top–Bottom)/(1 + 10^[(Log EC₅₀–X)*HillSlope])

(2) Antagonist response = Bottom + (Top–Bottom)/(1 + (Antag/FixedAg)^{HillSlope})

(3) Antag = (10^{Log EC₅₀})*(1 + ((10^X)/(10^{–1}* pA_2)))^{HillSlope}

where X is the Log concentration of the agonist, Bottom is the response when X = 0, Top is the response for an infinite concentration of X, EC₅₀ is the concentration of the agonist that produce half of the response, Hill Slope is the slopes of the curves, Fixed Ag is the initial fixed concentration of the agonist used in the determination of the antagonist inhibition curve. The concentration–response curves of platelet aggregation were analysed by Prism-5 software utilizing the four-parameter logistic model as described in the ALLFIT program [33]. Parameter errors are all expressed in percentage coefficient of variation (% CV) and calculated by simultaneous analysing at least three different independent experiments performed in duplicate or triplicate. $P < 0.05$ was set as the statistical level of significance. All curves shown are computer generated.

3. Results

3.1. Chemistry

For the synthesis of compounds **7** (2-[(2-chloro-6-fluorophenyl) amino]-5-methyl-benzoic acid) and **32** (2-[(4-chlorophenyl) sulfonyl] amino)-5-methyl-benzoic acid) the procedure reported in Scheme 1 (see Supplementary materials) was used. The synthesis of lumiracoxib analogue **7** was accomplished as previously described [24]. Briefly, the Chan–Lam coupling was used by reacting 2-amino-5-methylbenzoic acid with 2-chloro-6-fluorophenylboronic acid in the presence of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) and a stoichiometric amount of copper acetate in dioxane solution. Compound **32** was synthesized by reacting 2-amino-5-methylbenzoic acid with 4-chlorobenzensulfonyl chloride in the presence of excess Na₂CO₃ in water at 60–80 °C. The product was isolated and recrystallized from ethanol. Compounds **18** (N-(2-chloro-6-fluorophenyl)-4-methyl-2-(1H-tetrazol-5-ylmethyl)-benzenamine) and **20** (N-[[2-[(2-chloro-6-fluorophenyl) amino]-5-methylphenyl]methyl]-1,1,1-trifluoromethanesulfonamide) were obtained as previously reported [24] (Fig. 1).

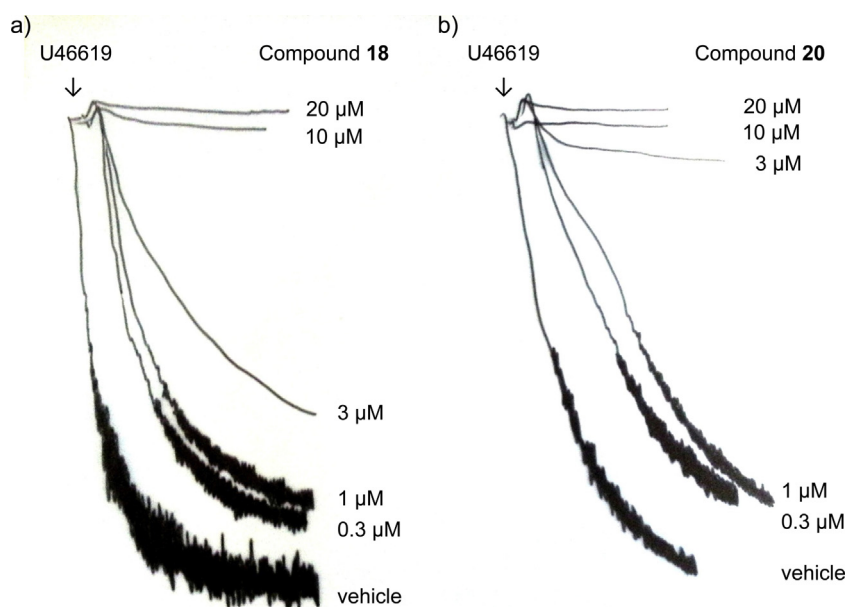


Fig. 2. Representative original traces of acetylsalicylic acid-treated human platelets demonstrating the effect of compounds **18** and **20** on the TP-dependent aggregation. Washed platelets were challenged with 0.1 μM U46619 (arrow) in the presence of vehicle (0.2% DMSO) or the indicated concentrations of compounds **18** (a) and **20** (b).

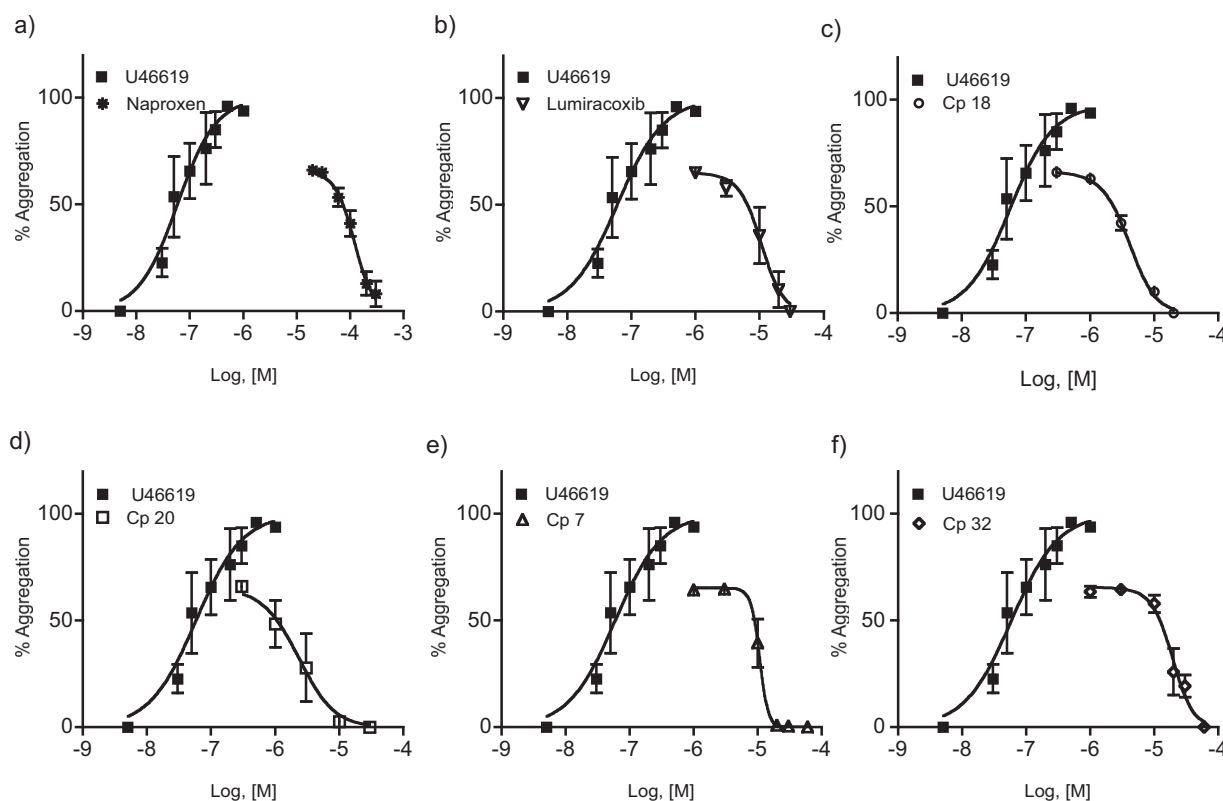


Fig. 3. Antagonism of human platelet aggregation induced by U46619 by the indicated compounds. Concentration–response curves of U46619-induced washed platelet aggregation from human blood and inhibition curves of: naproxen (a), lumiracoxib (b), compound **18** (c), compound **20** (d), compound **7** (e) and compound **32** (f). EC_{50} 's were calculated using a four parameters logistic model, while pA_2 's were calculated accordingly to the set of Eqs. (1)–(3) as described in materials and methods. Values shown represent platelet aggregation (mean $\pm$ SE) expressed as % maximal aggregation induced by $0.1 \mu M$ U46619. Blood was treated with $100 \mu M$ acetylsalicylic acid. Experiments have been performed at least three times in duplicates. All curves shown were computer generated.

3.2. Physico-chemical characterization of compounds

The compounds selected for this study present most of the structural characteristics predicted for lumiracoxib analogs suitable as COX-2 inhibitors from a recent *in silico* study. [34]. Their structures contain: (1) two aromatic rings; (2) at least two oxygen atoms (with the exception of compound **18**); (3) at least one carboxyl group or a carboxyl isosteric group; (4) at least one $-OH$ or $-NHn$ group. Inspection of data reported in Table 1 evidences that the topological polar surface area (tPSA) of the studied compounds is similar or higher than that of lumiracoxib; in particular, the value of tPSA for the tetrazole analog **18** (61.1) is very close to the mean value found for a set of 36 NSAIDs (63.2) and of lumiracoxib analogs (70.5) [34,35]. The measured solubility indicates that **18** is more soluble than lumiracoxib both in simulated gastric fluid and in phosphate buffered saline. Its solubility double that of the reference drug when the simulated gastric fluid is considered as the medium. This may be an advantage following oral administration. Derivatives **7** and

32 also show a similar solubility with respect to lumiracoxib at physiological pH, their solubility is slightly lowered when the pH is brought down to 1.5. Compound **20** is the least soluble derivative of the series at pH 7.4, nevertheless it retains a fair solubility in SGF medium (0.59 fold that of lumiracoxib).

The pK_a values of **7**, **18** and **32** are slightly higher than that of lumiracoxib. Derivative **20** presents a significantly lower acidity with respect to that of the reference drug, this property reflects in a distribution coefficient measured at pH 7.4 greater than 3.5, consequently **20** is more lipophilic than lumiracoxib in physiological condition. Compounds **7** and **18** are 1.56 and 1.34 fold more lipophilic than lumiracoxib and this may favor cellular permeability. All the compounds were also shown to be stable in human serum with >98% unchanged form detected after 24 h incubation (data not shown). From this preliminary characterization we could assume that the tetrazole derivative **18** shows the best drug-like properties among the compounds of this series.

Table 3

COX-2 and COX-1 inhibitory activities determined by *in vitro* assay in lympho-monocytes and washed human platelets.

Compound	COX-2 inhibition IC_{50} (μM) $\pm$ % CV	COX-1 inhibition IC_{50} (μM) $\pm$ % CV	COX-2/COX-1 selectivity
Lumiracoxib	0.0035 ± 26	3.22 ± 22	910
Diclofenac	0.0011 ± 30	0.0083 ± 6.2	7.6
Naproxen	0.19 ± 66	0.11 ± 10	0.58
Terutroban	inactive at $10 \mu M$	N.D.	N.D.
18	0.014 ± 23	13.2 ± 22	942
20	0.42 ± 32	16.1 ± 6	38
7	0.025 ± 46	25.5 ± 10	1020
32	1.20 ± 45	inactive at $60 \mu M$	N.D.

N.D.: not determined.

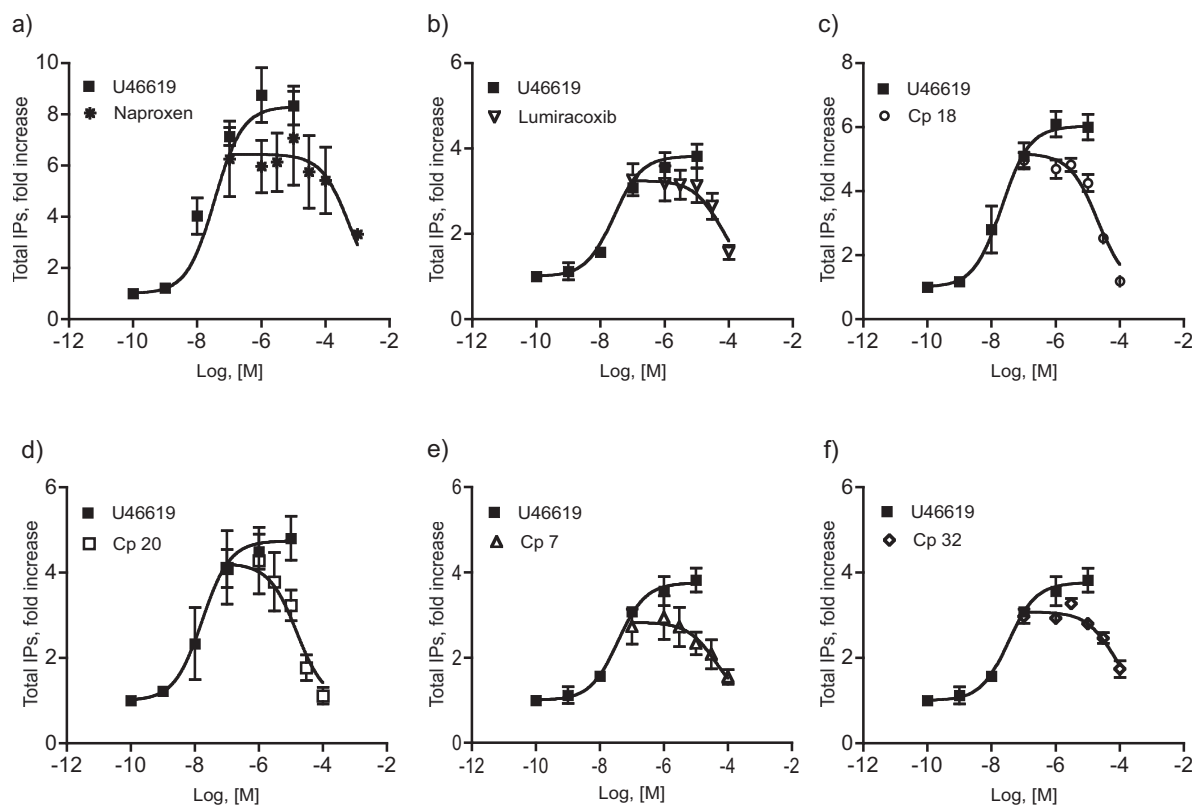


Fig. 4. Antagonism by the indicated compounds of total IP production induced by the TXA₂ analog U46619 in HEK cells transiently transfected with the alpha isoform of the human TP receptor. Concentration–response curves of U46619-induced total IP production and inhibition curves of naproxen (a), lumiracoxib (b), compound **18** (c), compound **20** (d), compound **7** (e), and compound **32** (f), obtained in the presence of 0.1 μ M U46619. EC₅₀'s were calculated using a four parameters logistic model, while pA₂'s were calculated accordingly to the set of Eqs. (1)–(3) as described in materials and methods. Values shown represent the mean percentage of fold increase over basal $\pm$ SE. Experiments were performed at least three times in duplicates. All curves shown were computer generated.

3.3. Inhibition of TP receptor functional activity in human platelets

A series of newly synthesized compounds, *i.e.*, compound **18**, **20**, **7**, and **32**, as well as a non-selective COX inhibitor (naproxen) and a potent and selective COX-2 inhibitor (lumiracoxib) (Fig. 1) were studied for TP receptor antagonism in platelets from healthy human volunteers. The extent of aggregation was detected by Born-turbidimetric assay. Blood was collected in the presence of 100 μ M acetylsalicylic acid to render the platelets unresponsive to the challenge with arachidonic acid (1–3 μ M), but fully responsive to the calcium ionophore A-23187 (3 μ M; data not shown). Representative traces of washed platelet aggregation obtained with 0.1 μ M of the stable TXA₂ analogue U46619 in the presence of increasing concentrations (0.3–20 μ M) of compounds **18** and **20** are portrayed in Fig. 2. When platelets were challenged with increasing concentrations of U46619, a concentration-dependent platelet aggregation occurred, which revealed a potency value of 59 nM $\pm$ 19% CV (Fig. 3), in perfect agreement with previous results [17]. This response was thus truly independent of endogenous TXA₂ formation. The sensitivity of platelets to U46619 did not change during the time required for the experiment and none of the tested compounds caused any aggregator response by itself. Fig. 3 also shows the inhibition curves of U46619-induced (0.1 μ M) platelet aggregation due to increasing concentrations of the newly synthesized compounds as well as the reference compounds. Table 2 reports their respective pA₂ values calculated accordingly to Eqs. (1)–(3) (see methods section). Among the different molecules tested, compounds **18** and **20** were found to be the most potent in terms of TXA₂ antagonism with pA₂ values comparable to that of diclofenac, but, at least for compound **20**, statistically different from that of lumiracoxib (pA₂ = 5.9,

95% CI-confidence interval 5.4–6.4 for compound **20** –pA₂ = 5.0, 95% CI-confidence interval 4.7–5.2 for lumiracoxib).

3.4. Inhibition of TP α functional activity in HEK293

All compounds were also tested for their ability to inhibit the total inositol phosphate (IP) production following classic TP receptor coupling with Gq. The human TP α receptor transiently expressed in HEK293 cells was activated by the stable TXA₂ analogue U46619 (0.1 μ M, 30 min) in the absence and presence of 30 min pretreatment with increasing concentrations of the reported antagonists (Fig. 4). TP receptor activation by U46619 resulted in a robust increase in total IP production with a calculated EC₅₀ of 29.3 nM $\pm$ 10% CV, as previously reported [28–30]. No response has been obtained from mock-transfected cells (Fig. 1S). The pA₂ values calculated for each compound are presented in Table 2. It is worth notice here that the results obtained in total IP production inhibition are in full agreement with those obtained in aggregation studies. Compounds **18** and **20** were again the most potent molecules, with pA₂ values similar to that of diclofenac and both statistically different from that of lumiracoxib (pA₂ = 5.5, 95% CI, 5.2–5.8 for compound **18** –pA₂ = 5.7, 95% CI, 5.4–6.0 for compound **20** –pA₂ = 4.6 95% CI, 4.1–5.1 for lumiracoxib), in good agreement with affinity binding data previously obtained in HEK293 cells [24].

3.5. COX-2/COX-1 selectivity

Newly synthesized compounds should maintain the COX-2 selectivity of the parent lumiracoxib and therefore their capacity to act as COX-2 inhibitors was determined on isolated human

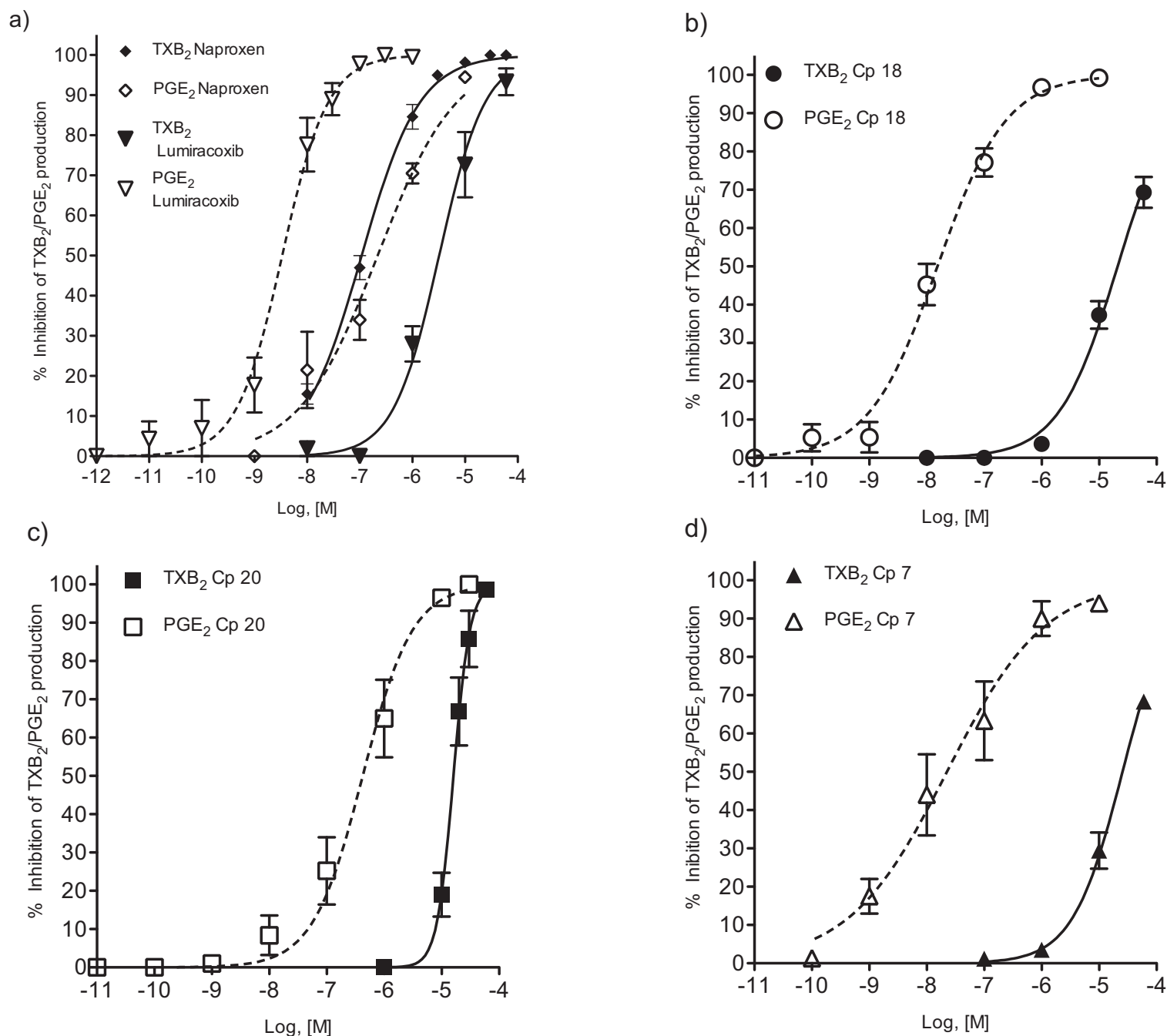


Fig. 5. Inhibition of COX-1 and COX-2 activity by the indicated compounds in comparison to the reference compounds naproxen and lumiracoxib (a). COX-1 activity was assessed in terms of inhibition of TXB₂ production induced by calcium ionophore in human washed platelets; COX-2 activity was assessed in terms of inhibition of PGE₂ production induced by LPS in isolated human monocytes. Data are expressed as percent inhibition of TXB₂ or PGE₂ release versus untreated controls. Error bars represent mean $\pm$ SE of at least three independent experiments, each performed in duplicate.

lympho-monocytes following treatment with acetylsalicylic acid. COX-2 expression was stimulated overnight with 10 μ g/mL of LPS, and the PGE₂ produced was determined by enzyme immunoassay and mass spectrometry, whereas COX-1 inhibitory activity was determined in washed human platelets as TXB₂ production by mass spectrometry.

All the compounds tested inhibit the COX-2 enzyme in a concentration-dependent manner with lumiracoxib and diclofenac displaying the highest absolute potency (Table 3), while compound 32, containing the 4-chlorobenzenesulfonamide moiety present in

the very potent TP receptor antagonist terutroban [25], being the less potent molecule of the series (Table 3). Among the other molecules, the tetrazole derivative 18 and compound 7 inhibited COX-2 in a very similar way with potencies of 0.014 and 0.025 μ M, respectively, while compound 20 shows a potency similar to that of naproxen (Fig. 5 and Table 3).

Diclofenac displayed by far the highest potency as COX-1 inhibitor in washed platelets, with values in the nM range, followed by naproxen (Table 3). Once again the tetrazole derivative 18 and compound 7 behaved very similarly with IC₅₀ values of 13.2 μ M

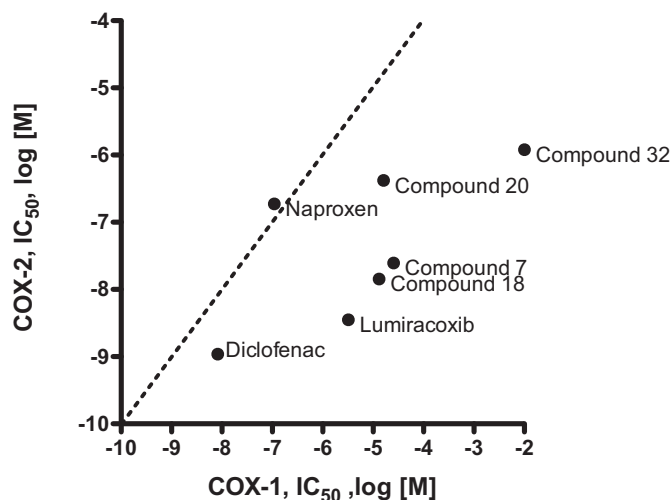


Fig. 6. COX-2/COX-1 selectivity. IC_{50} values obtained for the various compounds are plotted to appreciate their selectivity: the diagonal line indicates equivalence, therefore compounds with high selectivity for COX-2 over COX-1 are plotted below the line.

and 25.5 μ M, respectively, while this time compound **20** showed an IC_{50} of 16.1 μ M, close to that of **18** and **7**; compound **32**, resulted inactive in terms of COX-1 inhibition (Fig. 5 and Table 3).

Naproxen and lumiracoxib were expected to be, respectively, the less and the most selective COX-2 inhibitors (Fig. 5 and Table 3) in perfect agreement with previously published data summarizing the selectivity profiles of various NSAIDs, including coxibs (Fig. 6) [4]. Furthermore, Fig. 5 and Table 3 clearly indicate that compounds **18** and **7** are the most selective coxibs among the multitarget molecules synthesized of notice; the sulfonamide derivative **20**, despite being only about 40 times more selective for COX-2 with respect to COX-1 inhibition at calculated IC_{50} , presented a very steep slope of the concentration-response curve (Hill coefficient »1). This characteristic has no direct impact on the Therapeutic Index of the molecule, and, thus, on its safety, but rather suggest that it is possible to find a dose of compound **20** that is selective for COX-2. Thus, at concentrations ten times its IC_{50} for COX-2 inhibition, about 95% of COX-2 is inhibited, while no inhibition of COX-1 are observed (Fig. 5c).

4. Discussion

In this contribution we report a full *in vitro* pharmacological characterization of selected molecules from a series of compounds recently synthesized [24] and two additional molecules with dual COX-2 inhibitor activity and TP receptor antagonism. As reference compounds we have considered the non selective first generation NSAID naproxen, the most selective COX-2 inhibitor lumiracoxib and the TP antagonist terutroban. This work directs to a new generation of multitarget compounds among which two of the lumiracoxib derivatives, the tetrazole compound **18** and the trifluoromethansulfonamido-isoster compound **20**, are the most active and with a fairly balanced COX-2 inhibitor activity and TP receptor antagonism. However, TP receptor inhibitory potency remain in the micromolar range, still far from optimal values to be considered around the nanomolar range, preventing, at this stage, a real *in vivo* relevance for these molecules. However, these data will be used as the base to adopt a new and different strategy to design more potent molecules that might change the way we treat chronic inflammatory diseases.

Today, there is no safe NSAIDs, their main side effects including GI and renal toxicity [36]. Thus, coxib therapy is currently used in chronic pain control in selected patients with higher risks of

GI complications but lower risk of CV events as an alternative to conventional NSAIDs associated to gastroprotective therapy. However, just because taking a coxib, these patients have an increase in CV risk, and therefore must undergo wash-out periods from the drug, and even if treated with low-dose aspirin they are not protected from non-enzymatic AA products such as isoprostanes. On the other hand, there is still an unmet need for an adequate pain therapy combined with minimal GI damage and CV toxicity. Anyone who is at risk for or who is experiencing a vascular thrombotic diseases (diabetes, venous thromboembolism, etc.), may still suffer from a further increase in vascular risk even treated with a traditional NSAIDs [9], with the possible exception of naproxen [8]. In addition, traditional NSAIDs have also been observed to interfere with the cardioprotective effect of low-dose aspirin, somehow endangering the benefit of their association [37].

It is worth mentioning here that a CV safer coxib may also be a valuable drug in chemoprevention, considering that all the colon adenoma prevention trials yielded encouraging results on cancer prevention, in spite of the divergent incidence of CV events in their long term use [5,6,38]. Indeed, cardiac toxicity during colon chemoprevention trials was the main reason for withdrawing rofecoxib from the market, despite the threshold of the risk/benefit ratio should be particularly low in this particular case, considering the risk of developing a particularly aggressive form of cancer. More to this, recent finding have highlighted a role for TXA_2 synthesis in colon tumorigenesis, corroborating the use of a molecule endowed with TP antagonist properties in selected form of cancer [39]. Thus, a dual COX-2 inhibitor and a TP receptor antagonist might be a valuable solution to both the problem of long-term pain control and cancer colon prevention.

Indeed, a number of TXA_2 biosynthesis inhibitors and/or receptor antagonists have been developed and studied providing evidence of their efficacy on top of aspirin [40,41], even though they have always suffered from being perceived as too similar to the economical and efficacious aspirin. In particular, the potent and selective TP antagonist terutroban [42] has been demonstrated to be an effective antithrombotic agent in peripheral arterial disease [43] and to improve endothelial function in atherosclerotic patients [44]. Somehow unexpectedly, the PERFORM Phase III clinical trial with terutroban in patients with cerebral ischemic events did not meet the predefined criteria for non-inferiority to aspirin, while showing similar rates of primary endpoint between the two drugs [45]. Of notice, however, patients with a history of ischemic stroke before the qualifying event had a statistically significant lower recurrence rate with terutroban than with aspirin. Considering that some of these patients might have been receiving aspirin before their qualifying event, these data seems to suggest an aspirin treatment failure and support the use of a TP receptor antagonist as an alternative strategy to pursue in selected subgroups of patients, particularly those unresponsive to aspirin, such as diabetics [46].

Furthermore, it has been demonstrated that dimerization of the prostacyclin receptor (IP) with $TP\alpha$ promotes a strong TP-induced cAMP generation in a mechanism of control over TP pro-aggregating activity [47]. However, when the naturally occurring IP receptor mutant R212C is co-expressed with $TP\alpha$, TP response is normalized [48] and patients carrying this polymorphism seem to develop more intimal hyperplasia than control individuals [49]. Therefore, we are confident that our approach to solve the CV issue of coxibs by designing a multitarget drug [23] is an effective way to retain inhibition of the COX-2 dependent synthesis of the inflammatory PG and extraplatelets TXA_2 combining the block of all TP agonists, both aspirin-sensitive and aspirin-insensitive (isoprostanes), thus minimizing risks to the individuals.

The data presented here have shown that compounds **7** and **32** did not possess better TP antagonist properties than the reference compound lumiracoxib, while losing most of their potency as COX

inhibitors albeit preserving COX-2 selectivity. The replacement of 2-fluoro-6-chloro-phenyl substituent present on the amino nitrogen in compound **7** with the *p*-chlorophenylsulfonyl substructure, typical of terutroban, generated compound **32** where two phenyl rings are linked by a sulfonamide group instead of the classic amino group. However, this modulation did not accomplish our expectations, as compound **32** did not show better properties with respect to compounds **18**, **20** and **7**. These data suggest that COX-2 selectivity might be determined by the presence of the 2-amino-5-methyl benzen carboxylic acid moiety, while potency could be regulated by the nature of the substituent present on the amino nitrogen. The first observation is in agreement with literature data highlighting the importance of methyl substitution for COX-2 selectivity [50].

In turn, compounds **18** and **20** have gained in TP receptor antagonist activity, with compound **20** statistically different from lumiracoxib in both inhibition of human platelets aggregation and IP generation in a transfected system, despite, as mentioned before, still far from required values for an in vivo application. These functional results are in good agreement with affinity binding data previously reported for these compounds [24], and support the strategy of isosteric replacement of the carboxylic function of lumiracoxib, either with linear or cyclic substructures for the efficacious generation of molecules endowed with improved TP antagonism. Compound **18** and particularly compound **20** displayed better balanced activities diverging less from the ideal value of 1 when considering the ratio between the calculated potencies at both pharmacological targets (pA_2 TP/ IC_{50} COX-2 = 179 and 3, respectively) with respect to lumiracoxib or diclofenac (pA_2 TP/ IC_{50} COX-2 = 2269 and 3619, respectively). However, compound **20** has lost most of its COX-2 inhibitory activity and COX selectivity comparing to compound **18** suggesting that a higher acidity of carboxylic acid isoster is needed to maintain high level of COX-2 inhibition. Concerning COX-2 activity, it is interesting to note that shortening of the alkyl chain connecting the acidic moiety to the benzene ring reflects in a loss of activity (compare derivative **7** to lumiracoxib). This observation clearly indicates that the acidic moiety should be separated from the benzene ring by one carbon atom to reach optimal binding to COX-2 pocket, in agreement with the postulated binding pose of diclofenac and lumiracoxib at the COX-2 binding site [51]. Collectively our data indicate that both pK_a values and correct positioning of the acidic group are important to maintain a high degree of COX-2 inhibition in this class of compounds. Interestingly the distance of the acidic unit from the benzene ring might also influence the antagonism at the TP receptor (compare derivatives **18** and **20** vs **7** and **32** in Table 2). The stringent structural requirements for COX-2 inhibition are apparently in contrast with the need of compounds endowed with more flexible and extended structures, which proved more efficient as TP receptor antagonists. In this context, the use of different carboxylic acid bioisosters, together with docking studies on both COX-2 and TP receptor models could be envisaged for the development of new molecules [52–55].

Naproxen is a non-selective COX inhibitor (Fig. 6) and it appears to have a better CV risk profile than other NSAIDs [8,19,20] despite it is a very weak TP receptor antagonist. Interestingly, naproxen has been demonstrated to inhibit not only platelet-derived TXA_2 but also systemic PGI_2 biosynthesis [56], thus, not disrupting the balance between the opposing actions of TXA_2 and PGI_2 . This balance, part of the intricate cross-talk of lipid mediators generated from AA seems to be a fundamental feature when designing an anti-inflammatory drug with a safe CV profile, and should be the target of any therapeutical intervention that might affect eicosanoid metabolism. Our strategy is precisely intended to correct the unbalance between PGI_2 and TXA_2 synthesis that has been observed in patients exposed to a coxib [12].

5. Conclusions

We show here that structure-activity analysis allowed us to obtain prototypes of new chemical entities endowed with higher TP antagonist potencies and a more balanced COX-2 selectivity. The modification of existing drugs targeted against COX-2 (lumiracoxib) can lead to new bivalent molecules with obvious advantage in the pharmacological properties. The main goal of a multitarget drug is to have both activities at the same concentration range with a consistent pharmacodynamic and single pharmacokinetic profile with respect to the co-administration of two different molecules. Further studies will be certainly necessary to improve the pharmacodynamic profile of these molecules before a careful evaluation of our hypothesis could take place in an in vivo animal model.

We believe that it is more and more evident that treatment of inflammation by a generalized inhibition of eicosanoid synthesis is a strategy far to be optimal. These new compounds will be critical for the innovation of the pharmacological treatment of patients that require long-term therapy with anti-inflammatory drugs and are at high CV risk for the primary importance of the TP receptor in the CV setting (platelets aggregation, endothelial dysfunction and plaque stabilization) [57]. This 'third generation' NSAID could be safely used also in other chronic diseases such as Alzheimer [58,59] or even in selected form of colon cancer where NSAIDs have already been proven to have a clinical efficacy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phrs.2015.11.012>.

References

- [1] W.L. Smith, D.L. DeWitt, R.M. Garavito, Cyclooxygenases: structural, cellular, and molecular biology, *Annu. Rev. Biochem.* 69 (2000) 145–182.
- [2] Morita I. Distinct functions of COX-1 and COX-2. *Prostaglandins Other Lipid Mediat.* 2002; 68–69, 165–175.
- [3] L. Laine, K. Takeuchi, A. Tarnawski, Gastric mucosal defense and cytoprotection: bench to bedside, *Gastroenterology* 135 (1) (2008) 41–60.
- [4] G.A. FitzGerald, C. Patrono, The coxibs, selective inhibitors of cyclooxygenase-2, *N. Engl. J. Med.* 345 (6) (2001) 433–442.
- [5] R.S. Bresalier, R.S. Sandler, H. Quan, J.A. Bolognese, B. Oxenius, K. Horgan, C. Lines, R. Riddell, D. Morton, A. Lanis, M.A. Konstam, J.A. Baron, Adenomatous polyp prevention on viox trial I. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial, *N. Engl. J. Med.* 352 (11) (2005) 1092–1102.
- [6] S.D. Solomon, J.J. McMurray, M.A. Pfeffer, J. Wittes, R. Fowler, P. Finn, W.F. Anderson, A. Zauber, E. Hawk, M. Bertagnolli, Adenoma prevention with celecoxib study I. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention, *N. Engl. J. Med.* 352 (11) (2005) 1071–1080.
- [7] J. Hippisley-Cox, C. Coupland, Risk of myocardial infarction in patients taking cyclo-oxygenase-2 inhibitors or conventional non-steroidal anti-inflammatory drugs: population based nested case-control analysis, *BMJ* 330 (7504) (2005) 1366.
- [8] P.M. Kearney, C. Baigent, J. Godwin, H. Halls, J.R. Emberson, C. Patrono, Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials, *BMJ* 332 (7553) (2006) 1302–1308.
- [9] T.D. Warner, J.A. Mitchell, COX-2 selectivity alone does not define the cardiovascular risks associated with non-steroidal anti-inflammatory drugs, *Lancet* 371 (9608) (2008) 270–273.
- [10] G.A. FitzGerald, Coxibs and cardiovascular disease, *N. Engl. J. Med.* 351 (17) (2004) 1709–1711.

- [11] F. Catella-Lawson, B. McAdam, B.W. Morrison, S. Kapoor, D. Kujubu, L. Antes, K.C. Lasseter, H. Quan, B.J. Gertz, G.A. FitzGerald, Effects of specific inhibition of cyclooxygenase-2 on sodium balance, hemodynamics, and vasoactive eicosanoids, *J. Pharmacol. Exp. Ther.* 289 (2) (1999) 735–741.
- [12] B.F. McAdam, F. Catella-Lawson, I.A. Mardini, S. Kapoor, J.A. Lawson, G.A. FitzGerald, Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2, *Proc. Natl. Acad. Sci. U. S. A.* 96 (1) (1999) 272–277.
- [13] E. Ahehart, J. Stitham, F.W. Asselbergs, K. Douville, T. MacKenzie, K.M. Fetalvero, S. Gleim, Z. Kasza, Y. Rao, L. Martel, S. Segel, J. Robb, A. Kaplan, M. Simons, R.J. Powell, J.H. Moore, E.B. Rimm, K.A. Martin, J. Hwa, Acceleration of cardiovascular disease by a dysfunctional prostacyclin receptor mutation: potential implications for cyclooxygenase-2 inhibition, *Circ. Res.* 102 (8) (2008) 986–993.
- [14] J.A. Mitchell, R. Lucas, I. Vojnovic, K. Hasan, J.R. Pepper, T.D. Warner, Stronger inhibition by nonsteroid anti-inflammatory drugs of cyclooxygenase-1 in endothelial cells than platelets offers an explanation for increased risk of thrombotic events, *FASEB J.* 20 (14) (2006) 2468–2475.
- [15] R.P. Mason, M.F. Walter, C.A. Day, R.F. Jacob, A biological rationale for the cardiotoxic effects of rofecoxib: comparative analysis with other COX-2 selective agents and NSAIDs, *Subcell. Biochem.* 42 (2007) 175–190.
- [16] L.P. Audoly, B. Rocca, J.E. Fabre, B.H. Koller, D. Thomas, A.L. Loeb, T.M. Coffman, G.A. FitzGerald, Cardiovascular responses to the isoprostanes iPF₂(α)-III and iPE₂(2)-III are mediated via the thromboxane A₂ receptor in vivo, *Circulation* 101 (24) (2000) 2833–2840.
- [17] E. Selg, C. Buccellati, M. Andersson, G.E. Rovati, M. Ezinga, A. Sala, A.K. Larsson, M. Ambrosio, L. Lastbom, V. Capra, B. Dahlen, A. Ryrfeldt, G.C. Folco, S.E. Dahlen, Antagonism of thromboxane receptors by diclofenac and lumiracoxib, *Br. J. Pharmacol.* 152 (8) (2007) 1185–1195.
- [18] G.H. Gislason, J.N. Rasmussen, S.Z. Abildstrom, T.K. Schramm, M.L. Hansen, E.L. Fosbol, R. Sorensen, F. Folke, P. Buch, N. Gadsboll, S. Rasmussen, H.E. Poulsen, L. Kober, M. Madsen, C. Torp-Pedersen, Increased mortality and cardiovascular morbidity associated with use of nonsteroidal anti-inflammatory drugs in chronic heart failure, *Arch. Intern. Med.* 169 (2) (2009) 141–149.
- [19] E.L. Fosbol, G.H. Gislason, S. Jacobsen, F. Folke, M.L. Hansen, T.K. Schramm, R. Sorensen, J.N. Rasmussen, S.S. Andersen, S.Z. Abildstrom, J. Traerup, H.E. Poulsen, S. Rasmussen, L. Kober, C. Torp-Pedersen, Risk of myocardial infarction and death associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) among healthy individuals: a nationwide cohort study, *Clin. Pharmacol. Ther.* 85 (2) (2009) 190–197.
- [20] W.A. Ray, C. Varas-Lorenzo, C.P. Chung, J. Castellsague, K.T. Murray, C.M. Stein, J.R. Daugherty, P.G. Arbogast, L.A. Garcia-Rodriguez, Cardiovascular risks of nonsteroidal antiinflammatory drugs in patients after hospitalization for serious coronary heart disease, *Circ. Cardiovasc. Qual. Outcomes* 2 (3) (2009) 155–163.
- [21] P. McGettigan, D. Henry, Use of non-steroidal anti-inflammatory drugs that elevate cardiovascular risk: an examination of sales and essential medicines lists in low-, middle-, and high-income countries, *PLoS Med.* 10 (2) (2013) e1001388.
- [22] I.S. Mackenzie, L. Wei, T.M. Macdonald, Cardiovascular safety of lumiracoxib: a meta-analysis of randomised controlled trials in patients with osteoarthritis, *Eur. J. Clin. Pharmacol.* 69 (2) (2013) 133–141.
- [23] G.E. Rovati, A. Sala, V. Capra, S.E. Dahlen, Folco G. Dual, COXIB/TP antagonists: a possible new twist in NSAID pharmacology and cardiovascular risk, *Trends Pharmacol. Sci.* 31 (3) (2010) 102–107.
- [24] M. Bertinaria, M.A. Shaikh, C. Buccellati, C. Cena, B. Rolando, L. Lazzarato, R. Fruttero, A. Gasco, M. Hoxha, V. Capra, A. Sala, G.E. Rovati, Designing multitarget anti-inflammatory agents: chemical modulation of the lumiracoxib structure toward dual thromboxane antagonists-COX-2 inhibitors, *ChemMedChem* 7 (9) (2012) 1647–1660.
- [25] S. Simonet, J.J. Descombes, M.O. Vallez, T. Dubuffet, G. Lavielle, T.J. Verbeuren, S 18886, a new thromboxane (TP)-receptor antagonist is the active isomer of S 18204 in all species, except in the guinea-pig, *Adv. Exp. Med. Biol.* 433 (1997) 173–176.
- [26] V. Capra, A. Veltri, C. Foglia, L. Crimaldi, A. Habib, M. Parenti, G.E. Rovati, Mutational analysis of the highly conserved ERY motif of the thromboxane A₂ receptor: alternative role in G protein-coupled receptor signaling, *Mol. Pharmacol.* 66 (4) (2004) 880–889.
- [27] C. Buccellati, A. Sala, G. Rossoni, V. Capra, G.E. Rovati, A. Di Gennaro, G. Folco, S. Colli, C. Casagrande, Pharmacological characterization of 2NTX-99 [4-methoxy-N1-(4-*trans*-nitrooxycyclohexyl)-N3-(3-pyridinylmethyl)-1,3-benzenedicarboxamide], a potential antiatherothrombotic agent with anti-thromboxane and Nitric Oxide donor activity in platelet and vascular preparations, *J. Pharmacol. Exp. Ther.* 317 (2) (2006) 830–837.
- [28] M. Ambrosio, F. Fanelli, S. Brocchetti, F. Raimondi, M. Mauri, G.E. Rovati, V. Capra, Superactive mutants of thromboxane prostanoid receptor: functional and computational analysis of an active form alternative to constitutively active mutants, *Cell. Mol. Life Sci.* 67 (17) (2010) 2979–2989.
- [29] F. Fanelli, M. Mauri, V. Capra, F. Raimondi, F. Guzzi, M. Ambrosio, G.E. Rovati, M. Parenti, Light on the structure of thromboxane A₂ receptor heterodimers, *Cell. Mol. Life Sci.* 68 (18) (2011) 3109–3120.
- [30] V. Capra, M. Busnelli, A. Perenna, M. Ambrosio, M.R. Accomazzo, C. Gales, B. Chini, G.E. Rovati, Full and partial agonists of thromboxane prostanoid receptor unveil fine tuning of receptor superactive conformation and G protein activation, *PLoS One* 8 (3) (2013) e60475.
- [31] A. Avdeef, J.E.A. Comer, S.J. Thomson, pH-metric log P. 3. Glass electrode calibration in methanol–water, applied to pK_a determination of water-insoluble substances, *Anal. Chem.* 65 (1) (1993) 42–49.
- [32] E.H. Kerns, L. Di, G.T. Carter, In vitro solubility assays in drug discovery, *Curr. Drug Metabol.* 9 (9) (2008) 879–885.
- [33] A. De Lean, P.J. Munson, D. Rodbard, Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose–response curves, *Am. J. Physiol.* 235 (1978) E97–E102.
- [34] R. Bartzatt, Drug analogs of COX-2 selective inhibitors lumiracoxib and valdecoxib derived from in silico search and optimization, *Antiinflamm. Antiallergy Agents Med. Chem.* 13 (1) (2014) 17–28.
- [35] R. Bartzatt, Anti-inflammatory drugs and prediction of new structures by comparative analysis, *Antiinflamm. Antiallergy Agents Med. Chem.* 11 (2) (2012) 151–160.
- [36] F. Salvo, A. Fourrier-Reglat, F. Bazin, P. Robinson, N. Riera-Guardia, M. Haag, A.P. Caputi, N. Moore, M.C. Sturkenboom, A. Pariente, Investigators of safety of non-steroidal anti-inflammatory drugs SOSP. Cardiovascular and gastrointestinal safety of NSAIDs: a systematic review of meta-analyses of randomized clinical trials, *Clin. Pharmacol. Ther.* 89 (6) (2011) 855–866.
- [37] F. Catella-Lawson, M.P. Reilly, S.C. Kapoor, A.J. Cucchiara, S. DeMarco, B. Tournier, S.N. Vyas, G.A. FitzGerald, Cyclooxygenase inhibitors and the antiplatelet effects of aspirin, *N. Engl. J. Med.* 345 (25) (2001) 1809–1817.
- [38] L.J. Marnett, The COXIB experience: a look in the rearview mirror, *Annu. Rev. Pharmacol. Toxicol.* 49 (2009) 265–290.
- [39] H. Sakai, T. Suzuki, Y. Takahashi, M. Ukai, K. Tauchi, T. Fujii, N. Horikawa, T. Minamimura, Y. Tabuchi, M. Morii, K. Tsukada, N. Takeguchi, Upregulation of thromboxane synthase in human colorectal carcinoma and the cancer cell proliferation by thromboxane A₂, *FEBS Lett.* 580 (14) (2006) 3368–3374.
- [40] A.J. Cayatte, Y. Du, J. Oliver-Krasinski, G. Lavielle, T.J. Verbeuren, R.A. Cohen, The thromboxane receptor antagonist S18886 but not aspirin inhibits atherogenesis in apo E-deficient mice: evidence that eicosanoids other than thromboxane contribute to atherosclerosis, *Arterioscler. Thromb. Vasc. Biol.* 20 (7) (2000) 1724–1728.
- [41] K.S. Sakariassen, E.A. Femia, F.M. Daray, G.M. Podda, C. Razzari, M. Pugliano, A.E. Errasti, A.R. Armesto, W. Nowak, P. Alberts, J.P. Meyer, A.S. Sorensen, M. Cattaneo, R.P. Rothlin, EV-077 in vitro inhibits platelet aggregation in type-2 diabetics on aspirin, *Thromb. Res.* 130 (5) (2012) 746–752.
- [42] P. Gaussem, J.L. Reny, C. Thalamas, N. Chatelain, M. Kroumova, B. Jude, B. Boneu, J.N. Fiessinger, The specific thromboxane receptor antagonist S18886: pharmacokinetic and pharmacodynamic studies, *J. Thromb. Haemost.* 3 (7) (2005) 1437–1445.
- [43] J.N. Fiessinger, H. Bounameaux, M.A. Cairols, D.L. Clement, S. Coccheri, J.P. Fletcher, U. Hoffmann, A.G. Turpie, Thromboxane antagonism with terutroban in peripheral arterial disease: the TAIPAD study, *J. Thromb. Haemost.* 8 (11) (2010) 2369–2376.
- [44] P.F. Lesault, L. Boyer, G. Pelle, A. Covali-Noroc, D. Rideau, S. Akakpo, E. Teiger, J.L. Dubois-Rande, S. Adnot, Daily administration of the TP receptor antagonist terutroban improved endothelial function in high-cardiovascular-risk patients with atherosclerosis, *Br. J. Clin. Pharmacol.* 71 (6) (2011) 844–851.
- [45] M.G. Bousser, P. Amarenco, A. Chamorro, M. Fisher, I. Ford, K.M. Fox, M.G. Hennerici, H.P. Mattle, P.M. Rothwell, A. de Cordoue, M.D. Fratacci, Terutroban versus aspirin in patients with cerebral ischaemic events (PERFORM): a randomised, double-blind, parallel-group trial, *Lancet* 377 (9782) (2011) 2013–2022.
- [46] M. Lee, B. Ovbiagele, Vascular events after stroke: terutroban fails to PERFORM, *Lancet* 377 (9782) (2011) 1980–1982.
- [47] S.J. Wilson, A.M. Roche, E. Kostetskaia, E.M. Smyth, Dimerization of the human receptors for prostacyclin and thromboxane facilitates thromboxane receptor-mediated cAMP generation, *J. Biol. Chem.* 279 (51) (2004) 53036–53047.
- [48] S. Ibrahim, M. Tetruashvily, A.J. Frey, S.J. Wilson, J. Stitham, J. Hwa, E.M. Smyth, Dominant negative actions of human prostacyclin receptor variant through dimerization: implications for cardiovascular disease, *Arterioscler. Thromb. Vasc. Biol.* 30 (9) (2010) 1802–1809.
- [49] P. Patrignani, C. Di Febbo, S. Tacconelli, K. Douville, M.D. Guglielmi, R.J. Horvath, M. Ding, K. Sierra, J. Stitham, S. Gleim, G. Baccante, V. Moretta, L. Di Francesco, M.L. Capone, E. Porreca, J. Hwa, Differential association between human prostacyclin receptor polymorphisms and the development of venous thrombosis and intimal hyperplasia: a clinical biomarker study, *Pharmacogenet. Genomics* 18 (7) (2008) 611–620.
- [50] A.L. Blobaum, L.J. Marnett, Molecular determinants for the selective inhibition of cyclooxygenase-2 by lumiracoxib, *J. Biol. Chem.* 282 (22) (2007) 16379–16390.
- [51] S.W. Rowlinson, J.R. Kiefer, J.J. Prusakiewicz, J.L. Pawlitz, K.R. Kozak, A.S. Galgutkar, W.C. Stallings, R.G. Kurumbail, L.J. Marnett, A novel mechanism of cyclooxygenase-2 inhibition involving interactions with Ser-530 and Tyr-385, *J. Biol. Chem.* 278 (46) (2003) 45763–45769.
- [52] Y. Yamamoto, K. Kamiya, S. Terao, Modeling of human thromboxane A₂ receptor and analysis of the receptor–ligand interaction, *J. Med. Chem.* 36 (7) (1993) 820–825.
- [53] A. Krishna, A. Yadav, Lead compound design for TPR/COX dual inhibition, *J. Mol. Model* 18 (9) (2012) 4397–4408.
- [54] X. Wang, L. Liu, L. Huang, K. Herbst-Robinson, A.S. Cornec, M.J. James, S. Sugiyama, M. Bassetto, A. Brancale, J.Q. Trojanowski, V.M. Lee, A.B. Smith 3rd, K.R. Brunden, C. Ballatore, Potent long-acting cyclopentane-1,3-dione

- thromboxane (A₂)-receptor antagonists, *ACS Med. Chem. Lett.* 5 (9) (2014) 1015–1020.
- [55] C. Ballatore, J.H. Soper, F. Piscitelli, M. James, L. Huang, O. Atasoylu, D.M. Huryn, J.Q. Trojanowski, V.M. Lee, K.R. Brunden, A.B. Smith, Cyclopentane-1,3-dione: a novel isostere for the carboxylic acid functional group. Application to the design of potent thromboxane (A₂) receptor antagonists, *J. Med. Chem.* 54 (19) (2011) 6969–6983.
- [56] M.L. Capone, S. Tacconelli, M.G. Sciulli, M. Grana, E. Ricciotti, P. Minuz, P. Di Gregorio, G. Merciaro, C. Patrono, P. Patrignani, Clinical pharmacology of platelet, monocyte, and vascular cyclooxygenase inhibition by naproxen and low-dose aspirin in healthy subjects, *Circulation* 109 (12) (2004) 1468–1471.
- [57] C. Giannarelli, M.U. Zafar, J.J. Badimon, Prostanoid and TP-receptors in atherothrombosis: is there a role for their antagonism? *Thromb. Haemost.* 104 (5) (2010) 949–954.
- [58] J.C. Breitner, P.P. Zandi, Do nonsteroidal antiinflammatory drugs reduce the risk of Alzheimer's disease? *N. Engl. J. Med.* 345 (21) (2001) 1567–1568.
- [59] B.A. Veld, A. Ruitenberg, A. Hofman, L.J. Launer, C.M. van Duijn, T. Stijnen, M.M. Breteler, B.H. Stricker, Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease, *N. Engl. J. Med.* 345 (21) (2001) 1515–1521.