

A New Gateway for Rheumatoid Arthritis: COXIBs with an Improved Cardiovascular Profile

M. Hoxha, V. Capra, C. Buccellati, A. Sala, C. Cena, R. Fruttero, M. Bertinaria, G.E. Rovati

Abstract—Today COXIBs are used in the treatment of arthritis and many other painful conditions in selected patients with high gastrointestinal risk and low cardiovascular (CV) risk. Previously, we have identified an unexpected mechanism of action of a traditional non-steroidal anti-inflammatory drug (NSAID) (diclofenac) and a specific inhibitor of cyclooxygenase-2 (COXIB) (lumiracoxib) demonstrating that they possess weak competitive antagonism at the thromboxane receptor (TP). We hypothesize that modifying the structure of a known COXIB so that it becomes also a more potent TP antagonist will preserve the anti-inflammatory and gastrointestinal safety typical of COXIBs and prevent the CV risk associated with long term therapy.

Keywords—Cyclooxygenase, inflammation, lumiracoxib, thromboxane A₂.

I. INTRODUCTION

NONSTEROIDAL anti-inflammatory drugs (NSAIDs) are a group of medications that provide anti-inflammatory/analgesic and anti-pyretic activities. Their main mechanism of action is based on the inhibition of the cyclooxygenases (COXs), which are responsible of the transformation of arachidonic acid in a series of prostanoids [1] (Fig. 1). COX-1 is the constitutive isoform expressed in most tissues and is involved in gastric cytoprotection and haemostatic integrity [2]. COX-2, on the other hand, is the inducible isoform mainly expressed in response to inflammatory stimuli and is involved in inflammation, tumorigenesis and inhibition of platelet aggregation and vasodilatation [2]. In the 1990s after the identification of the second COX isoform a new class of NSAIDs or COX-2 selective inhibitors (COXIBs) was developed [3] to improve the gastrointestinal safety of the traditional NSAIDs. The therapeutic use of COXIBs is however limited by potential cardiovascular (CV) hazard associated with their long term

therapy [4]. These effects have been hypothesized by the imbalance theory, which assumes that any COXIB that reduces endothelial-derived prostacyclin (PGI₂, a platelet aggregation inhibitor and vasodilator) without affecting platelet-derived thromboxane A₂ (TXA₂, a platelet aggregation stimulator and vasoconstrictor) will predispose to a prothrombotic state [5]. Therefore, the increasing CV hazard led to the withdrawn from the market of rofecoxib or Vioxx by Merck and Valdecoxib (Bextra) by Pfizer [6] with significant economic and image loss for the companies [7]. Yet, there is great interest in designing new COXIBs with reduced CV adverse effects to extend their use to patients that are at high CV risk.

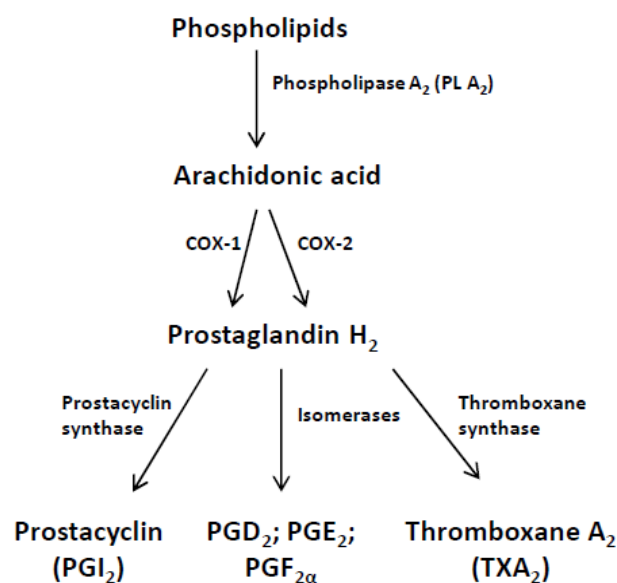


Fig. 1 The arachidonic acid cascade: COX-1 is the constitutive isoform, whereas the COX-2 is the inducible isoform responsible for the transformation of arachidonic acid in prostanoids [15]

M. Hoxha is a PhD Candidate at the University of Milan, Department of Pharmacological Sciences, Milan, Italy (phone: 00390250318369; fax: 00390250318284; e-mail: malvina.hoxha@unimi.it)

V. Capra is an Assistant Professor at the University of Milan and a Researcher at the Department of Health Sciences, San Paolo, Milan, Italy (e-mail: valerie.capra@unimi.it)

C. Buccellati is an Associate Researcher at the University of Milan, Italy, (e-mail: carola.buccellati@unimi.it)

A. Sala and G. Enrico Rovati are Associate Professors of Pharmacology at the University of Milan, Department of Pharmacological Sciences, Italy (e-mail: angelo.sala@unimi.it, genrico.rovati@unimi.it)

C. Cena is an Associate Professor at the University of Turin, Italy (e-mail: clara.cena@unito.it)

R. Fruttero is a Professor of Medicinal Chemistry at the University of Turin, Italy (e-mail: Roberta.Fruttero@unito.it)

M. Bertinaria is an Associate Professor of Medicinal Chemistry at the Bertinaria@unito.it)

Recently we observed an unexpected mechanism of action of diclofenac, a potent and widely used anti-inflammatory drug, with a non selective COX profile, as well as of its derivative, lumiracoxib, a selective COX-2 inhibitor. Both compounds displayed a weak competitive antagonism at the TXA₂ receptor (TP) [8], however not sufficiently potent for therapeutic use.

TP receptor is a G-protein coupled receptor, which exists in 2 isoforms named TP α and TP β . It is mainly coupled to G α protein [9] but also to G_{12/13} and G α s, [10]. Activation of TP receptors induces platelet shape change and aggregation, constriction of vascular smooth-muscle cells, providing a

stimulus for causing thrombus formation, contraction of pulmonary venous and arterial smooth muscle [11]-[14].

We hypothesize that modifying the structure of a known COXIB so that it becomes also a potent TP receptor antagonist will preserve the anti-inflammatory and gastrointestinal safety profile typical of COXIBs, while preventing the reported increase in CV risk associated with long term therapy resulting from the imbalance between the decrease in endothelial PGI₂ and the platelet TXA₂ production [15].

These findings lead us to a new study, the modulation of the structure of lumiracoxib in order obtain new NSAIDs with a more balanced COXIB and TP receptor antagonist properties leading to a third generation of NSAIDs with an improved CV profile [16].

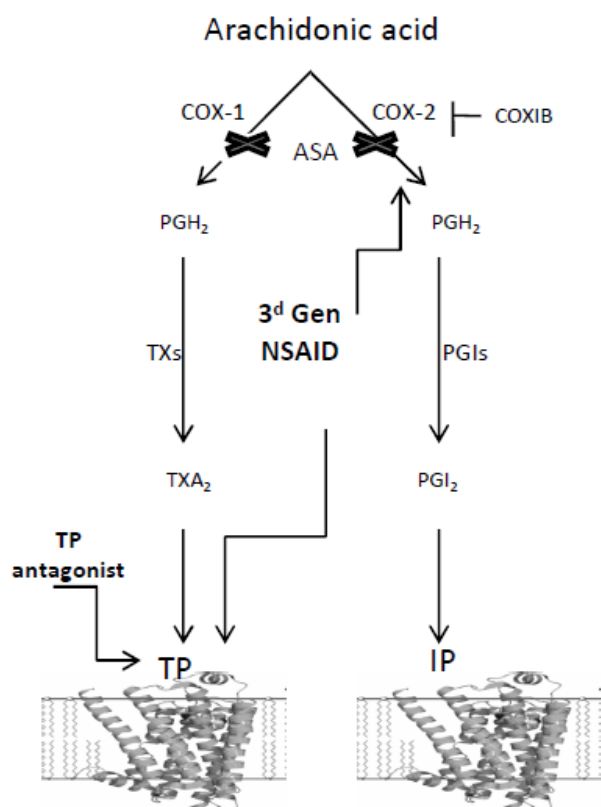


Fig. 2 Pharmacological modulators of thromboxane A₂ (TXA₂) and prostacyclin (PGI₂). Aspirin (ASA) inhibits COX-1 and COX-2 suppressing the production of both TXA₂ and PGI₂. TP antagonist prevents activation of the TP receptor by all possible mediators, including isoprostanes. COXIBs block COX-2 and inhibit the production of inflammatory prostaglandins, but also suppress the endogenous formation of PGI₂ in endothelial cells without affecting TXA₂ generation. This might increase the risk of a major cardiovascular event. A third-generation NSAID might possess anti-inflammatory properties, together with an improved gastrointestinal (GI) and CV profile [15]

II. METHODS

The carboxylic function of the lead was replaced by non-classical isosteres of acid groups giving rise to different compounds.

The anti-aggregatory potencies of the compounds, expressed as IC₅₀ values were assessed in washed human platelets by Born-turbidimetric assay. Platelets were isolated from human blood of healthy volunteers with no history of cardiovascular diseases in the presence of the anticoagulant CPD (Citrate Phosphate Dextrose solution: sodium citrate, dihydrate, 26.3g/L; dextrose, monohydrate, 25.5g/L; citric acid, anhydrous 3.27g/L; monobasic sodium phosphate, monohydrate, 2.22g/L). Buffy coat was treated with 100μM acetylsalicylic acid and buffy coat was centrifuged at 280g for 15min at room temperature to obtain the platelet-rich plasma (PRP). PRP was centrifuged at 650g for 10min at room temperature. The pelleted platelets were suspended in washing buffer (mM composition: citric acid monohydrate 39, glucose monohydrate 5, KCl 5, CaCl₂ 2, MgCl₂ x 6H₂O 1, NaCl 103, pH 6.5), recentrifuged at 650g for 15min at room temperature, and resuspended in 15ml of HBSS (Hank's Balanced Salt Solution: CaCl₂·2H₂O 0.185g/L; KCl 0.40g/L; KH₂PO₄ 0.06 g/L; MgCl₂·6H₂O 0.10g/L; MgSO₄·7H₂O 0.10g/L; NaCl 8.00 g/L; NaHCO₃ 0.35g/L; Na₂HPO₄ 0.048g/L; D-glucosio 1.00 g/L). After the incubation with drug or vehicle (DMSO, maximum 0.2%, v:v) for 5min at 37°C, the platelet aggregation was induced by U46619 (0.1-0.5μM) under continuous stirring. The aggregation was monitored for 6 minutes.

The TP receptor binding were assessed by radioligand binding assay in Human Embryonic Kidney cells (HEK 293) cells (ATCC, Manassas, VA) transfected with human TPα using the transfecting agent Lipofectamine 2000 (Invitrogen, Carlsbad, CA). 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. The cells were labeled with the specific antagonist of the receptor [³H]-SQ29,548 (Perkin-Elmer, Boston, MA) and the radioactivity was measured by liquid scintillation counting (Ultima Gold; Packard Instruments, Meriden, CT).

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 50 U ml⁻¹ penicillin, 100μg ml⁻¹ streptomycin, and 20mM HEPES buffer, pH 7.4, at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

COXIB activity was studied in isolated human lymphomonocytes. The expression of COX-2 was induced by lipopolysaccharide (LPS) (from *E. coli*, serotype 0111:B4; 10 μg ml⁻¹, overnight, 37°C). The activity of COX-2 was assessed through the Prostaglandin E₂ (PGE₂) production, a marker of activity of COX-2 detected by immunoenzymatic assay (EIA) (PGE₂ EIA kit, Cayman Chemical). Different concentrations of the tested compounds were used in order to evaluate the IC₅₀ values. Prostanoid concentrations in each sample were calculated from PGE₂ standard curves. IC₅₀ of the compounds were calculated versus the maximal PGE₂ production.

COX-1 inhibition was performed in washed human platelets suspension (2 x 10⁸ cells ml⁻¹). Platelets were treated (15min) with increasing concentration of the tested compounds, and incubated at 37°C in a Dubnoff bath. Calcium ionophore (A23187, 2μM) was added to test tube samples. After an incubation period of 10min at 37°C, the samples were

centrifuged at 5000g for 5min at 4°C. In the supernatant obtained after centrifugation, the TXB₂ production was evaluated through the EIA.

III. RESULTS

All the synthesized compounds were tested for their COX-2 inhibitory activity, TP binding affinity as well as antagonist property, and the results are reported in Table I. PGE₂ production was used as a marker of COX-2 activity in human lympho-monocytes following treatment with 10µg/mL aspirin and overnight pretreatment with 10µg/mL LPS to induce COX-2 expression. As expected, our data confirmed that lumiracoxib is a rather potent COX-2 inhibitor with a reported IC₅₀ of 0.0046 µM, while compound 1, 2 and 3 showed similar IC₅₀s in the range of 0.025-0.016 µM (Fig. 3 (a)). In addition, while compounds 1 was almost inactive at the TP receptor, compound 2 was shown to display a slight (less than two-fold) more potent antagonistic activity at the TP receptor with respect to lumiracoxib, however less potent than compound 4 (IC₅₀ = 12.8 and 3.37µM, respectively). These data were determined by measuring the inhibition of human washed platelet aggregation stimulated with 0.5µM of U46619 as TP agonist. Compound 4 resulted to possess the highest affinity of all the derivatives tested for the TP receptor with a K_i value of 1.4 µM, more than fifty-fold more potent than lumiracoxib (K_i value= 73.5 µM). K_i values were determined in competition studies using the specific TP receptor antagonist [³H]SQ29,548 as labeled ligand for the human TPα receptor expressed in recombinant cells.

TABLE I

COX-2 INHIBITORY ACTIVITY, THROMBOXANE A₂ ANTAGONISM AND TPα RECEPTOR AFFINITY FOR DICLOFENAC, LUMIRACOXIB AND SYNTHESIZED COMPOUNDS 1-4

Compound	COX-2 IC ₅₀ ± %CV [µM]	Aggregation IC ₅₀ ± %CV [µM]	TPα K _i ± %CV [µM]
Diclofenac	0.0015 ± 40	16.00 ± 17	42 ± 45
Lumiracoxib	0.0046 ± 35	21.3 ± 10	73.5 ± 54
^a Compound 1	0.025 ± 95	10% inhib. (60 µM)	Not tested
^a Compound 2	0.016 ± 44	12.8 ± 5	61 ± 45
^a Compound 3	0.025 ± 54	26.7 ± 15	Not tested
^a Compound 4	0.65 ± 91	3.37 ± 16	1.4 ± 20

a **1**: 2-[(2-chloro-6-fluorophenyl)amino]-N-hydroxy-5-methyl-benzeneacetamide; **2**: N-(2-chloro-6-fluorophenyl)-4-methyl-2-(1H-tetrazol-5-ylmethyl)-benzamine; **3**: 2-[(2-chloro-6-fluorophenyl)amino]-5-methyl-benzoic acid; **4**: N-[[2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl]methyl]-1,1,1-trifluoro-methanesulfonamide. Synthesis of compounds 1-4 is reported in [16].

COX-2/COX-1 selectivity of diclofenac, lumiracoxib and the two most interesting compounds of the series, compound 2 and 4 were further investigated. COX-1 activity was assessed in terms of inhibition of TXB₂ production, a stable metabolite of TXA₂, induced by calcium ionophore. As expected, diclofenac resulted, by far, to be the most potent COX-1 inhibitor, while concentration-response curves of Fig. 3 (b) clearly show that all the other derivatives possess a COX-1 potency similar to lumiracoxib.

Overall, among of all the compounds under investigation

only compound 4 displayed rather balanced properties as TP antagonist and COX-1 inhibitor (IC₅₀ TP/COX-2 = 5.2 versus IC₅₀ TP/COX-2 = 4630 for lumiracoxib). In addition, compound 4 also displays about 25-fold COX-2 versus COX-1 selectivity.

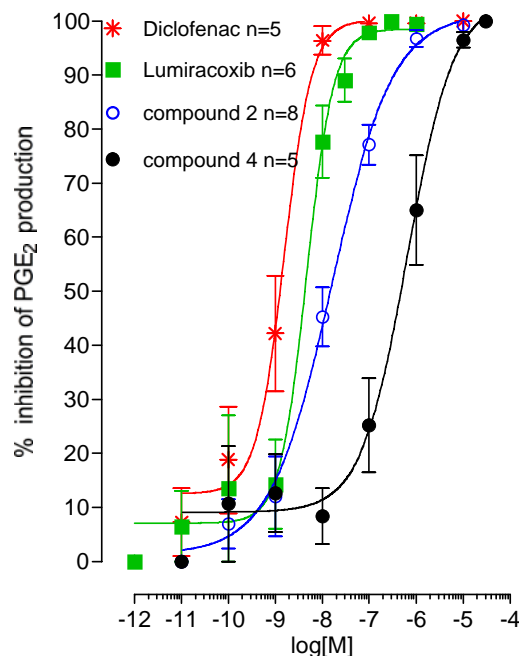


Fig. 3 (a) Evaluation of COX-2 activity in terms of release of PGE₂ in isolated human monocytes. Data are expressed as percent inhibition of PGE₂ release vs. untreated controls. Error bars represent mean ± SE of at least three independent experiments performed in duplicate.

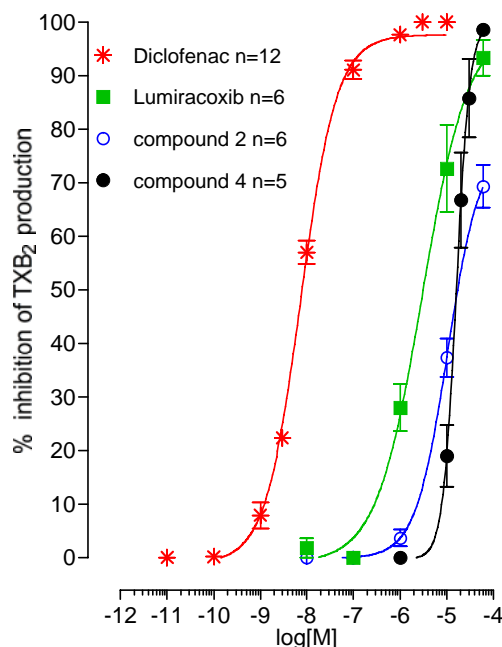


Fig. 3 (b) Evaluation of COX-1 activity in terms of release of TXB₂ (metabolite of TXA₂) from washed platelets during clotting. Data are expressed as percent inhibition of TXB₂ release vs. untreated controls. Error bars represent mean ± SE of at least three independent experiments performed in duplicate.

IV. CONCLUSION

Our results show 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 compared to lumiracoxib. The modification of existing drugs targeted against COX-2 (lumiracoxib) can lead to a new molecule with obvious advantage in the pharmacodynamic properties. Further studies will be certainly necessary to confirm these results *in vivo* animal models.

These compounds are 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. In rheumatoid arthritis COXIBs are used in long term therapy, typically in a regimen that consists of the administration for 15 days followed by a washout period of 1-2 months, in order to reduce the CV hazard. A third generation NSAID with a safer CV profile could have an impact on the duration and ameliorate the management of therapy not only in patients affected by rheumatoid arthritis or other painful conditions but also by selected form of cancer.

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M. Hoxha was born in Vlore, Albania on 22 June 1988. She is a PhD Candidate at the University of Milan, Graduate School in Pharmacological Sciences. She is a graduate of United World College of South East Asia, Singapore and she received her degree in Pharmacy in 2012 from the University of Milan, Italy.

She actually attends her PhD studies at the Laboratory of Molecular Pharmacology, Department of Pharmacological Sciences, University of Milan. Her interests have focus on Cardiovascular and Inflammation field in particular COXIBs with dual activity: TP antagonist/COXIB; study of thromboxane prostanoid receptor (TP) and characterization of its highly functional mutants with respect to homo/heterodimer formation and their regulation by different agonists.

Dr. Hoxha is a member of EPSA and in 2009 she was given the Research Award in memory of Professor Elena Sottocornola from the University of Milan, Italy, whereas in 2006 she obtained the Bilingual Award from United World College of South East Asia, Singapore.