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# **Nonsteroidal Anti-Inflammatory Drugs: Exploiting Bivalent COXIB/TP Antagonists for the Control of Cardiovascular Risk**

Silvia Carnevali<sup>a\*</sup>, Carola Buccellati<sup>a\*</sup>, Chiara Bolego<sup>b</sup>, Massimo Bertinaria<sup>c</sup>, G.Enrico Rovati<sup>a</sup> and Angelo Sala<sup>a,d</sup>

*<sup>a</sup>Department of Pharmacological and Biomolecular Sciences University of Milan, Milan, Italy; <sup>b</sup>Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Padua, Italy; <sup>c</sup>Department of Drug Science and Technology, University of Turin, Turin, Italy; <sup>d</sup>IBIM, National Research Council, Palermo, Italy.*

Address all correspondence to:

G.Enrico Rovati, Laboratory of Molecular and Eicosanoid Pharmacology, Dept. of Pharmacological and Biomolecular Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy.

Telephone: +390250318312, Email: [GEnrico.Rovati@unimi.it](mailto:GEnrico.Rovati@unimi.it)

\* Equally contributed

## **Abstract**

**Background.** Nonsteroidal anti-inflammatory drugs (NSAIDs) are some of the most widely prescribed or dispensed analgesics and antipyretics that act by inhibiting prostaglandins (PGs) and thromboxane synthesis. After the identification of a second isoform of COX, the pharmaceutical research focused on developing COX-2-selective drugs (COXIBs) considered as second generation NSAIDs that would retain the anti-inflammatory and analgesic activities of traditional NSAID without blunting the gastrointestinal cytoprotection sustained by COX1-derived products such as PGE<sub>2</sub>. However, while several clinical trials confirmed a gastrointestinal safer profile of COXIBs vs unselective COX inhibitors, increasing evidence for potential cardiovascular risk associated with COXIBs rapidly emerged. Today, there is no really safe NSAIDs to be used in chronic pain and anti-inflammatory treatments, as an adequate therapy associated with a minimal gastrointestinal damage and cardiovascular toxicity is yet to be developed. **Objective.** Here we present evidences that combining the anti-aggregating and anti-atherotrombotic activities of a thromboxane receptor antagonist with the anti-inflammatory activity of a COXIB we could obtain a new multitarget drug providing protection against the harmful activities mediated by the COXIB component, yet exploiting its recognized therapeutic advantages as a gastrointestinal-safer anti-inflammatory drug. We also summarize recent progress achieved in this field of research and possible new strategies to obtain a new bivalent compound. **Conclusion.** This possible third-generation NSAID with a safer pharmacological profile, will have all the pharmacological characteristics for the long-term therapy of chronic disorders such as inflammatory diseases or selected forms of cancer.

**Keywords:** Non steroidal anti-inflammatory drug, arachidonic acid, COX-2 selective inhibitor, thromboxane receptor antagonist, cardiovascular risk, mutitarget drug.

## 1. INTRODUCTION

Anti-inflammatory drugs characterized by nonsteroidal activity (NSAIDs), are some of the most widely prescribed or dispensed over the counter (OTC) analgesics and antipyretics that act by inhibiting prostaglandins (PGs) and thromboxanes (TXs) generated from the common precursor arachidonic acid (AA) [1]. AA is a polyunsaturated fatty acid with 20 carbon atoms and 4 double bonds (20:4) of the  $\omega$ -6 series released from membrane phospholipids by a wide range of stress-activated stimuli, from chemical to mechanical or immunological, and rapidly metabolized by key enzymes such as cyclooxygenases (COXs) [2, 3], lipoxygenases (LOXs) [4, 5] or cytochrome P450 [6] to a variety of products, most of which exert pro-inflammatory activities. Within this complex network of lipid mediators (LMs) the inhibition of one pathway can be responsible for an extra production of metabolites deriving from other pathways, the so called shunting in AA metabolism [7, 8], causing an increased synthesis of specific products and, consequently, the enhanced activation of their molecular targets, i.e. one or more G protein coupled receptors (GPCRs) [9-11] (see also IUPHAR/BPS Guide to Pharmacology: <http://www.guidetopharmacology.org/GRAC/ReceptorFamiliesForward?type=GPCR>).

In the late '80s the identification of two COX isoforms responsible for the synthesis of prostanoids [12], initiated a new era for the anti-inflammatory drugs: the opportunity to selectively target one of the two isoforms gave rise to a line of pharmacological research aimed at the identification of a safer NSAID. Given the multifaceted involvement of COX products in the physiological homeostasis of gastrointestinal (GI) epithelium and in the water and electrolyte balance in the kidney, GI lesions and impaired renal function are considered unavoidable consequence of COX-1 inhibition and therefore the typical side effects of all the first generation NSAIDs [13-17]. Thus, NSAIDs, initially classified on the basis of their chemical and pharmacological properties, were later reconsidered mainly on a COX-1/2 selectivity base [18, 19].

The research was, therefore, focused on COX-2-selective drugs (COXIBs) considered as second generation NSAIDs ([20] that would retain the anti-inflammatory and analgesic activities without blunting the GI cytoprotection sustained by COX1-derived mucosal PGE<sub>2</sub> and PGI<sub>2</sub> synthesis [21, 22]. As platelet COX-1 was demonstrated to be the major responsible for the synthesis of pro-aggregating TXA<sub>2</sub>, a marked COX-2 inhibition was considered to be an ideal choice, because GI bleeding often associated with the lack of gastric cytoprotection can also be enhanced by a suppressed formation of TXA<sub>2</sub> [23].

However, while on one side several clinical trials including VIGOR [24], CLASS [25] and TARGET [26] largely confirmed a GI safer profile of COXIBs, on the other side increasing evidence for potential cardiovascular (CV) risk associated with COXIBs emerged [27, 28]; this was mainly ascribed to inhibition of the

COX-2-dependent prostacyclin (PGI<sub>2</sub>) generation by endothelial cells (ECs), which is responsible for the anti-thrombotic properties of vessel walls [29]. Indeed, the so called ‘balance-tipping’ or “imbalance” theory suggests that any drug that reduces endothelial COX-2-derived PGI<sub>2</sub>, without affecting platelet COX-1-derived TXA<sub>2</sub>, will predispose to a prothrombotic state [30]. As a consequence of this rather unanticipated but potentially fatal side effect, rofecoxib from Merck and valdecoxib from Pfizer were voluntarily withdrawn from the market [19, 31].

## **2. PROSTANOID PRODUCTION BY ENDOTHELIAL COXS**

Although there is now a general agreement that PGI<sub>2</sub> in vascular ECs is generated mainly by COX-2 possibly as a result of COX-2 expression induced by laminar flow shear stress [32], evidence of the *in vivo* vascular expression of COX-2 under normal flow condition is still controversial [33, 34]. For example, a recent study on the effects of COX-2 inhibition in humans shows a minimal contribution of COX-2-derived PGI<sub>2</sub> to endothelial-dependent vasodilation [35]. At variance, it has also been reported that in unstimulated human ECs COX-1 was mainly responsible for TXA<sub>2</sub> production, whereas COX-2, detectable only after activation with an inflammatory stimulus, was solely involved in PGI<sub>2</sub> and PGE<sub>2</sub> synthesis [36]. Considering that the housekeeping COX-1 historically has been considered to be the major isoform responsible for endothelial PGI<sub>2</sub> production, the question as to the relative role of COX isoforms toward the synthesis of endothelial prostanoids remains open [37-39]. However, it is worth mentioning that studies in cells expressing both COX isoforms show that both enzymes could contribute to prostaglandin synthesis, while striking differences between the two isoform exist in the regulatory mechanisms by hydroperoxide tone [40]. In line with these considerations, our group showed that both COX isoforms appear to be important for PGI<sub>2</sub> production in ECs under normal conditions. However, upon increased hydroperoxide tone, such as in pathological conditions associated with platelet adhesion and activation, COX-1 represents the main player responsible for PGI<sub>2</sub> generation. Accordingly, selective COX-2 inhibition did not significantly affect the production of endothelial PGI<sub>2</sub> in this pathological setting [41, 42].

Although this observation should be confirmed in pathological conditions characterized by increased oxidative stress, the role of COX-2 in CV protection is far from being elucidated. Notably, based on recent data from atherosclerotic COX-2 knock-out animals, COX-2 may protect the vascular wall through mechanisms independent from local production of PGI<sub>2</sub> [43]. Thus, overall, the “imbalance” theory seems to provide only a partial explanation for the association between COX-2 inhibitor use and CV events [44].

## **3. THE THROMBOXANE PROSTANOID RECEPTOR**

The two lipid mediators mostly involved in CV risk, TXA<sub>2</sub> and PGI<sub>2</sub>, act both through specific G-protein coupled receptors (GPCR) [9], causing intracellular Ca<sup>2+</sup> [Ca<sup>2+</sup>]<sub>i</sub> increase and enhanced cAMP formation, respectively; these transduction mechanisms promote opposite effects on platelet activation and CV homeostasis: increased concentrations of cAMP following activation of PGI<sub>2</sub> receptor (IP) inhibits platelet aggregation and cause vasodilation [45], while [Ca<sup>2+</sup>]<sub>i</sub> increase following activation of TXA<sub>2</sub> receptors (TP $\alpha$ ), promotes platelet aggregation and vasoconstriction [46]. TP receptor, which can be also activated by other lipid mediators such as PGF<sub>2 $\alpha$</sub>  [47] or PGD<sub>2</sub> [48], is therefore considered a target that, once blocked, could potentially limit the undesirable CV side effects of COXIBs. Of notice, TP receptor has also been identified as the *in vivo* molecular target of the non enzymatic AA metabolites isoprostanes [49, 50], that, by their nature, are insensitive to the action of aspirin or traditional NSAIDs (tNSAIDs), and, whose production as a result of oxidative stress cannot be easily inhibited. Indeed, isoprostanes have been identified as potential markers of oxidative stress in atherothrombotic disease [51-54], and an important, if not the most abundant and significant, compound observed in plaques, suggesting that nonenzymatic lipid peroxidation predominates in advanced atherosclerosis and may promote plaque instability [55]. In addition, it has been demonstrated that TP $\alpha$  is able to dimerize with the IP receptor and that this heterodimerization shift TP pharmacological profile from Ca<sup>++</sup> generation to cAMP production, a mechanism that has been interpreted as way to control TP pro-aggregating activity [56]. On the other hand, when a naturally occurring IP receptor ‘loss-of-function’ mutant is co-expressed with TP $\alpha$ , TP response is switched back to classical inositolphosphate generation and [Ca<sup>2+</sup>]<sub>i</sub> increase [57]. Accordingly, patients carrying this R212C polymorphism displayed exaggerated loss of platelet IP responsiveness and accelerated CV disease [57], and seem to develop more venous thrombosis and intimal hyperplasia than control individuals [58].

#### **4. BLUNTING PGS’ SIDE EFFECTS**

Many are the reasons that induce to believe that COXIBs alone should not fully be considered safer anti-inflammatory agents: COX-2 enzyme generates PGI<sub>2</sub> in vascular ECs (see paragraph above), and, thus, this mediator is required to balance TXA<sub>2</sub> pro-thrombotic activity and to maintain CV homeostasis at least in particular conditions [30, 59, 60], while COX-2 is also constitutively expressed in the kidney, where it plays part in regulation of renal function [61].

In addition, through COX-2 enzyme, circulating monocytes generate PGE<sub>2</sub>, a metabolite that might even mimic CV effects of PGI<sub>2</sub> [62, 63], and finally, COX-2 may also be involved in the ‘adaptive cytoprotection’ of an ulcerated gastric mucosa, where is rapidly induced to form healing prostanoids [64]. Thus, today, COXIB

therapy is restricted mainly to patients with high risk of GI complications, but lower risk of CV events. However, because these patients have, nevertheless, an increase in CV risk [65], they 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 are also positive effect that can be obtained with COXIB therapy and that must be taken into consideration in designing a new drug, such as the role of COX-2 as a possible target in colon cancer prevention [66-70] associated to a significantly limited GI damage [71]. Indeed, all the colon adenoma prevention trials generated positive results on cancer prevention [72, 73], while CV concerns were the main reason to prematurely stop these trials [28, 74] despite the risk/benefit ratio could have been expected to be particularly favourable in these cases [75].

Today, we do not have a really safe NSAIDs to be used in chronic pain and anti-inflammatory treatments, and an adequate therapy associated with a minimal GI damage and CV toxicity is yet to be developed [76]. Indeed, retrospective analysis showed that CV risk is linked not only to COXIBs but also to first generation anti-inflammatory tNSAIDs [44, 77-82], and, in addition, tNSAIDs have also been observed to interfere with the cardio-protective effect of low-dose aspirin, somehow jeopardizing the benefit of their association [83]. [Interestingly, in a recent clinical trial in patients not affected by any previous CV disease the rate of CV events, despite infrequent, resulted similar in subjects on celecoxib or on tNSAIDs \[84\].](#) Thus, given the benefits [and the vast use](#) of tNSAIDs, it seems necessary to develop a new strategy to design innovative pharmacological tools to contrast the CV risk associated to first and second generation anti-inflammatory molecules. To this point, an interesting observation arose from naproxen, a non-selective COX-1 and COX-2 inhibitor, that appears to have a better CV risk profile than other tNSAIDs [77, 82, 85], [despite its protective effect was recently questioned against another widely prescribed tNSAID, i.e. ibuprofen,](#) [86]. Intriguingly, [naproxen](#) has been demonstrated to efficiently inhibit both platelet-derived TXA<sub>2</sub> and systemic PGI<sub>2</sub> biosynthesis [87]. This observation suggests that maintaining the balance between the opposing actions of TXA<sub>2</sub> and PGI<sub>2</sub> may be one of the key features to avoid CV toxicity. A new anti-inflammatory drug with a safer CV profile should avoid or compensate any imbalance in the intricate cross-talk of lipid mediators generated from AA. Thus, we believe that an alternative strategy for molecules such COXIB, known to inhibit PGI<sub>2</sub> biosynthesis, could be a hybrid compound with COX-2 inhibitory/TP antagonistic activities, a convenient way to deal with the unaffected TXA<sub>2</sub> synthesis associated with COXIBs therapy.

## 5. WHY AN HYBRID COMPOUND WITH COX-2 INHIBITORY/TP ANTAGONISTIC ACTIVITIES?

Aspirin, in particular the so called 'cardio-aspirin' or low-dose aspirin, is still the gold-standard for secondary prevention of atherothrombosis [88] due to its efficacy, unequalled by other non-selective COX inhibitors, in inhibiting platelet activation [89]. Certainly, the most important and best characterized mechanism of action of aspirin is its covalent inactivation of COX-1 [90], which, in turn, leads to a permanent suppression of platelet TXA<sub>2</sub> production [91]. Thus, inhibition of TP receptor-mediated platelet activation and aggregation seems to be the key step for the prevention of a major CV event such as vascular occlusion, stroke or myocardial infarct (MI).

Over the years a number of TXA<sub>2</sub> biosynthesis inhibitors and/or receptor antagonists have been developed as a possible alternative to COX-1 inhibition by low dose aspirin [92-107]. Interestingly, some of these compounds have demonstrated their efficacy on top of aspirin both in mice [108] and in humans [109, 110]. For one of these compounds, the potent and selective TP antagonist S-1886 terutroban [111], enough evidence has been accumulated to make this drug a promising treatment for atherothrombosis [112-115], and for blocking the progression of atherosclerosis and transforming lesions towards a more stable phenotype [116, 117]. To this point, Egan and colleagues have shown that terutroban delays spontaneous atherogenesis in the Apobec1/LDLR double knockout mice more than combined inhibition of COX-1 and COX-2, likely reflecting activation of the receptor by multiple ligands [118]. In addition, it prevented the negative effects of rofecoxib treatment on cardiac function in APOE3Leiden mice [119]. However, co-administration of terutroban with another COXIB, the Merck Frosst (MF) tricyclic, led to changes in plaque morphology that has been interpreted by the authors as suggestive of lesion destabilization [118]. This rather unexpected, because it was not observed in animals given each drug alone, and unexplained finding might well depend on the specific COXIB utilized as well as on the model used, that is unsuited for the evaluation of plaque destabilization [120].

In humans terutroban has been shown to have an anti-thrombotic activity superior to aspirin in the prevention of ischemic stroke [121] and to improve endothelial function in patients with coronary [109] or peripheral arterial disease [122] as well as in patients with high-CV risk [123]. Conversely, terutroban, in a very large 28 months double-blind clinical trial, did not perform better than aspirin in patients of both gender with a recent history of stroke or arterial retinal ischemia or transient ischemic attack [124]. However, an interesting observation was present in the original report, ~~was somehow overlooked by the authors~~: patients with an history of ischemic stroke, had a statistically lower recurrence rate with terutroban than with aspirin. These patients, that were likely on aspirin treatment before the qualifying event, might have had therefore a further CV event despite

aspirin treatment, the so-called ‘aspirin treatment failure’, [an all too common clinical scenario that, surprisingly, has barely been investigated](#) [125]. This information (i.e. the aspirin treatment before the qualifying event) was inexplicably not recorded by the investigators, likely because omitted in the drafting of the trial protocol. In this particular subgroup, the terutroban ‘non inferiority’ criteria was certainly met, but apparently underestimated, supporting the use of terutroban as a possible surrogate to aspirin regimen, especially in the presence of aspirin-resistant subjects.

For these reasons, we believe that the synthesis of a bivalent compound with multitarget activities, i.e. COX-2 inhibitor/TP antagonist, may provide protection against all the harmful activities mediated by the COXIB component, yet exploiting its recognized therapeutic advantages as a GI-safer anti-inflammatory drug. Of notice, celecoxib (CELEBREX<sup>R</sup>), the only selective COX-2 inhibitor available in U.S. market, showed a substantial equivalence in CV risk with tNSAID or placebo in a series of clinical trials [126, 127]. [These results have been recently confirmed by a noninferiority trials of celecoxib vs. two different tNSAID, i.e. ibuprofen and naproxen, in patients at increased CV risk, a study mandated by the Food and Drug Administration in the aftermath of the withdrawal of rofecoxib](#) [86]. [The results of these huge \(24,222 patients\) eight years long trial demonstrate a non inferiority of ‘moderate’ doses of celecoxib, as compared with the two other tNSAIDs with respect to the primary CV composite outcome. Similarly, a nation-wide population-based cohort study on 7 million Swedish residents did not find the use of COXIB \(mainly celecoxib\) associated with MI or ischemic stroke, but unveiled a potential effect of both COXIBs and tNSAIDs on atrial fibrillation, with the highest risk associated with the more selective etoricoxib](#) [128].

[To this point,](#) despite celecoxib pharmacology *in vitro* has unveiled only a 7-30 fold selectivity for COX-2 vs. COX-1 [129, 130], it was, nevertheless, associated with a lower incidence of ulcers and ulcer complications compared with traditional non-selective NSAIDs [25] [and in lower rates of renal adverse events than ibuprofen](#) [86]. On this basis we believe that a moderate COX-2 selectivity in association with a balanced TP receptor antagonistic activity are properties that, whenever present in a single molecule, can lead to a promising family of new compounds, a third generation anti-inflammatory drugs with a better GI and CV profile than the drugs currently available for long-term anti-inflammatory therapies, chronic diseases or, even more importantly, in selected forms of cancer [70, 131]. Interestingly, a greater selectivity for COX-2 does not seem to confer an additionally lower risk of upper GI bleeding [132], while it might further negatively affect the CV profile of the drug [127, 133, 134].

The studies involving COXIBs evolved over time from ignoring potential CV side-effects, to the knowledge that not only COXIBs but also tNSAIDs may contribute to the overall CV risk of subjects. Additionally, a better understanding of confounding factors now allow us to more precisely understand the risk associated with the more general class of non steroidal anti-inflammatories [128].

## 6. WHAT'S NEW

Results obtained with serendipity from an unrelated research project demonstrated a previously unknown and rather unexpected mode of action of diclofenac, one of the most widely used anti-inflammatory drugs [135]. In an *ex vivo* preparation of guinea pig airways, it was found that diclofenac concentration-dependently and selectively inhibited the contraction responses to TP receptor agonists such as PGD<sub>2</sub> or TXA<sub>2</sub> stable analog U-46619 [48]. As the chemical structure of diclofenac resembles that of lumiracoxib, a highly selective COXIB, it was decided to evaluate whether TP receptor antagonism was a feature shared by both molecules. Indeed, *in vitro* human platelet aggregation and second messenger formation experiments in a recombinant system showed that also lumiracoxib possesses a dual COX-2 inhibitory/TP receptor antagonistic activity. However, the effect of lumiracoxib and diclofenac as a TP receptor antagonist was found to be significant only in the high micromolar concentration range, which, nonetheless, is not far from the concentrations reached following therapeutic administration of these drugs [135, 136]. In contrast, results obtained with other well known COXIBs, such as celecoxib and rofecoxib, or other tNSAIDs, demonstrated that these molecules do not possess TP receptor antagonistic activity, thus indicating that TP receptor is not a target shared by the whole class of NSAIDs, whether unselective or COX -2 selective [48].

We therefore started a research effort to demonstrate that hybrid structures can be developed to obtain a multitarget drug that, ideally, would display COX-2 inhibitory and TP receptor antagonistic activity in the same concentration range [137]. Our initial approach to design a multitarget anti-inflammatory agent was to synthesize a series of lumiracoxib derivatives by chemical modulation of its structure, aimed to balance the two desired activities [138]. Indeed, lumiracoxib, despite its withdrawal from the market due to adverse liver toxicity [139], possibly associated to specific HLA alleles [140], has been recently the subject of a meta-analysis including eighteen clinical trials of patients with osteoarthritis treated with this COXIB, placebo or other tNSAIDs. Intriguingly, no significant differences was evident in the CV outcomes among the different groups [141].

A series of lumiracoxib derivatives were obtained substituting the carboxylic function with non-classical isosteres of acid groups [142]. While most of the compounds synthesized to date maintained the TP antagonist

activity within the lumiracoxib potency range, just a few preserved the desired COX-2 potency and selectivity [138]. Indeed, some of the new compounds, such as the tetrazole and trifluoromethansulfonamido-isostere derivatives, showed a TP receptor antagonist profile slightly better than the lead compound, that, together with a reduction in COX inhibitory potency, resulted in a more balanced hybrid molecules [143]. Yet, their potency as TP receptor antagonists remain in the micromolar range, almost two order of magnitude far from the desired values for a receptor antagonist (nanomolar range).

Considering the set of close lumiracoxib analogues studied, their Structure-Activity Relationships (SAR) for TP $\alpha$ -antagonism and COX-2 inhibition can be summarized as follows (Figure 1):

- COX-2 inhibition in human lympho-monocytes was correlated to their ionization degree. The most acidic the terminal acid isostere, the more potent COX-2 inhibition was obtained. The optimal  $pK_a$  value for the synthesized derivatives ranged from 4 to 7. One notable exception was represented by the use of the hydroxamic acid moiety that, in spite of a low acidity ( $pK_a = 8.9$ ) proved only 8 fold less active than lumiracoxib in COX-2 inhibition. This result might be attributed to other properties of this residue (e.g. complexing ability?) and is worth of further investigations. Conversely, the ionization degree of the acidic residue could not be the major player in regulating antagonism at the TP $\alpha$  receptor in lumiracoxib derivatives. The ability of the terminal moiety to behave as hydrogen bond donor (HBD) or hydrogen bond acceptor (HBA), its lipophilicity and steric hindrance might be relevant parameters for TP $\alpha$  receptor blockade.

- The optimal length of the alkyl chain linking the acidic residue to the 2-amino-5-methyl-substituted benzene ring is represented by one methylene unit. Longer chains led to the loss of both the desired activities. Interestingly, the suppression of this chain in the structure of lumiracoxib brought to a slight decrease (7 fold) of COX-2 inhibition while conserved the ability to inhibit TP $\alpha$ -dependent platelet aggregation thus suggesting that this modulation can be further explored in the design of dual TP $\alpha$ /COX-2 antagonists.

- Methyl-substitution at the amino nitrogen bridge led to an expected decrease of COX-2 blockade and to the total loss of TP $\alpha$  antagonism, indicating that hydrogen bond by the -NH group might be important either for conformational stabilization of the molecular architecture (through internal hydrogen bonding) or for direct interaction with the TP $\alpha$  receptor.

- Finally, the replacement of the methyl group in position 5 of the benzene ring, which is the best substitution for conferring COX-2 vs COX-1 selectivity in lumiracoxib analogues [144] with a propionic acid chain led to a potent TP $\alpha$  antagonist with the major drawback of losing any COX-2 inhibition. This could be

expected taking into account the stringent structural future of the COX-2 pocket. Nevertheless, this observation can suggest that less sterically demanding acidic residues might be introduced at this position.

Thus, so far, structure-activity analysis led to new more balanced chemical entities retaining both the requested activities. However, the pharmacodynamic profile of these molecules have yet to be improved before the evaluation in an *in vivo* model can be planned. In the future, the obtained preliminary SAR together with molecular docking studies, could allow a more efficient design of improved dual TP $\alpha$  antagonists/COX-2 inhibitors. Moreover, a recently published *in silico* analysis of the physico-chemical properties of a series of lumiracoxib analogues [145] should be of great help in the design of new lumiracoxib-based multitarget drugs with optimized pharmacokinetics properties.

Another research group, following a completely different approach, identified one compound derived from a natural component of garlic, with interesting affinity for both COX-2 enzyme and TP receptor [146]. Thus, [alternative strategies are possible](#), such as the modification of more tNSAID with intrinsic TP antagonism activity [in a more COX-2 selective molecule, that, hopefully, will result in the identification of](#) lead compound [with the desired pharmacodynamics characteristics](#).

Despite different strategies have been proposed over the year to control the CV hazard of COXIBs and tNSAIDS [147, 148], we believe that the goal for a third-generation NSAID with a safer pharmacological profile, could lie in reaching a reasonable COX-2/COX-1 selectivity, something in a range comparable to that of celecoxib (CELEBREX<sup>®</sup>), and a balanced high nanomolar range potency for COX-2 inhibition/TP antagonism within a single molecule. This compound, [at least in our opinion, should](#) have the pharmacological characteristics for the long-term therapy of chronic disorders such as inflammatory diseases or even cancer.

## 7. METHODOLOGICAL APPROACHES

Our approach was to test the activity of new molecules on circulating cells relevant to inflammation and CV homeostasis such as human isolated monocytes and platelets. Monocytes isolated from human blood and resuspended in saline buffer were stimulated with a pro-inflammatory challenge, such as LPS, and newly synthesized compounds tested for their capacity to reduce PGE<sub>2</sub> production, a marker of COX-2 expression and activity [149]. When whole blood aliquots were used instead of isolated monocytes, potency of all inhibitors was drastically reduced [138], likely depending on plasma protein binding [150]. In washed human platelets, TXA<sub>2</sub> production was induced by Ca<sup>++</sup>-ionophore (A-23187) challenge [150, 151], and potency for COX-1 inhibition obtained by a concentration-dependent pre-treatment of platelets with the tested compounds.

PGE<sub>2</sub> and TXB<sub>2</sub> (the stable TXA<sub>2</sub> metabolite) production in supernatants from pelleted cells were evaluated by liquid chromatography-tandem mass spectrometry using the isotopic dilution of deuterated internal standards [d<sub>4</sub>] PGE<sub>2</sub> and [d<sub>4</sub>] TXB<sub>2</sub>, respectively [143]. To define a compound as a COXIB, a ratio of at least 10-20 fold between the calculated IC<sub>50</sub>s for COX1 and COX2, respectively, was required.

TP receptor antagonism was tested in washed human platelets, a cellular type in which the TP $\alpha$  isoform is extensively expressed. Platelets were challenged with U46619 and aggregation measured by the Born turbidimetric assay [152]. Compounds antagonism was revealed by a concentration dependent reduction of the U46619-dependent aggregation, typical of a competitive antagonism [48].

The most promising compounds were also tested in a vascular pharmacological *in vitro* model, to assess their ability to reduce U46619-induced rat aortic ring contraction. A rightward shift of the agonist cumulative concentration curve, obtained in presence of the tested compound demonstrates a typical competitive antagonism at the TP vascular receptor [48, 138].

Finally, we also tested the ability of the different compounds to compete for the specific binding site labelled by the TP antagonist [<sup>3</sup>H]SQ29,548 in human embryonic kidney cells (HEK293) transiently transfected with wild-type human TP $\alpha$  receptor [153]. The functional activity of the TP $\alpha$  receptor was also assessed by measuring the accumulation of total inositol phosphate (IPs) in transfected HEK293 cells labelled with myo-[2-<sup>3</sup>H]inositol [143, 154-157].

## CONFLICT OF INTEREST

Authors declare no conflict of interest

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## FIGURES

**Figure 1.** SAR for a series of dual TP $\alpha$ -antagonist / COX-2 inhibitors