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 Therefore, the increasing CV hazard led to the withdrawn from the market of rofecoxib or Vioxx by Merck and Valdecoxib (Bextra) by Pfizer [6] with significant economic and image loss for the companies [7].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.

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, gennrico.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 University of Turin, Italy (e-mail: 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 TPα and TPβ. It is mainly coupled to Gq protein [9] but also to Gi1,2/3 and Gs, [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].

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), recenrifuged 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 assay 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

![Diagram of thromboxane A2 (TXA2) and prostacyclin (PGI2) production](image-url)
centrifuged at 5000g for 5min at 4°C. In the supernatant obtained after centrifugation, the TXB$_2$ 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. PGE$_2$ 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 IC$_{50}$ of 0.0046 µM, while compound 1, 2 and 3 showed similar IC$_{50}$ 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 (IC$_{50}$ = 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 posses 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 TP$_x$ receptor expressed in recombinant cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>COX-2 IC$_{50}$ ± %CV [µM]</th>
<th>Aggregation IC$_{50}$ ± %CV [µM]</th>
<th>TP$\alpha$ Ki ± %CV [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diclofenac</td>
<td>0.0015 ± 40</td>
<td>16.00 ± 17</td>
<td>42 ± 45</td>
</tr>
<tr>
<td>Lumiracoxib</td>
<td>0.0046 ± 35</td>
<td>21.3 ± 10</td>
<td>73.5 ± 54</td>
</tr>
<tr>
<td>Compound 1</td>
<td>0.025 ± 95</td>
<td>10% inhib. (60 µm)</td>
<td>Not tested</td>
</tr>
<tr>
<td>Compound 2</td>
<td>0.016 ± 44</td>
<td>12.8 ± 5</td>
<td>61 ± 45</td>
</tr>
<tr>
<td>Compound 3</td>
<td>0.025 ± 54</td>
<td>26.7 ± 15</td>
<td>Not tested</td>
</tr>
<tr>
<td>Compound 4</td>
<td>0.65 ± 91</td>
<td>3.37 ± 16</td>
<td>1.4 ± 20</td>
</tr>
</tbody>
</table>

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-methansulfonamide. 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 TXB$_2$ production, a stable metabolite of TXA$_2$, 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 posses 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 COXIB inhibitor (IC$_{50}$ TP/COX-2 = 5.2 versus IC$_{50}$ 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 PGE$_2$ in isolated human monocytes. Data are expressed as percent inhibition of PGE$_2$ 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 TXB$_2$ (metabolite of TXA$_2$) from washed platelets during clotting. Data are expressed as percent inhibition of TXB$_2$ release vs. untreated controls. Error bars represent mean ± SE of at least three independent experiments performed in duplicate.

TABLE I

COX-2 INHIBITORY ACTIVITY, THROMBOXANE A$_2$ ANTAGOISM AND TP$_x$ RECEPTOR AFFINITY FOR DICLOFENAC, LUMIRACOXIB AND SYNTHESIZED COMPOUNDS 1-4

-12
t-11
t-10
t-9
t-8
t-7
t-6
t-5
t-4

-12
t-11
t-10
t-9
t-8
t-7
t-6
t-5
t-4

0
10
20
30
40
50
60
70
80
90
100

Diclofenac n=5
Lumiracoxib n=6
compound 2 n=8
compound 4 n=5

% inhibition of PGE$_2$ production

log[µM]

compounds 1-4 is reported in [16].
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 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.

ACKNOWLEDGMENTS

This work was supported in part by a grant from Regione Lombardia (Progetto Ex-ASTIL ID 16755-Rif. SAL-02 to G.E.R.) and Fondazione Banca Monte di Lombardia (Funding programme 2010 to G.E.R.). This work was partially supported by a MIUR Grant (PRIN 2009) to RF.

REFERENCES