Pharmacological Research 103 (2016) 132–143

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

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

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ARTICLE INFO

Article history:
Received 30 October 2015
Accepted 15 November 2015
Available online 24 November 2015

Keywords:
Cyclooxygenase
Thromboxane
Inflammation
NSAID
Coxib
Multitarget drugs

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 A_2 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 (pA_2) 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 A_2 (TXA_2) 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 IC_{50} 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 pA_2 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.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) provide analgesic and anti-inflammatory properties by virtue of cyclooxygenase (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) E_2 production [3]. Celecoxib (Celebrex) and rofecoxib (Vioxx) were the first COX-2

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; TXA_2, thromboxane A_2; TP, thromboxane prostanoid receptor.

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http://dx.doi.org/10.1016/j.phrs.2015.11.012
1043-6618/

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 TPα 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

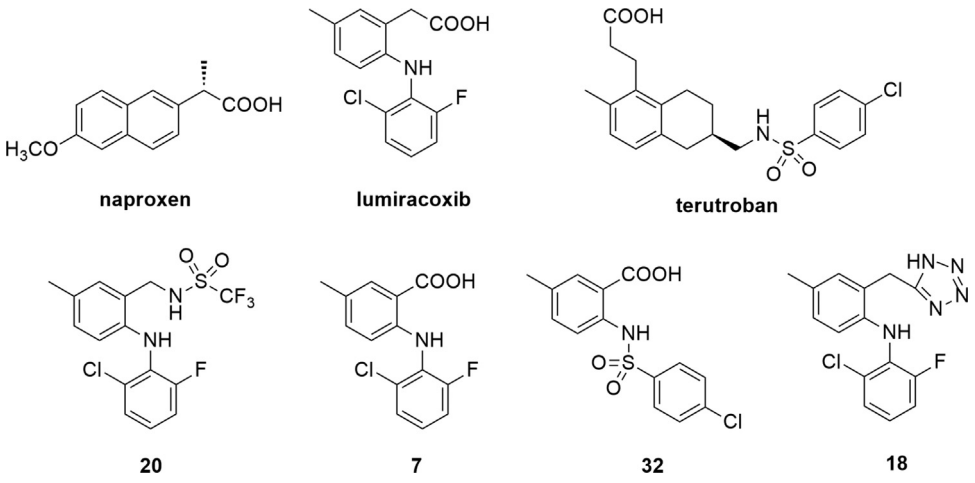

Fig. 1. Chemical structures of reference compounds naproxen, lumiracoxib and terutroban, as well as of the newly synthetized compounds **7, 18**, **20,** and **32**.