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**THE POTENTIAL THERAPEUTIC ROLE OF MONTELUKAST AND NEW
HYBRID AGENTS, TXA₂ ANTAGONIST-COX-2 INHIBITORS IN
CARDIOVASCULAR EVENTS**

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I- INTRODUCTION

1.1 Arachidonic acid cascade

Arachidonic acid (AA) is a polyunsaturated fatty acid (AA, 5, 8, 11, 14-eicosatetraenoic acid) present in cell membrane phospholipids and released upon activation of phospholipase A2. AA can be re-incorporated in the membranes or distributed outside the cell and it is a substrate for cyclooxygenases (COXs) and lipoxygenases (LOXs).

COXs are responsible for the conversion of AA into cyclic endoperoxide intermediates prostaglandin G2 and H2 (PGG₂-PGH₂), which are the precursor for the biosynthesis of the following prostanoids: other PGs (PGD₂, PGE₂, PGF_{2α}), prostacyclin (PGI₂) and thromboxane (TXA₂) biosynthesis (Fig 1). Prostanoids exert diverse functions as: pain response, maturation for ovulation and fertilization, fever generation, inhibition of gastric acid secretion, bone resorption (PGE₂), modulation of platelets aggregation (PGI₂ and TXA₂), alteration of smooth muscle tone (PGE₂, PGI₂ and TXA₂), leukocytes infiltration at the inflammatory site (PGD₂), uterine smooth muscle contraction (PGF_{2α}) [Funk CD., 1993].

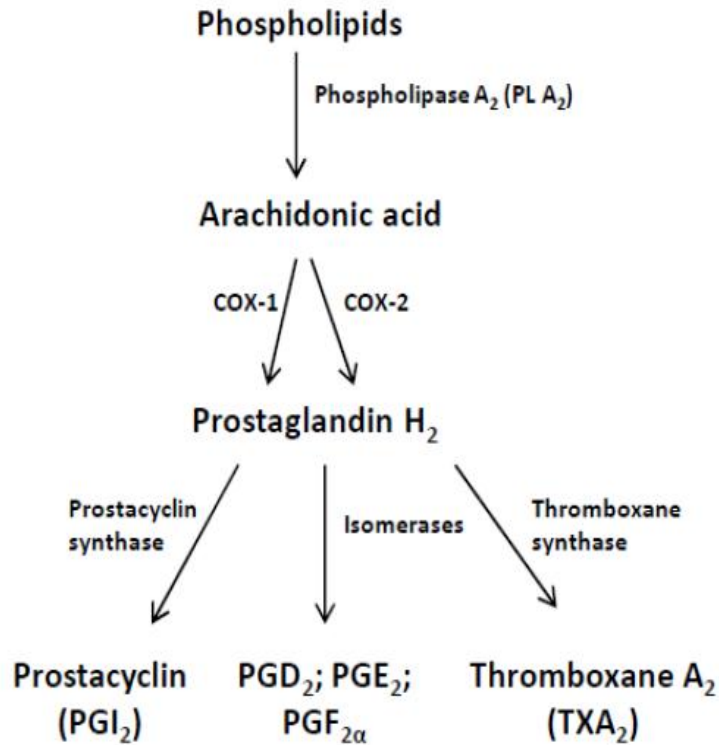


Fig 1. The arachidonic acid cascade [Hoxha M. et al., 2014]

LOX are classified according to the number of the carbon subjected to dioxygenation as well as their stereoselectivity that can be either “S” or “R”. The main human LOXs are 5-LOX, 12-LOX and 15-LOX. 5-LOX, in particular, transform the AA in 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by oxygenation at carbon 5, which, in turn, can bring to further production of leukotrienes (LTs) (Fig 2). 5-LO-activating protein (FLAP) should be co-localized with 5-LOX at the nuclear envelope for the LTs synthesis [Evans JF. et al., 2008]. LTA₄ is a precursor of the production of LTs, a product of removal of hydrogen at carbon 10 from 5-HpETE [Shimizu T. et

al., 1984; Rådmark O. and Samuelsson B., 2009]. LTA_4 can follow two different pathways, as it can be a substrate for LTA_4 hydrolase leading to the production of LTB_4 [Haegström JZ., 2004] or be a substrate for LTC_4 synthase (LTC_4S) leading to the production of LTC_4 , product of conjugation of LTA_4 with glutathione [Austen KF., 2007]. LTD_4 is formed by metabolism of LTC_4 by glutamyl transpeptidase, and subsequently a serum dipeptidase can bring to the production of LTE_4 from LTD_4 . LTs are classified in two groups: the first represented by LTB_4 , and the second by peptide leukotrienes (cysteinyl-LTs) such as LTC_4 , LTD_4 , and LTE_4 , based on the fact that they display a cysteinyl group at carbon 6.

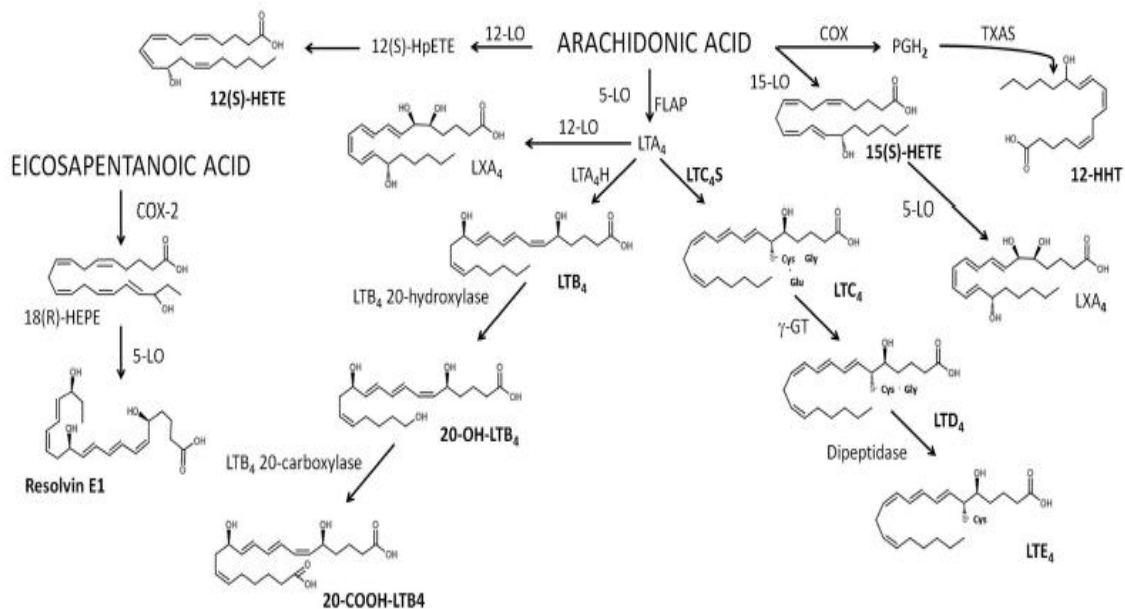


Fig 2. Biosynthesis and chemical structures of endogenous leukotriene receptor agonists, derived from arachidonic acid. [Bäck M. et al., 2007]

1.2 NSAIDs: Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs are a class of drugs that display anti-inflammatory, analgesic and antipyretic activity due to the inhibition of the COX enzymes. There are two isoforms of COX, that vary by two aminoacid at their COXs catalytic sites [Vane JR. et al., 1998]; COX-1 the constitutive enzyme, recognized also as the housekeeper enzyme is believed to be responsible for the haemostatic integrity and gastric cytoprotection and the COX-2, the inducible enzyme, which is also constitutively expressed in some types of cells, like, kidney and endothelial cells [Smith W.L. et al., 2000, Morita I., 2002] is mainly expressed in response to inflammatory stimuli. The development and commercialization of Aspirin, the gold standard NSAID, which is an irreversible inhibitor of both isoforms of COX, disclosed an important side effect that belongs to NSAIDs, i.e. the gastric-lesivity, like, bleeding and ulcers [Laine L. et al., 2008]. These effects are mainly related to the inhibition of COX-1 enzyme, in particular to the inhibition of the local synthesis of the gastric mucosa cytoprotective PGE₂, as well as to the NSAID storage inside the intestinal enterocytes [Matsui H. et al., 2011] and to the local lesion caused by the acidic properties of the drugs.

1.3 COXIBs: COX-2 selective inhibitors

The necessity to minimize the gastrointestinal (GI) toxicity induced by the conventional NSAIDs and the discovery of a second isoform of COX lead to the quick development of a new class of drugs. The '90s were very significant years for the development of a second generation of NSAIDs, which were named as COX-2 selective inhibitors or COXIBs. A new alternative for patients having osteoarthritis and rheumatoid arthritis was proposed, so Celecoxib (Celebrex) and Rofecoxib (Vioxx) were the first COXIBs used in therapy [FitzGerald GA. and Patrono C., 2001]. The enthusiasm that accompanied the COXIB development, did not last longer. In fact, in 2004 VIOXX was withdrawn from the market for increasing the risk of cardiovascular (CV) events, such as myocardial infarction (MI), and stroke. In a short time, this increase in CV adverse effects was related not only to COXIBs but also to the use of classical unselective NSAIDs [Hippisley-Cox J. et al., 2005][Kearney PM. et al., 2006][Warner TD. and Mitchell JA., 2008][Bresalier R.S. et al., 2005][Solomon S.D. et al., 2005], which led to a re-evaluation of the whole class of NSAIDs. Eventhough COXIBs reached their goal in reducing the GI side effects caused by conventional NSAIDs, as evidenced by several clinical studies [Bombardier C. et al., 2000; Silverstein FE. et al., 2000], they led to inevitable CV warnings. The withdrawn of

VIOXX, based on the Adenomatous Polyp Prevention on Vioxx (APPROVe) trial [Bresalier R.S. et al., 2005] that highlighted the severe CV effects [Grosser T. et al., 2006], was followed by the withdrawal of Valdecoxib (Bextra), and moreover, other COXIBs like etoricoxib never reached the FDA for further approval.

In an effort to answer the question, why these compounds gave rise to CV side effects, several hypothesis were proposed, one of which, known as the imbalance theory, correlated the COXIBs with the decrement of the PGI₂ synthesis, possessing antiaggregatory properties, while maintaining unaltered TXA₂ level, a potent platelet activator [Fitzgerald GA., 2004]. The reason behind this hypothesis was related to a study conducted in patients taking celecoxib and rofecoxib [Mc Adam B.F. et al., 1999] that presented decreased urinary excretion of the main PGI₂ metabolite, 2,3 – dinor 6-keto PGF_{1α}, and on the other hand, unaltered level of the TXB₂, the urinary metabolite of TXA₂ [Catella-Lawson F. et al., 1999]. Other potential mechanism can also be related to the shunt of AA towards the 5-LOX pathway, leading to the production of specific mediators concerned to CV events, or even the increase in LDL, as observed in patients treated with rofecoxib, taking also into consideration the fact that the effect and toxicity depend on the individual response.

Later on, it was suggested that the CV risk was not only an issue of COXIB alone, but also of traditional NSAIDs. The Arthritis Research and Gastrointestinal Event Trial (TARGET study) found that the CV risk, such as stroke, MI as well as CV death, was quite the same between patients taking a COXIB, like, lumiracoxib (0.65%) or a classical NSAID (0-55%) [Schnitzer TJ. et al., 2004]. Moreover, both NSAIDs and COXIBs can display side effects on kidney, bringing to fluid retention and consequently to increased blood pressure [Hao CM. et al., 2008] or hazard could depend upon differences in the levels of lipid peroxides or in the supply of AA substrate between platelets and endothelial cells [Mitchell JA. et al., 2006]. Another report suggests that the CV toxicity of rofecoxib could be due to its intrinsic physical-chemical properties and primary metabolism that increase Low Density Lipoproteins and membrane lipids oxidation thus promoting formation of isoprostanes, a characteristic feature of atherogenesis [Mason RP. et al., 2007 ; Shapiro MS., 2009]. As a result, the CV events may not be ascribed to a class of drugs, but rather to 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 [Rovati GE. et al., 2010]. In order to overcome this problem, several

COXIBs combined with moieties that release nitric oxide, an inhibitor of platelet aggregation, were developed [Wallace JL. et al., 2009]. But we believe this is not the best approach to solve these concerns, due to isoprostanes (like 8-iso-PGF_{2α} or 8-iso-PGE₂) and TXA₂ implication in CV events [Rovati GE. et al., 2010]. 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 TXA₂ and, finally act through the TXA₂ prostanoid (TP) receptor [Audoly LP et al., 2000].

The Selg et. al study marked a turning point in the NSAIDs saga. In this study it was found that diclofenac and lumiracoxib displayed a new mechanism of action, the competitive antagonism of the TXA₂ receptor (TP) [Selg E. et al., 2007], initially shown using guinea-pig lung strips, and later on, with receptor binding experiments in human platelets. But, the main issue that spoils this finding was the balance between two activities; the low potency as TP receptor antagonist does not permit to exploit this characteristic in therapy [Selg E. et al., 2007].

1.4 Cysteinyl Leukotrienes and their receptors

Cysteinyl-LTs (LTC_4 , LTD_4 , and LTE_4) are important mediators of asthma and have a significant role in the earliest stages of bronchoconstriction, as well as in the late chronic inflammatory component [Nicosia S. et al., 2001]. They increase the endothelial cell permeability [Capra V. et al., 2015] and are also involved in the leukocyte recruitment and activation as well as other inflammatory situations, as immune disorders, CV diseases, several cancers, atopic dermatitis, or even GI and hepatic problem as cholestasis, portal hypertension [Capra V., 2004][Rovati GE. and Capra V., 2007; Capra V., 2007]. In addition cysteinyl-LTs are produced in the liver during reperfusion and may enhance cytotoxicity [Takamatsu Y. et al., 2004].

LTs receptors belong to the rhodopsin family of the G-protein-coupled receptors (GPCR) and are classified in BLT and CysLT receptors based on the ligand, leukotriene B_4 (LTB_4) and cysteinyl-LTs [Bačk M. et al., 2011], respectively. Two subtypes of the human Cys-LT receptor have been cloned known as CysLT₁R and CysLT₂R, sharing 38% amino acid identity [Capra V., 2007][Bačk M. et al., 2011].

New recent data suggest the existence of supplementary leukotriene receptor subtypes, which respond to both, cysteinyl-LTs and uracil nucleosides [Rovati GE. and Capra V. 2007; Austen KF. et al., 2009; Back M. et al., 2014]. It is also hypothesized that CysLTRs can form homo or heterodimers [Capra V. et al., 2005; Jiang Y. et al., 2007], or that can cross talk with other receptors of the membrane [Capra V. et al., 2005][Rovati GE. and Capra V., 2007]. CysLTRs may also be localized at the nuclear level, influencing cell function and signaling [Bandeira-Melo C. et al., (2002b)], therefore be a potential target for a new pharmacological treatment [Dalrymple MB. et al., 2008; Kenakin T., 2002].

There is a wide distribution in the human tissues of the CysLTRs, particularly CysLT₁ in cells with a significant implication in asthma, such as smooth muscle cells along the respiratory tract [Figueroa DJ. et al., 2001], monocytes/macrophages, neutrophils, or in tumors like colorectal carcinomas, but also in the human mast cells of non asthmatic patients [Ohd JF. et al., 2003; Yoshisue H. et al., 2007].

Whereas CysLT₂ receptor is widely expressed in the brain like thalamus, hypothalamus, and medulla [Heise CE. et al., 2000]; in heart, adrenals, basophils [Gauvreau G.M. et al., 2005;

Rovati GE. and Capra V., 2007]. Interestingly, CysLT₂ receptors are not expressed in cells containing high levels of CysLT₁ receptor, like both undifferentiated or differentiated promyelocytic HL-60 and promonocytic U937 cells [Murray J. et al., 2003; Back M. et al., 2011]. Cys-LTRs, as well as cysteinyl-LTs, can be also expressed and synthesized, respectively, at the atherosclerotic site.

1.5 Cysteinyl-leukotriene receptor antagonists, montelukast

Asthma is a very common based inflammatory pathology, in both adults and children, characterized by airway reversible alterations. The pharmacological control of patients having asthma is based on the administration of a bronchodilator, which should be combined also with an anti-inflammatory drug, like corticosteroids, in order to prevent chronic inflammation. Based on the fact that cysteinyl-LTs play a crucial role in asthma, the CysLTR antagonists drugs (LTRAs) are used since the late 1990s as an alternative therapy in patients having asthma, and later on, also in other inflammatory conditions as chronic obstructive pulmonary disease (COPD) or allergic rhinitis and urticaria [Capra V. et al., 2006]. At the same time, few studies have reported that some patients taking a LTRA do not respond to the treatment [Drazen JM. et al., 1999; Israel E., 2005], which suggest that in some asthmatics, the symptoms are due to other mediators than cysteinyl-LTs, therefore indentifying a particular phenotype resistant to LTRAs [Asano K. et al., 2002; Tantisira KG. et al., 2009; Langmack EL. and Martin RJ., 2010] is of particular interest.

Several studies have indicated that LTRA can also decrease the intimal hyperplasia following vascular injury, as well as can prevent the atherosclerosis progression, and have a protective role after cerebral ischemia [Riccioni G. et al., 2008][Bäck M., 2009][Capra V. et al., 2013].

Patients having COPD are characterized by chronic pulmonary inflammation which is different than in asthmatic patients. Evidences have shown increased levels of PGE₂ in COPD, and increased levels of LTE₄ in asthma [Montuschi P. et al., 2003; Gaki E. et al., 2007]. However, zafirlukast seems to improve the lung function in smoker with COPD [Cazzola M. et al., 2000; Nannini LJ. and Flores DM., 2003], but, on the other hand, due to relevant neutrophilic inflammation in COPD, LTB₄ may have a role in this disease, hence, taking a BLT antagonist or 5-LO/FLAP inhibitor can be a better pharmacological approach in these patients.

Allergic rhinitis (AR) is one of the diseases that may be target by LTRAs. Cysteinyl-LTs are produced in some of the cells responsible for AR, and CysLTRs are also involved in its pathology [Peters-Golden M. and Henderson WR Jr., 2005; Peters-Golden M. et al., 2006]. At the same time some studies indicate a possible role of cysteinyl-LTs and their receptors in chronic rhinosinusitis [Arango P. et al., 2002; Higashi N. et al., 2004; Sousa AR. et al., 2002], so LTRA can be a potential therapeutic alternative in this disease.

Atopic dermatitis (AD) and chronic urticaria are two pathologies that can as well be treated with LTRAs. Increased levels of urinary LTE₄ were detected in patients with AD [Adamek-Guzik T. et al., 2002; Øymar K. and Aksnes L., 2005], and LTRAs taken at the same doses as for asthma treatment have shown a symptomatology improvement, in both adult and children with different stages of AD, even though some controversial studies have been published [Veien NK. et al., 2005, Capra V. et al., 2006; Leonardi S. et al., 2007; Broshtilova V. and Gantcheva M., 2010].

Other diseases like cystic fibrosis are characterized by increased levels of cysteinyl-LTs due to the inflammatory process responsible of lung damage [Reid DW. et al., 2007], and LTRAs have shown to improve cystic fibrosis especially in the long-term [Schmitt-Grohe' S. et al., 2007].

Several antagonists of the CysLTRs, i.e. pranlukast, zafirlukast, montelukast, and pobilukast, were designed as selective CysLT₁R antagonists [Brink C. et al., 2003; Evans JF., 2003; Capra V., 2004]. At variance, BAY u9773, differs from the other compounds by acting as an antagonist of both CysLT₁ and CysLT₂ receptors, even if with poor potency and selectivity [Labat C. et al.,

1992; Tudhope S.R. et al., 1994]. Generally speaking, the first generation of CysLTR antagonists did not demonstrate sufficient potency for endogenous ligands [Bač k. M, et al., 2011].

Montelukast, a potent antagonist of the CysLT₁R subtype is used in patients having different stages of asthma severity. Furthermore, montelukast, as well as other LTRAs like zafirlukast or pranlukast can be used in AR. Montelukast can be used in children [Bisgaard H. et al., 2009], and to alleviate the symptoms present in AR, like eye and throat symptoms [Grainger J. and Drake-Lee A., 2006; Nayak A. and Langdon RB., 2007; Bousquet J. et al., 2009] as a monotherapy or in association with other antihistamic drugs [Meltzer EO. et al., 2000; Nayak AS. et al., 2002]. Montelukast was approved by the Food and Drug Administration (FDA) in 2003 for the treatment of seasonal AR and in 2005 for the treatment of perennial AR [Bač k. M. et al., 2011].

The Rubeinstein group studied the functional scores of COPD patients taking montelukast, and came to the conclusion that there was an improvement of nocturnal symptoms, (shortness of breath, wheezing, use of other drugs, as inhaled bronchodilators, corticosteroids, the number and

duration of hospitalizations, and emergency visits [Rubinstein I. et al., 2004; Celik P. et al., 2005].

In addition to these uses, montelukast is a choice in patients that do not respond to antihistaminic therapy [Erbagci Z., 2002; Bagenstose SE. et al., 2004].

1.6 Inflammation and cardiovascular events

Inflammation is a physiological reaction, triggered by a number of agents, involving, both blood cells (macrophages, monocytes), and vessel wall (vascular endothelium), characterized by the release of chemical mediators, and associated with the elimination of the invading host after identification. Increase in vascular permeability, leukocyte infiltration (consisting in margination, rolling, adhesion and diapedesis) in the injured district are some of the features of edema.

Initially, prostaglandins, LTs, and other inflammatory mediators such as histamine, serotonin, nitrogen oxide, and bradykinin are released, while successively the migration of leukocytes and intensification of the inflammatory response dominates, and eventually failure of organ functions and tissues repair induced by proliferation of connective tissue, particularly fibroblasts. IL-1 and TNF are two main inflammatory mediators, which enhance the synthesis of several proteins and the activation of neutrophils, cytokines, COX, LOX, B and T lymphocytes.

Inflammation is associated to several diseases, like atherosclerosis or asthma [Inglesson E. et al., 2012], highlighting the fact that CV events are among the most common cause of death worldwide. Endogenous ligands can be present at the inflammatory and myocardial damage site.

Even though several NSAIDs have been designed and commercialized, all of them can increase the CV risk. The development of COXIBs seemed to be an exalting invention in the NSAIDs field, but the triumph did not last longer, as Vioxx was withdrawn and followed by other frustrations due to CV toxicity associated to COXIB and traditional NSAIDs, including paracetamol [Ritter JM. et al., 2009].

At the same time, other AA metabolite, such as LTs, are associated to inflammation [Samuelsson B., 1983] and can enhance the CV risk.

LTs are part of the inflammatory process of several pathologies including CV diseases characterized by the increase of capillary permeability, vasoconstrictor properties, reduction of coronary blood flow [Capra V. et al., 2007, Brink C. et al., 2003]. MI, stroke, atherosclerosis, aortic aneurysms are some of the CV events characterized by generation of LTs from 5-LO pathway, a substantial part of vascular inflammation and disease progression [Lötzner K. et al., 2005; Bačk M., 2009, Bačk M. et al., 2007]. Several controversial studies claim the role of cysteinyl-LTs in the amplification of myocardial ischemic injury [Toki Y. et al., 1988; Hock CE.

et al., 1992], eventhough challenged by others studies, which claim their marginal or no role in ischemic and myocardial dysfunction [Ito T. et al., 1989; Hahn RA. et al., 1992]. Interestingly both CysLT receptors are found in injured human arteries [Allen S. et al., 1998; Spanbroek R. et al., 2003]. Given their association with the inflammatory onset and amplification, LTs synthesis inhibitors or LTRAs can be consider as potential approach for CV diseases. In addition, *in vivo* mouse data's revealed the effect of montelukast in reducing the vascular reactive oxygen species production, improving endothelial cells function [Mueller CF. et al., 2008], inhibiting atherosclerotic damaged area and intimal hyperplasia [Kaetsu Y. et al., 2007; Jawien J. et al., 2008], improving atherosclerotic plaque generation [Mueller CF. et al., 2008]. Moreover, Allayee group revealed an important reduction of C-reactive protein in asthmatic patients treated with montelukast [Allayee H. et al., 2007]. Montelukast can inhibit the MCP-1, and is responsible for the antiatherogenic effects *in vivo* in the rabbit carotid injury model [Ge S. et al., 2009], while it has been demonstrated to be useful in preventing hepatic ischemia-reperfusion injury in rats [Daglar G. et al., 2009].

Whereas, CysLT₂R are principally present in brain and selective CysLT₂R antagonists can be used to reduce the blood permeability in the brain [Capra V. et al., 2015; Di Gennaro A. et al.,

2004], hence cerebral ischemia [Biber N. et al., 2009; Yu GL. et al., 2005a,b; Qian XD. et al., 2006]. In a different perspective the overexpression of CysLT₂R in mice, can intensify the myocardial ischemia-reperfusion damage [Hui Y. et al., 2004]. Yet, BAY U9773, a CysLT₁/CysLT₂ receptor antagonist is more effective than other CysLT₁R antagonist in preventing the changes in brain permeability [Di Gennaro A. et al., 2004]. Allen group sustained that cysteinyl-LTs enhanced human atherosclerotic coronary arteries contractions, but at the same time arteries lacking the atherosclerosis plaque were unresponsive [Allen S. et al., 1998]. These solid data indicate that CysLT₂R is an essential pharmacological target to face the CV events.

Therefore, we are currently exploring two potential alternatives to approach and reduce the CV risk in inflammatory diseases:

- 1) to use a LTRAs, which has already been demonstrated to prevent the inflammatory response present in pathologies like asthma, AR, AD or chronic urticaria, and that might be considered as a new therapeutic approach in several other inflammatory diseases including carotid and coronary atherosclerosis to improve their CV outcome;

2) to use a multitarget drug possessing a dual COXIB and TP antagonist activity within a single molecule to reduce inflammation, and to inhibit TP receptor activation, including that from isoprostanes, which are known to be generated during atherogenesis and that cannot be prevented using a traditional NSAID or a COXIB.

II- AIM OF THE STUDY

Approaching the CV issues is a delicate matter, and the aim of our study was to propose alternative solutions targeting the specific AA metabolites for reducing the CV risk in patients suffering from inflammatory disease, like rheumatoid arthritis or asthma.

The first aim of our study was the design and the pharmacological characterization of new compounds possessing the anti-inflammatory and TP antagonist activity within a single compound [Bertinaria M. et al., 2012]. New molecules were synthesized at the University of Turin, starting from a COXIB, i.e. lumiracoxib, modifying its structure to increase TP antagonist potency and introducing some functional groups of the TP antagonist terutroban, in order to combine these two activities in a multitarget drug. Lumiracoxib and naproxene were used as reference drugs and their anti-aggregating activity was assessed in both human platelets stimulated by U46619, a specific stable agonist of the TP receptor, and in HEK293 cells transfected with the TP α receptor in terms of inhibition of inositol phosphate production. COX-1 and COX-2 activity were studied in washed platelets and human monocytes, respectively. By developing these dual compounds we can speculate not only to give rise to a new generation of

NSAID with reduced GI and CV side-effects, but also to achieve a novelty in terms of preventing the interaction of isoprostanes with the TP receptor.

The second aim of our study was to perform an observational retrospective study to determine whether there is a protective role of the LTRA drug montelukast in the prevention of a major CV events (i.e. stroke or MI) in subjects exposed to the drug for a different pathology. As discussed above, montelukast is a pharmacological alternative for patients suffering from asthma or AR.

Asthmatic patients exposed or non exposed to montelukast participated in the study, and they were further classified in subject with or without prior CV events such as ischemic stroke (IS) or MI. Data were collected regarding patients details, as well as the drugs use, age, income, residence, education level, and were analysed to determine whether or not there was a correlation between montelukast and the primary or secondary prevention of a major CV event.

III- MATERIALS AND METHODS

PROJECT I METHODS

3.1 Materials

U46619 ([1R-[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), and SQ29,548 ([1S-[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) were purchased from Cayman Chemical (Ann Arbor, MI).

Ultima Gold and myo-[2-³H]inositol were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA).

Lowry dye-binding protein reagents and anion exchange resin AG 1X-8 (formate form, 200–400 mesh) were purchased from Bio-Rad (Hercules, CA).

Antibiotics, Lipofectamine 2000, animal serum, molecular biology reagents and Opti-MEM I were bought from Invitrogen (Carlsbad, CA).

Inositol-free Dulbecco's modified Eagle's medium (DMEM) was acquired from ICN Pharmaceuticals Inc. (Costa Mesa, CA).

Highest purity reagents were supplied by Sigma-Aldrich (St. Louis, MO).

DMEM MP Biomedicals, was procured from Santa Ana, CA.

3.2 Human platelets isolation and platelet aggregation

Platelets were isolated from human blood of healthy volunteers (18-60 years old) lacking any CV disease, and not taking medications for at least 72 h. An anticoagulant, respectively, CPD solution (Citrate Phosphate Dextrose; 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 added to the blood. Platelet-rich plasma (PRP), was obtained after centrifugation of buffy coat treated with 100 μ M of acetylsalicylic acid at 280 g for 15 min at room temperature, and immediately afterwards at 650 g for 10 min at room temperature. 8 ml of washing buffer (mM composition: citric acid monohydrate 39, glucose monohydrate 5, KCl 5, CaCl₂ 2, MgCl₂ x 6H₂O 1, NaCl 103, pH 6.5), was used to suspend the platelet pellet. Furthermore the platelet pellet was centrifuged at 650 g for 15 min at room temperature and resuspended in 15 ml of HBSS (Hank's Balance Salt Solution: CaCl₂·2H₂O 0.185 g/L; KCl 0.40 g/L; KH₂PO₄ 0.06 g/L; MgCl₂·6H₂O 0.10 g/L; MgSO₄·7H₂O 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). Platelet aggregation was evaluated using a Chrono-Log aggregometer (Mascia Brunelli, Milano, Italy), after settling the washed platelets concentration at almost 2 x10⁸ cell ml⁻¹ and applying the Born turbidimetric assay [Born GV. and

Cross MJ., 1963] at 37°C in a 0.5 mL sample. The platelets were incubated with drug or vehicle (DMSO, maximum 0.2 %, v:v) for 5 min at 37°C, and induced by the specific TP receptor agonist, U46619 (0.1-0.5 µM) under continuous stirring for 6 minutes. Due to different platelet response, the anti-aggregating activity of each compound was compared with its respective control aggregation.

3.3 Isolation of lympho-monocytes, COX-2 expression and study of COX-2 inhibitory activity

Buffy coat obtained from healthy volunteers were used as a source of isolation of lympho-monocytes, by a Ficoll-Paque gradient density centrifugation (400 g for 30 min at 10°C), following dilution in a saline solution (NaCl 0.9%) and ricentrifugation (280 g for 15 min at 10°C). After the remaining suspended platelets were removed, in order to eliminate the remaining erythrocytes the lysis buffer (NaCl 0.2% weight/volume, w/v) was added and balanced with an equal volume of equilibrating solution (NaCl 1.6% + saccarose 0.2%, w/v). Lympho-monocytes were resuspended in HBSS and COX-2 inhibition assays were carried out. In order to avoid potential binding of the respective compounds with plasma proteins, COXIB activity was assessed in isolated human lympho-monocytes after treatment with 10 µg/mL of acetylsalicylic acid. Lipopolysaccharide (LPS) (from *E. coli*, serotype 0111:B4; 10 µg ml⁻¹) was used as a physiological inducer of the expression of COX-2 isoform. The samples were incubated overnight at 37°C and COX-2 activity was assessed through the Prostaglandin E₂ (PGE₂) production by mass spectrometry or enzyme immunoassay (PGE₂ EIA kit, *Cayman Chemical*). When the incubation phase was completed, the samples were centrifuged at 12000 g

for 2 minutes. The evaluation of PGE₂ content was carried out using the isolated supernatant
freezed at -20°C. IC₅₀ of the compounds was calculated versus the maximal PGE₂ production.

3.4 COX-1 inhibitory activity

COX-1 inhibitory activity of the tested compounds was assessed in washed human platelets suspension. Cells concentration was settled at 2×10^8 cells ml^{-1} . Increasing concentrations of the tested compounds, were used to treat platelets for 15 min, and thereafter incubated at 37°C in a Dubnoff bath. TXB_2 was used as a marker of activity of COX-1, and its production by platelet degranulation was enhanced by calcium ionophore (A23187 $2\mu\text{M}$). The samples were centrifuged at 5000g for 5min at 4°C , following an incubation period of 10 min at 37°C . TXB_2 production was assessed in the supernatant through immunoenzymatic assay of TXB_2 production and mass spectroscopy.

3.5 Mass spectrometry

Liquid chromatography-tandem mass spectrometry was used to determine PGE₂ and TxB₂ concentrations, making use of the deuterated internal standards [d4] PGE₂ and [d4] TxB₂.

Internal standards was added to the samples and injected in a liquid chromatography Agilent 1100 (Agilent Technologies, Santa Clara, CA). A reverse phase column (Synergi 4 µm Hydro-RP, 150x2 mm; Phenomenex, Torrance, CA) was used and the column was eluted with a gradient, made of 25 to 100% of solvent B (Methanol:Acetonitrile, 65:35) for 10 min and of Solvent A, consisting in 0.05% acetic acid pH 6 with ammonia. The current transition m/z 351>271 for PGE₂, m/z 355>275 for [d4] PGE₂, m/z 369>169 for TXB₂ and m/z 373>173 for [d4] TXB₂ were monitored after infusion of the effluent in an API4000 triple quadrupole operated in negative ion mode. Standard curves obtained from different standards (Cayman Chemical, Ann Arbor, MI) were used for quantization.

3.6 Culture and transfection of HEK293 cells

Human embryonic kidney cell line (HEK293) cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% of fetal bovine serum (FBS), 2 mM glutamine, 50 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, and 20 mM HEPES buffer, pH 7.4, at 37°C in a humidified atmosphere of 95% air and 5% CO₂. 12-well dishes, precoated with poly-D-lysine, were used to plate the cells, to ensure that at the time of transfection the confluence will be around 50–60%. HEK 293 cells were transfected with the TP α WT receptor, using the transfectant agent, Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, in an Opti-MEM I Medium with a 2:1 ratio, which was added to the cells after 20 min incubation at room temperature. Lowry dye binding procedure was used to confirm equal protein content for each respective analysis.

3.7 Total inositol phosphate determination in HEK293 cells

HEK293 cells transfected with the TP α WT receptor were labeled the day prior to the assay, with 0.5-1 μ Ci/ml of myo-[2- 3 H]inositol (PerkinElmer Life and Analytical Sciences, Boston, MA) for 24 h in DMEM (MP Biomedicals, Santa Ana, CA), free of inositol and serum, containing 20 mM HEPES buffer pH 7.4, 0.5% (w:v) Albumax, 2 mM glutamine, and 100U/ml penicillin-streptomycin. 48 h after transfection with the TP α WT receptor, its functional activity was evaluated through a column ion exchange chromatography by total labeled inositol phosphates production. 25 mM of LiCl were used to incubate the cells for 10 min at the day of the assay, accompanied with a pretreatment for 30 min at 37°C with the antagonist, and then stimulated with the specific agonist of TP receptor, compound U46619 0.1 μ M. Cells were lysed with 10 mM of formic acid for 30 min at 4°C, stopping the reaction and transferring the acidic phase in 5 mM NH $_4$ OH pH 8-9. Extraction of the total inositol phosphate production is accomplished by chromatography AG 1X-8 columns formate form, 200–400 mesh size (BioRad Laboratories, Hercules, CA).

3.8 Statistical analysis I

Prism 5 (GraphPad Software Inc., San Diego, CA) was used to calculate the pA_2 values based on the following equations:

$$1) \text{ Agonist response} = \text{Bottom} + (\text{Top}-\text{Bottom}) / (1 + 10^{((\text{LogEC50}-X) * \text{HillSlope})})$$

$$2) \text{ Antagonist response} = \text{Bottom} + (\text{Top}-\text{Bottom}) / (1 + (\text{Antag}/\text{FixedAg})^{\text{HillSlope}})$$

$$3) \text{ Antag} = (10^{\text{LogEC50}}) * (1 + ((10^X) / (10^{(-1 * pA_2)})))^{\text{SchildSlope}}$$

Hill Slope is the slope of the curves, X indicate the Log concentration of the agonist, Bottom is the response when $X = 0$, Top is the response for an infinite concentration of X, EC_{50} is the concentration of the agonist that induce half of the response, FixedAg is the initial fixed concentration of the agonist used to determine the antagonist inhibition curve.

The four-parameter logistic models were used to analyze the concentration-response curves of platelet aggregation and the parameter errors calculated by simultaneous analysis of at least three different independent experiments carried out in duplicate or triplicate, were expressed in percentage coefficient of variation (% CV). A value of $P < 0.05$ was used as the statistical level of significance.

PROJECT II METHODS

3.9 Data collection

Data from 18 years or older asthmatic patients were collected from different allergist/pathologist around the country based on patient medical records, patient register and the drug used.

Asthmatic patients were divided in two samples the first one, patients taking montelukast and the second sample consisting of asthmatic patients not taking montelukast. The sample size was 400

patients for each of two groups. The study was retrospective and the exposure to montelukast was considered to be at least 3 months of continual use of montelukast [Ingelsson E. et.al.,

2012]. Each of the two subjects sample was further classified in patients with or without MI or IS based on their diagnosis according to the International Classification of Diseases (ICD). The

drugs used for CV diseases were retained to be very important factor of monitoring in both two samples, in order to avoid a pre-predisposure of the monitored patients to an increased CV risk

for MI and stroke. From the other hand educational level, categorized as low (elementary and

primary school 0-9th grade), medium (high school 10th-12th grade) and high (university studies)

was evaluated together with the patients economic status, yearly income.

4.0 Statistical analysis II

R statistical software was used for the statistical analysis. Fisher's exact test was used to calculate and assess the difference from the null hypothesis, i.e. whether the treatment with montelukast affects or not the outcome, the MI or IS events. 2 x 2 contingency table was used both for myocardial events and IS events.

	Myocardial infarction/ischemic stroke events	Absence of myocardial infarction/ischemic stroke events	Raw total
Treatment with montelukast	a	b	a+b
No treatment with montelukast	c	d	c+d
Column total	a+c	b+d	a+b+c+d (=n)

n is the grand total and is equal to the sum of all total across columns and rows. Hypergeometric distribution was used to calculate the p value, in order to evaluate if the null hypothesis is accepted or rejected.

The null hypothesis implies that the two variables hence treatment or not with montelukast and presence or absence of MI/IS are independent from each other.

The p-value is calculated as follows:

$$p = \frac{\binom{a+b}{a} \binom{c+d}{c}}{\binom{n}{a+c}} = \frac{(a+b)! (c+d)! (a+c)! (b+d)!}{a! b! c! d! n!}$$

! corresponds to the factorial operator

$\binom{n}{k}$ corresponds to the binomial coefficient

The p value obtained from our data was compared to the significance level fixed at 5%; if the p

value was inferior to 5% the null hypothesis was rejected and vice versa.

IV- RESULTS

PROJECT I RESULTS

A number of compounds were synthesized at the University of Turin, from which the most interesting, resulted to be the compound 7, 18, 20 and 32 (Fig 3). Naproxene and lumiracoxib, a derivative of diclofenac were used as reference compounds, whereas terutroban sulfonyl functional group was inserted in the new synthesized compound, i.e. compound 20.

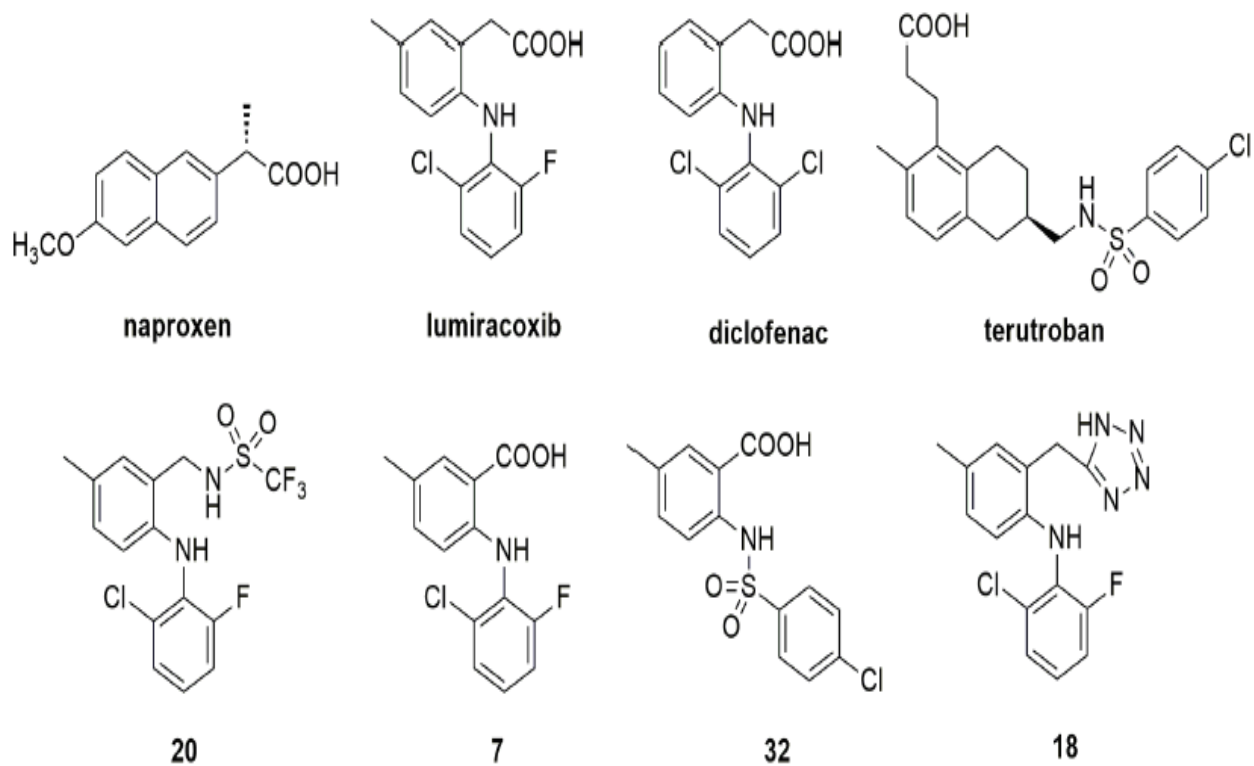


Fig 3. Chemical structures of new synthesized compound, 7, 18, 20, 32 and of terutroban and the reference compounds naproxen, lumiracoxib, diclofenac

4.1 Chemical Synthesis

For the synthesis of compounds **7** and **32** the procedure reported in Figure 4 was used. Chan-Lam coupling was applied for the synthesis of compound **7**, a lumiracoxib analogue, by the reaction of 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-chlorobenzenesulfonyl chloride in the presence of excess Na_2CO_3 in water at 60 – 80 °C. The product was isolated and recrystallized from ethanol.

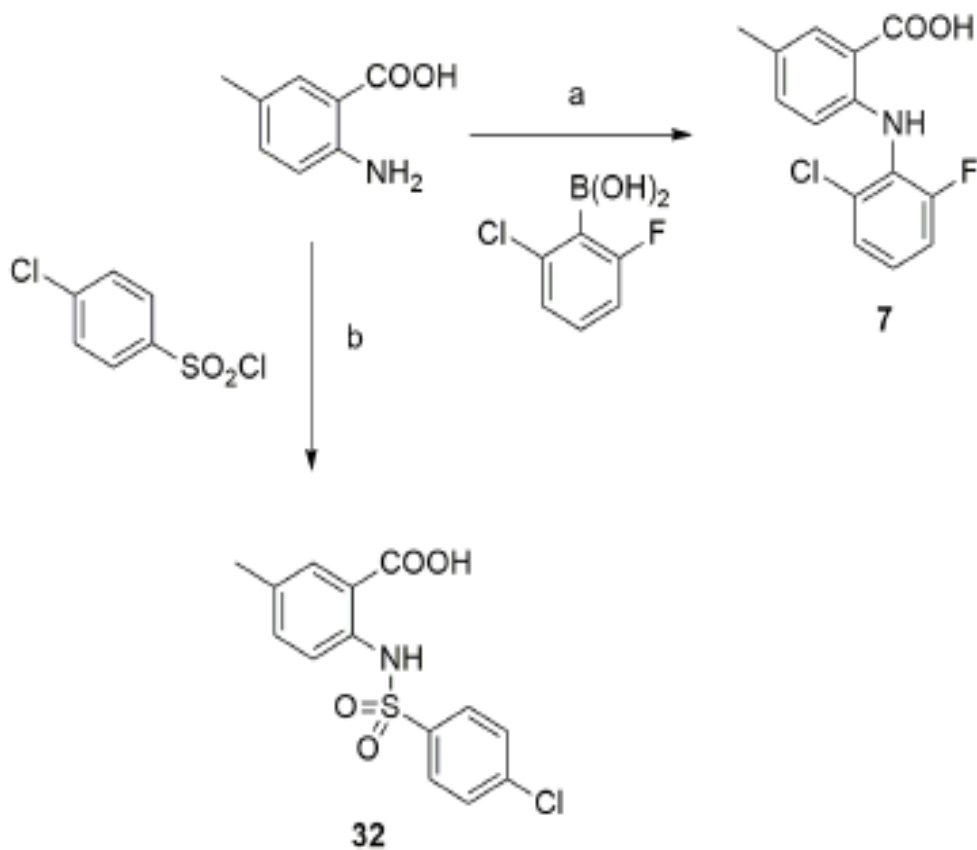


Fig 4. Chemical synthesis of compound 7 and 32

Compound 18, the tetrazole derivative, as well as compound 20 were obtained from a nitrile derivative. The latter reduction with THF complex in refluxing THF and subsequent sulfonylation in basic medium, using trifluoromethanesulfonic anhydride, gave rise to the sulfonamide compound 20 (N- [2-[2-[(2- chloro-6-fluorophenil) ammino]-5- methylphenyl] ethyl]- 1,1,1-trifluoromethansulfonamide) (Fig 5). The precursor nitrile derivative was treated

with excess NaN_3 and NH_4Cl in DMF at 120°C , which brought to the cyclization of the tetrazole

isoster 18 (N-(2-chloro-6-fluorophenyl)-4-methyl-2-(1H-tetrazol-5-ylmethyl)-benzenamine)

(Fig 5).

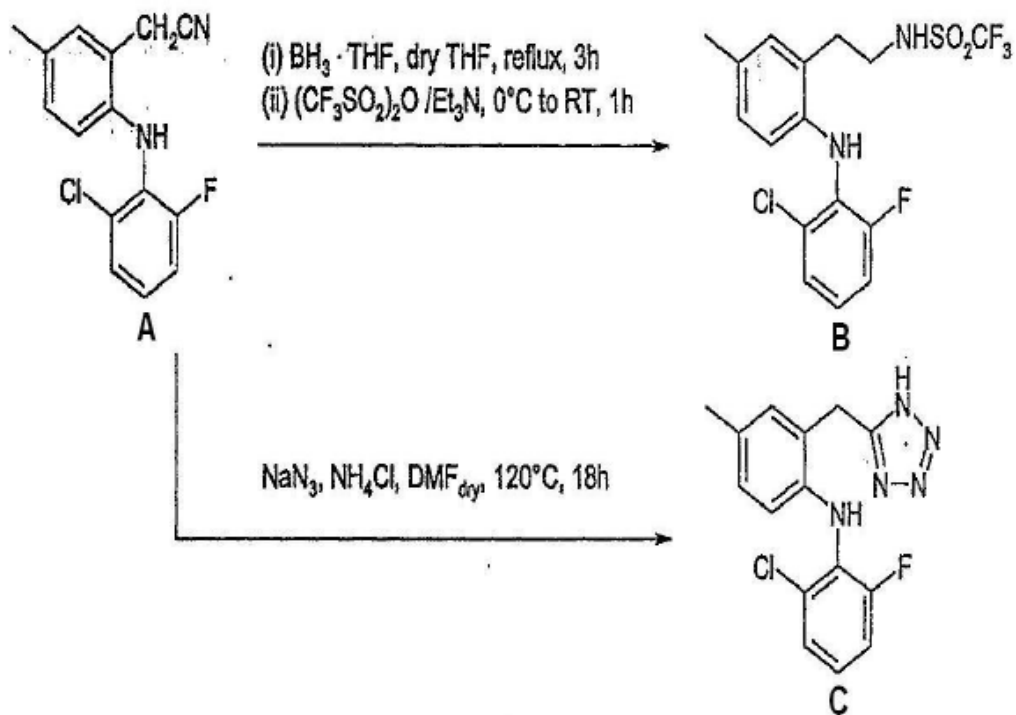


Fig 5. Chemical synthesis of compound 18 and 20

4.2 Antagonism of TP α functional activity in human platelets

Inhibition of TP receptor functional activity by the new synthesized compounds, as well as by the reference compounds, like, naproxen, lumiracoxib, diclofenac was assessed in human platelets from healthy volunteers. The platelet aggregation was studied through Born-turbidimetric assay. 100 μ M of acetylsalicylic acid was added to the blood to make platelets become unresponsive to AA stimulation (1-3 μ M), but responsive to the calcium ionophore A-23187 (3 μ M). Representative traces of platelet aggregation obtained from the stimulation with 0.1 μ M of U46619, and increasing concentrations (0.3-20 μ M) of compounds 18 and 20 are reported in Figure 6. When washed platelets were challenged with increasing concentrations of the stable TxA₂ analogue U46619 a concentration-dependent platelet aggregation occurred with a potency of 59 nM \pm 19 % CV (Figure 7). This response was thus truly independent of endogenous TxA₂ formation. The inhibition curves of U46619-induced (0.1 μ M) platelet aggregation in the presence of increasing concentrations of the tested compounds as well as the reference compounds are also reported in Figure 7. pA₂ values of the tested compounds were calculated (as described in method section) and reported in Table 1. Among all the tested

compounds, compound 18 and 20 resulted to be the most potent as TP antagonist, showing similar data as for diclofenac, while compound 20 displayed a pA_2 value statistically different from lumiracoxib ($pA_2 = 5.9$, 95% CI - Confidence Interval 5.5-6.4 for cp 20 - $pA_2 = 5.1$, 95% CI - Confidence Interval 4.8-5.4 for lumiracoxib) (Fig 7).

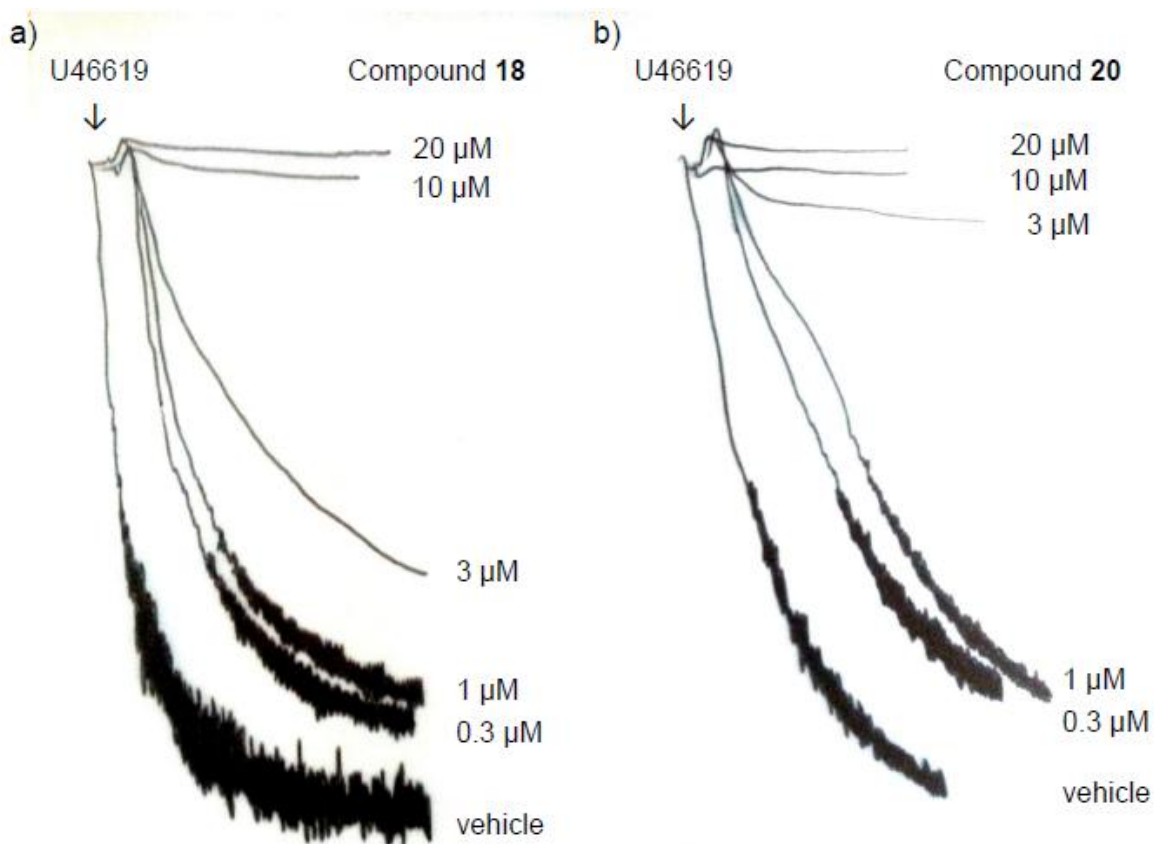


Figure 6. Platelet aggregation traces recovered from the stimulation with 0.1 μ M of U46619, and increasing concentrations (0.3-20 μ M) of compounds 18 and 20

Compound	pA ₂ ± %CV	
	Human washed platelet aggregation	Total IP production in HEK293 cells
Lumiracoxib	5.1 ± 3.4	4.6 ± 5.9
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* ± 2.2
20	5.9* ± 4.1	5.7* ± 2.3
7	5.0 ± 1.8	4.9 ± 5.1
32	4.8 ± 2.4	4.8 ± 2.5

* 95% CI vs. lumiracoxib

Table 1. TP receptor antagonism at human washed platelet aggregation and total IP production in transfected HEK 293 cells. pA₂ values were determined by measuring inhibition of aggregation response to the stable agonist U46619

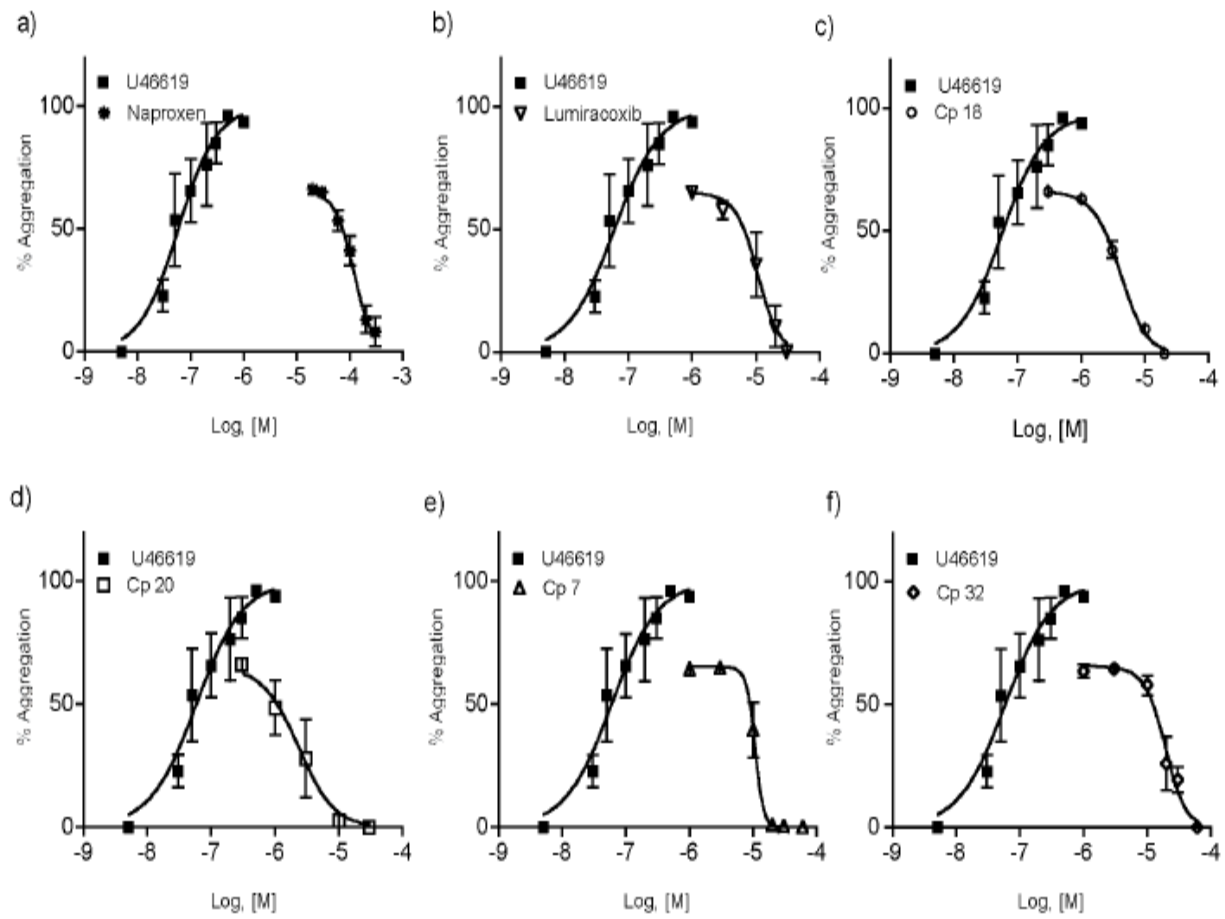


Figure 7. pA₂ values of the tested compounds determined by measuring inhibition of aggregation response to the stable agonist U46619

4.3 Antagonism of TP α functional activity in HEK293 transfected cells

HEK293 cells were transfected with the TP α receptor and the ability of the compounds to inhibit the total inositol phosphate (IP) production due to TP receptor coupling with Gq was assessed by stimulating cells with the specific receptor agonist U46619 (0.1 μ M, 30min), in the absence and presence of 30 min pretreatment with increasing concentrations of the reported compounds. The EC₅₀ calculated after the stimulation of TP α with U46619 was 29.3 nM \pm 10 % CV (Fig. 8).

The pA₂ values of the compounds calculated in HEK 293 cells similarly to human platelet aggregation are reported in Table 1. It is important to highlight that pA₂ values calculated for each compound in HEK 293 transfected cells are in full agreement with the results obtained in the aggregation assay. Once again, compounds 18 and 20 resulted to be the most potent compounds of the series, displaying pA₂ values similar to diclofenac (Fig. 8), but statistically different from lumiracoxib (pA₂ = 5.5, 95% CI, 5.2-5.8 for cp 18 - pA₂ = 5.7, 95% CI, 5.4-6.0 for cp 20 - pA₂ = 4.6 95% CI, 4.1-5.1 for lumiracoxib), matching the affinity binding data obtained in HEK293 cells [Bertinaria M. et al., 2012].

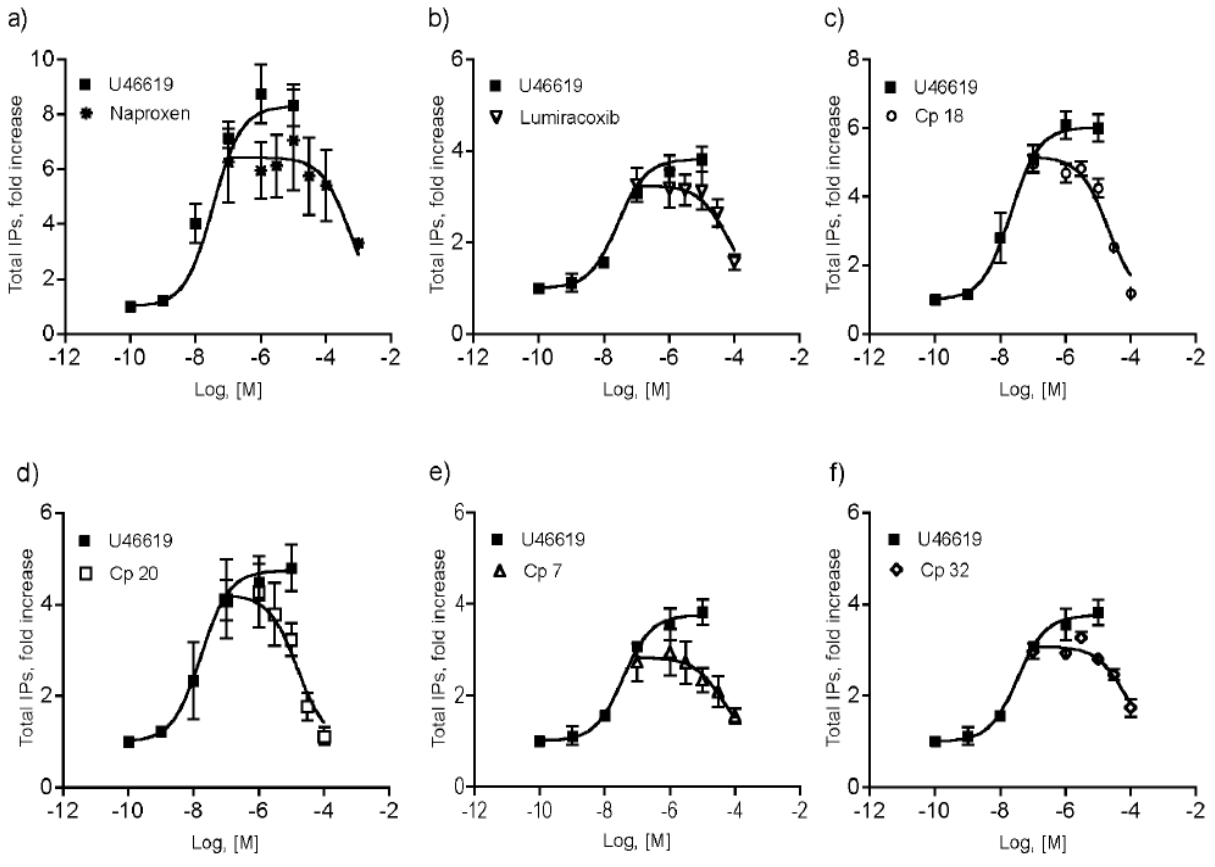


Figure 8. pA₂ values of the tested compounds obtained in total IP production inhibition in HEK293 cells transfected with TP α receptor

4.4 Evaluation of COX-2/COX-1 selectivity

The ability of the compounds to act as COX-2 inhibitors was determined in isolated human lympho-monocytes. All the new compounds should preserve a COX-2 selectivity similar to lumiracoxib, their reference compound. After treatment with acetylsalicylic acid, 10 µg/mL of lipopolysaccharide overnight was added to stimulate COX-2 expression, hence PGE₂ production, a marker of COX-2 activity, was determined by enzyme immunoassay and mass spectrometry.

Among all the tested compounds diclofenac and lumiracoxib possessed the highest absolute potency (Table 2) for inhibiting the COX-2 in a concentration-dependent manner, whereas compound 32, a derivative of TP receptor antagonist terutroban maintaining the 4-chlorobenzenesulfonamide moiety present in terutroban, resulted to be the less potent molecule (Table 2) of the series. Both compound 18, a tetrazole derivative, and compound 7 behaved very similarly, by inhibiting COX-2 with potencies of 0.014 and 0.025 µM, respectively, whereas compound 20 displayed a potency similar to naproxen (Figure 9 and Table 2).

On the other hand COX-1 inhibitory activity, assessed in washed human platelets as TXB₂ production, was determined by mass spectrometry. Out of all the tested compounds, diclofenac displayed the highest potency as COX-1 inhibitor in washed platelets, falling in the nM range, followed by naproxen (Table 2). In fully agreement with COX-2 inhibitory activity, the tetrazole derivative 18 and compound 7, behaved very similarly with IC₅₀ values of 13.2 μM and 25.5 μM, respectively, whereas, differently, the sulfonamide derivative 20 showed an IC₅₀ of 16.1 μM, close to that of compound 18 and 7. Compound 32, was inactive as COX-1 inhibitor (Figure 9 and Table 2).

The selectivity profile of all the tested compounds is reported in Figure 9, showing that naproxen and lumiracoxib were respectively, the less and the most selective COX-2 inhibitors (Table 2).

Among all the newly synthesized compounds, compound 18 and 7 resulted to be the most selective COXIBs (Figure 10, Table 2). Eventhough compound 20 is only 38 times more selective for COX-2 with respect to COX- 1 inhibition at calculated IC₅₀, it showed a very steep concentration-response curve (Hill coefficient >>1). Therefore, at a concentration ten times its

IC₅₀ for COX-2 inhibition, about 95% of COX-2 is inhibited, with no inhibition of COX-1 (Fig.

9c).

Compound	COX-2 inhibition IC ₅₀ (μM) ± %CV	COX-1 inhibition IC ₅₀ (μM) ± %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 μM	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 μM	-

N.D. - Not Determined

Table 2. COX-2 and COX-1 inhibitory activities determined by *in vitro* assay in lymphomonocytes and washed human platelets, respectively

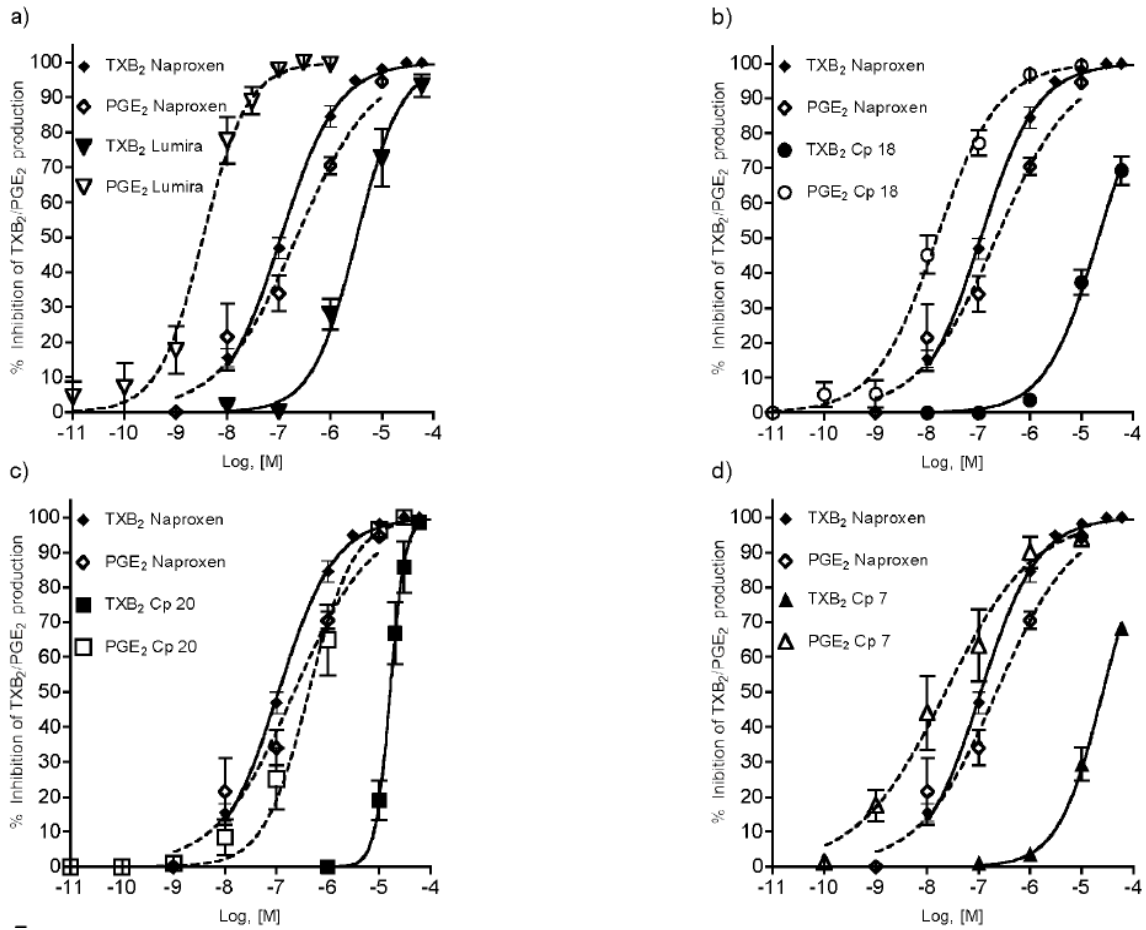


Figure 9. Inhibition of COX-1 and COX-2 activity by different compounds in comparison to the reference compound naproxen (a-d).

Data are expressed as percent inhibition of TXB₂ or PGE₂ release versus untreated controls. Error bars represent mean ± SE of at least three independent experiments, each performed in duplicate.

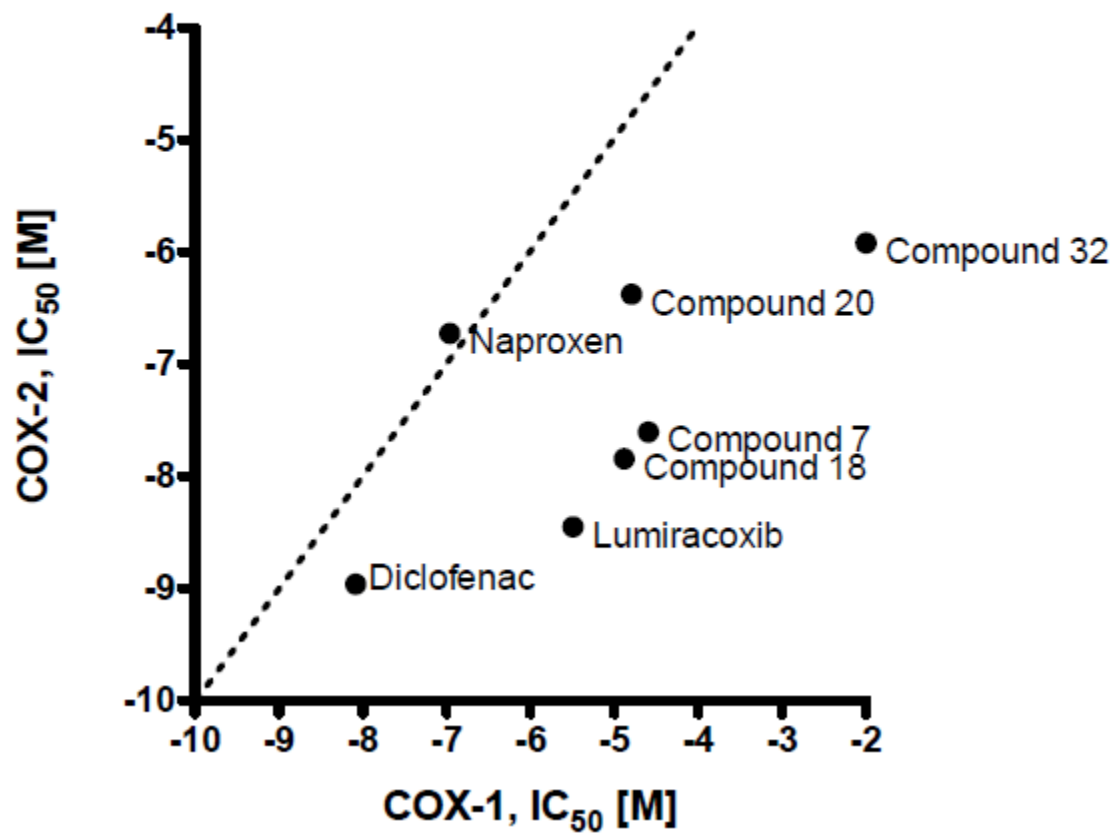


Figure 10. COX-2/COX-1 selectivity. The diagonal line indicates equivalence, hence, compounds with high selectivity for COX-2 over COX-1 are plotted beneath the line

PROJECT II RESULTS

The summary table containing all the patients characteristics, including mean age, gender, residence, educational level, income, and drug prescription is reported in Table 3.

The mean age of patients exposed to montelukast and having a history of MI is 68.50 years old, whereas that of patients suffering from MI but not exposed to montelukast is 71.40 years old, respectively. Regarding IS the mean age of patients with a history of IS using montelukast is 70 years old, with respect to 74.22 years old in patient not taking montelukast. The mean age of patients, exposed or not to montelukast, without a MI (57.1 and 61.84, respectively) or IS (57.18 and 62.16, respectively) event is relatively lower than that of patients having a history of CV events (as stated above), with at least 9 years difference, suggesting that age can contribute to the increase of CV risk, something not completely unexpected.

In case when at least one of the contingency table cells is less than five, or for small sample sizes, Fisher's Exact test was used to calculate the relationship between two categorical variables; otherwise Chi-squared test was implemented.

In subjects with MI exposed or not to montelukast the number of males was higher than that of females (75% males vs 25% females, in subjects using montelukast and 56% males vs 44% females in subjects not taking montelukast) whereas, interestingly, the proportion was inverted in IS (33% males vs 67% females in subjects unexposed to the drug with a prior IS event; the only IS in patients exposed to montelukast was indeed a female). Fisher exact test was used to calculate the two way interaction between gender and exposure to montelukast use in subjects with MI. The calculated p value was 0.622, so the null hypothesis is accepted and the two variables gender and exposure to the drug in subjects with MI are to be considered independent. The p-value of the Chi-square statistic test for subjects without prior MI exposed or not to montelukast and gender was 0.917, once again, suggesting no correlation between the two variables. The p value corresponding to Fisher exact test for gender and exposure to montelukast in patients with IS resulted to be less than 5%, which means that there is a correlation between the respective variables. Based on the data we obtained, although they are limited, it is possible

than females can be more predisposed to IS whether exposed or not to montelukast (1.62 % vs 0.82% males). Whereas the p value regarding the Chi square test for dependence of gender and exposure to the drug in patients without IS resulted to be 0.96, accepting the null hypothesis, hence no correlation is seen between two variables.

The data indicated that for both sample size exposed or not to montelukast without a prior CV event, such as MI or IS, the majority of subjects were resident in urban areas. 73% of all exposed patients are resident in urban areas, while 92% of all unexposed patients are resident in urban areas.

Subject samples education level was also studied, showing a 50% mid education level in the exposed patient with a prior MI, vs 60% of low education level in unexposed patients with a prior MI. Regarding IS events in both samples it was revealed that mid education level was predominant, 100% and 56% in exposed and unexposed patients, respectively. Furthermore, in subjects without prior CV events whether exposed or not to montelukast, it was evident a relatively higher number of mid education patients level.

Socioeconomic status was also analysed indicating overall a very low number of events in patients with high income in both sample sizes (4% vs 6% in exposed and unexposed patients respectively), and close data between mid and low yearly income (52.25% vs 45% for mid education and 43.75% vs 49% for low education in exposed and unexposed patients respectively).

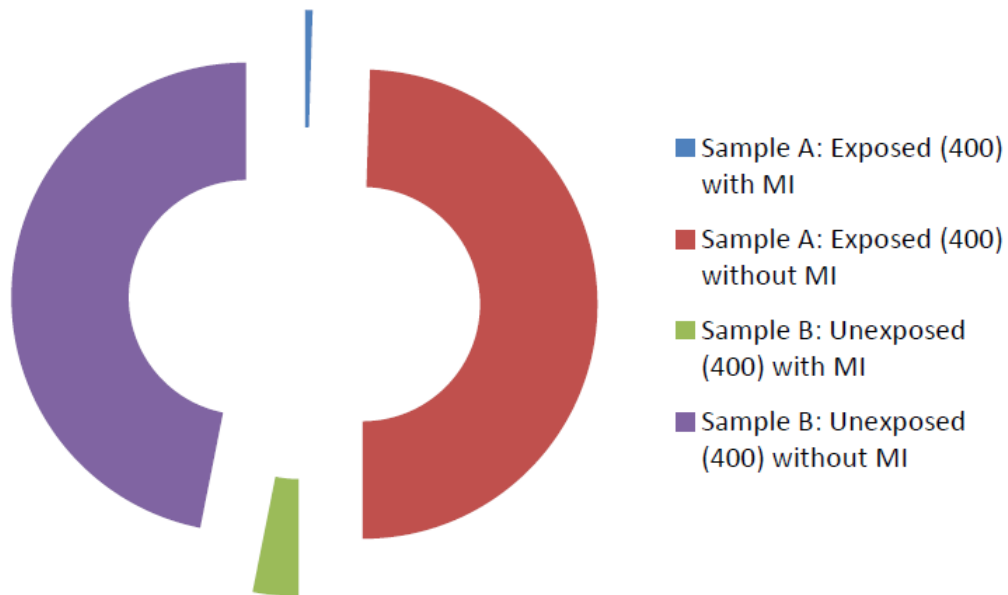
The monitoring of the drug used by each of the sample size was essential to exclude potential confounders of the results, the predisposition to CV events, hence the use of antihypertensive drugs, diuretics, antiplatelet, antihypercholesterolemic, hypoglycemics was very similar in both subjects sample, exposed or not to montelukast as shown in Table 3. Regarding the use of antihypertensive drugs 49.5% of patients using montelukast take antihypertensive drugs versus 50.25% of asthmatic non exposed patients. Similar proportions are also maintained for diuretics 21% vs 20.75% in exposed and unexposed patients respectively, whereas considering that diabetes is also a risk factor for CV diseases and therefore a potential confounder of the results, we observed that 5% and 7.25% of exposed and unexposed patients respectively, take hypoglycemic drugs.

Data recovered indicate that IS is present in 1.25% of our sample, consisting of eight hundred asthmatic patients whereas 3.62% suffered from MI. Considering that asthma can contribute to the increase of CV risk, we retain that these percentages are higher in respect to non asthmatic population.

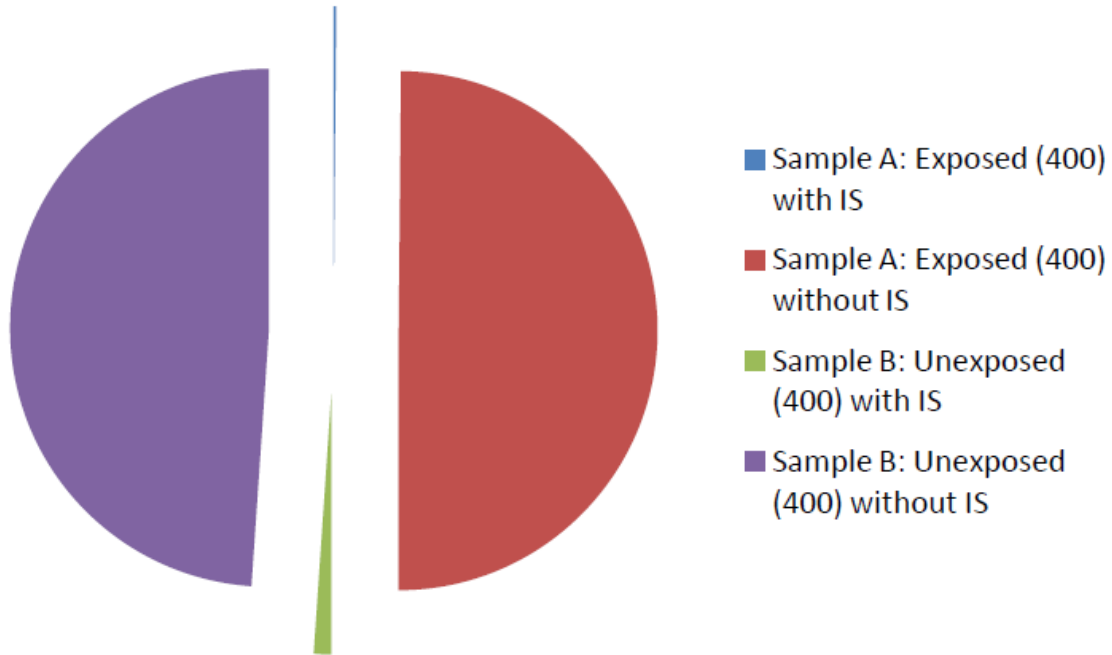
Table 3. The study sample characteristics, of both unexposed and exposed patients for MI and ischemic stroke (IS)

	Sample A: Exposed (400)		Sample B: Unexposed (400)		Sample A: Exposed (400)		Sample B: Unexposed (400)	
	With MI	Witho ut MI	With MI	Without MI	With IS	Without IS	With IS	Without IS
No.	4 (1%)	396 (99%)	25 (6.25%)	375 (93.75%)	1 (0.25%)	399 (99.75%)	9 (2.25%)	391 (97.75%)
Mean Age	68,50	57,10	71,40	61,84	70,00	57,18	74,22	62,16
Female	25%	54%	44%	55%	100%	54%	67%	54%
Male	75%	46%	56%	45%	0%	46%	33%	46%
Residence								
Urban	75%	73%	96%	92%	100%	73%	100%	92%
Rural	25%	27%	4%	8%	0%	27%	0%	8%
Educational Level								
Low	25%	17%	60%	42%	0%	17%	22%	44%
Mid	50%	64%	28%	49%	100%	64%	56%	47%
High	25%	19%	12%	9%	0%	19%	22%	9%
Income								
Low	25%	44%	48%	49%	0%	44%	67%	49%
Mid	75%	52%	44%	45%	100%	52%	22%	46%
High	0%	4%	8%	6%	0%	4%	11%	6%
Drug prescriptions								
Antihypertensives	100%	49%	100%	47%	100%	49%	100%	49%
Antiplatelets	100%	4%	100%	4%	100%	5%	100%	8%
Diuretics	100%	20%	100%	15%	100%	21%	100%	19%
Antipsychotics	0%	0%	0%	1%	0%	0%	0%	1%
Antiandrogens	25%	1%	4%	3%	0%	1%	0%	3%
Antithyroids	0%	1%	4%	1%	0%	1%	0%	1%
Antihypercholesterolemics	100%	14%	100%	13%	100%	14%	100%	17%
Antihistamines	0%	1%	0%	1%	0%	1%	0%	1%
Antipeptics	0%	0%	4%	1%	0%	0%	0%	1%
Antiarrhythmics	0%	0%	4%	1%	0%	0%	0%	1%
Antianemics	0%	0%	0%	1%	0%	0%	0%	1%
Hypoglycemics	0%	5%	8%	7%	0%	5%	0%	7%
Antiepileptics	0%	0%	0%	1%	0%	0%	0%	1%
Antitumorals	0%	0%	0%	2%	0%	0%	11%	2%
Antibiotics	0%	0%	0%	1%	0%	0%	0%	1%
Antidepressants	0%	0%	0%	100%	0%	0%	0%	0%

The pie chart (Graph 1) was used to indicate the MI events in patients exposed to montelukast 1% and in subjects not taking montelukast, 6.25%, respectively.



Graph 1. Graphic representation of MI events in sample A (asthmatic patients exposed to montelukast) compared to sample B (asthmatic patients not using montelukast)



Graph 2. Graphic representation of ischemic stroke (IS) events in sample A (asthmatic patients exposed to montelukast) compared to sample B (asthmatic patients not using montelukast)

The pie chart (Graph 2) was used to indicate ischemic stroke events in patients exposed to montelukast 0.25 % and in subjects not taking montelukast, 2.25%.

The MI event is 1% in patients exposed to montelukast and 6.25% in patients not taking montelukast, whereas ischemic stroke event in exposed patient is 0.25% with respect to 2.25% of unexposed patients.

Table 4. Fisher's exact test tables for both a) ischemic stroke in exposed and unexposed samples and b) myocardial infarction

a)

	No Ischemic stroke events	Ischemic stroke events	Total
Exposed	399	1	400
Unexposed	391	9	400
Total	790	10	800

The p value calculated from the Fisher's exact test equals 0.0207, less than 5%, and the null hypothesis is rejected, hence the treatment with montelukast affects the outcome, the predisposure to ischemic stroke event.

b)

	No Myocardial infarction events	Myocardial infarction events	Total
Exposed	396	4	400
Unexposed	375	25	400
Total	771	29	800

The p value calculated from the Fisher's exact test is less than $0.0001 < 5\%$, so the null hypothesis is rejected, the treatment with montelukast can reduce the predisposure to MI event.

V- DISCUSSION

This research illustrates two different approaches to CV risk reduction in inflammatory diseases.

From one side we were focused on studying a possible new NSAID, a multitarget compound, displaying two different pharmacological profiles, in order to provide clear benefits on its safety and efficacy. Starting from the findings of Selg et. al [Selg E. et al., 2007], demonstrating a new and unrecognized mechanism of action of a traditional NSAID like diclofenac, and of its derivative lumiracoxib, which instead is a potent COXIB, both acting as TP antagonist eventhough with a potency too low for their effects to be appreciated in therapy (pA_2 values in high micromolar range), new structural analogous of lumiracoxib were synthesized [Bertinaria M. et al., 2012] and pharmacologically characterized [Hoxha M. et al., 2015]. Our intent was to provide new compounds displaying the COXIB activity in order to be used as antiinflammatory drugs, but increasing their potency as TP antagonist, so that we can obtain new compounds with predicted reduced CV side effects. Here we reported the pharmacological profile of some of the newly synthesized compounds, compound 18, the tetrazole derivative of lumiracoxib, the trifluoro methansulfonamido-isoster compound 20, compound 7 and compound 32, along with

the reference compounds diclofenac, an excellent analgesic [Todd PA. and Sorokin EM., 1988], and its derivative lumiracoxib, a potent COX-2 inhibitor, as well as of a traditional, non selective NSAID, naproxen. Pharmacological activities studies have shown that both the terazole derivative 18, as well as compound 20 are the most active TP antagonists with a decent balanced activity as COXIB and TP antagonist.

We believe that this strategy would provide an additional benefit for patients with chronic pain and high GI risk taking a COXIB, as it will avoid the washed out period due to the increase in CV risk associated to COXIB use, or even in patients taking simultaneously a COXIB and low dose aspirin, due to the inhibition of the interaction of isoprostanes with TP receptor, something that is not provided from either of the treatments. Isoprostanes, non enzymatic products of AA, and extra-platelet TxA₂ are produced *in vivo*, they do not respond or have a very scarce response to aspirin action [Audoly LP. et al., 2000], but their activity can be blocked by preventing their interaction with the TP receptor. Moreover, traditional NSAID, with the possible exclusion of naproxen, have also shown an increase of CV risks. Patients with diabetes or other high-risk vascular thrombotic disease have a basic demand for analgesic drugs that provide a GI and CV

safer profile. In addition, a dual COXIB-TP antagonist could also have a valuable use in chemoprevention, like colon adenoma prevention [Reddy BS. et al., 2005] based on the contribution of TxA₂ synthesis in colon tumorigenesis [Sciulli MG. et al., 2005].

On the other hand, TP receptor antagonists and TxA₂ biosynthesis inhibitors were not considered to be superior to the gold standard drug, aspirin [Cayette AJ. et al., 2000]. In addition, terutroban, a potent TP antagonist, despite presenting antithrombotic properties in peripheral arterial disease and improvement of endothelial function in atherosclerosis, did not result to be superior, but only similar to aspirin in the Perform Phase III clinical trial.

Compound 32, studied in our lab was obtained by the replacement of 2-fluoro-6-chloro-phenyl substituent present on the amino nitrogen with the *p*-chlorophenylsulfonyl substructure, linking two phenyl rings by a sulfonamide group which, interestingly enough, is also present in terutroban. In the meanwhile, introducing a functional group of terutroban we expected that compound 32 would have displayed better properties, be a more potent TP antagonist with respect to other compound such as 7, 18 or 20; presumption

rejected by the results. In coherence with data in the literature, we observed that 2-amino-5-methyl benzen carboxylic acid moiety is essential for the COX-2 selectivity. In terms of the TP antagonist properties both compounds 18 and 20 have shown an increase in TP receptor antagonist activity, highlighting the fact that in two different systems, inhibition of human platelets aggregation and IP generation, compound 20 resulted to be statistically different with respect to lumiracoxib, matching the affinity binding data previously reported [Bertinaria M. et al., 2012]. In addition these data suggest that the isosteric replacement of carboxylic function of lumiracoxib give rise to higher TP antagonistic activities of the compounds. This replacement can either be with cyclic or linear structures.

The therapeutic effects of these new chemical entities endowed with a dualistic activity depend on the balance of two pharmacological profiles. Once again compound 18 and 20 resulted to be the most interesting compounds of the series, demonstrating better balanced activities (pA_2 TP/ IC_{50} COX-2 = 179 and 3, respectively) with respect to the reference compounds lumiracoxib and diclofenac (pA_2 TP/ IC_{50} COX-2 = 2269 and 3619, respectively). Although naproxen, a non selective COX inhibitor, has a very weak TP receptor antagonism, it resulted to be safer for CV events with respect to others NSAID [Grosser T. et al., 2006]. Several studies have evidenced

that naproxen inhibits both systemic PGI₂ biosynthesis [Graff J. et al., 2007], and platelet derived TxA₂, hence not interfering with the equilibrium between TxA₂ and PGI₂, essential for designing a safer antiinflammatory drug.

In conclusion, our data have shown that it is possible to obtain novel compounds starting from lumiracoxib, with higher TP antagonist potencies and better balanced COX-2 selectivity. In perspective, these new compounds may lead to a new class of NSAID with fewer CV side effects, with obvious pharmacokinetic and pharmacodynamic benefits displaying two of these activities at the same concentration range. After the bitter frustration and economic losses of pharmaceutical industries in the NSAID field due to the drawbacks of several COXIBs, we believe that the development of these dual compounds could represent a successful strategy to pursue. It is important to obtain innovative compounds with superior TP antagonist activity and better balanced COX activity to further investigate those in *in vivo* animal model. In particular, these dual compounds might have an impact in the therapeutic treatment of patients with higher CV risk that require a long term therapy with NSAIDs, like diabetics, or even in certain forms of cancer like colon cancer, or in chronic diseases like Alzheimer [Breitner JC. et al., 2011].

On the other side, targeting others AA metabolites, such as cysteinyl-LTs involved in asthma and other inflammatory conditions including CV disorders [Funk CD., 2005], we thought it could add a new dimension to CV event reduction. In this context, we studied the effects of montelukast, a LT receptor antagonist (LTRA) drug, in the prevention of CV events (stroke and MI) in asthmatic patients. Thus, we performed a retrospective study including a total of eight hundred asthmatic patients exposed or not exposed to montelukast to assess the potential effect of this drug in the primary and secondary prevention of a major CV event (manuscript in preparation).

Somehow expected, the mean age of patients without a prior CV event, both exposed or not to montelukast, was almost 9 years lower with respect to patients with prior IS or MI, suggesting that age is a factor for an increased CV risk rate. In addition it is possible that females (both exposed or not to montelukast) can be more predisposed to IS (1.62 % vs 0.82% males).

Data were recorded also for patients residence in urban or rural areas and accumulating results have shown that the majority of patients without prior CV event were resident in urban areas.

Regarding patients education level, overall, mid education level was predominant either in subjects without prior MI or IS, exposed or not to montelukast, or in patients with a history of IS events. Only patients unexposed to montelukast with a prior history of MI had a predominance of low education level, around 60%, whereas yearly income (mid and low) was very similar in the two patients groups, with a very low number of events in high income patients. Importantly, the percentages of the drug used in patients exposed or not to montelukast were very similar. The latter is essential to exclude potential confounders for the CV event rate.

Overall, these results suggest that the MI and IS event rates were more than 6 fold and 9 fold higher, respectively, in asthmatic patients non taking montelukast with respect to patients exposed to it. These encouraging results, however, should be expanded to a larger number of patients, possibly to the whole Albanian asthmatic population. In addition, extending the treatment period with montelukast as well as the total exposure, should be implemented in further studies in order to provide a better overview of the whole patients clinical background and the real impact of the use of this drug. Moreover, further studies should also include data

regarding other risk factors such as obesity or smoking, regardless of the drug used monitored in the present study.

Based on the results presented in this thesis we can foresee new innovative strategies for inflammatory disease like rheumatoid arthritis or carotid and coronary atherosclerosis by targeting specific components of the AA cascade. 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. Nowadays there is still an unmet need for an adequate pain therapy conferring minimal GI damage and maximal cardioprotection. Furthermore, our montelukast study seems to suggest a novel therapeutic alternative for the prevention of the CV risk in the general population. In general terms, the pharmacological importance of compounds interfering with the inflammatory component is crucial for a different approach to CV diseases

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