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Rationally Designed Multitarget Agents Against Inflammation and Pain

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Abstract

Arachidonic acid (ARA) undergoes enzyme-mediated oxidative metabolism, resulting in the formation of a number of biologically active metabolites. For over a century, these biochemical transformations have been the target of numerous pharmacological drugs for inflammation and pain. In particular, non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 (COX-2) selective inhibitors (coxibs) are widely used in the treatment of inflammation and pain. However, gastrointestinal (GI) and cardiovascular adverse effects of NSAIDs and coxibs, and recent findings demonstrating that there are significant risks from the disruption of oxylipin levels when pharmacologically inhibiting a single ARA cascade metabolic pathway, have led to studies involving the simultaneous inhibition of multiple pathways in ARA cascade. These studies suggest that multitarget inhibition represents a new and valuable option to enhance efficacy or reduce side-effects in the treatment of inflammation and pain. This review focuses on the crosstalk within the three pathways of the ARA cascade (cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450)), and summarizes the current and future approaches of multitarget inhibitors for the treatment of eicosanoid driven inflammation and pain.

Keywords

Cyclooxygenase (COX); lipoxygenase (LOX); cytochrome P450 (CYP); soluble epoxide hydrolase (sEH); multitarget inhibitors; dual inhibitors

1. INTRODUCTION

There are three major pathways associated with the arachidonic acid (ARA) cascade, cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) pathways [1, 2]. Since the synthesis of acetylsalicylic acid (Aspirin®) as the first COX inhibitor [3], non-steroidal antiinflammatory drugs (NSAIDs) have become the most widely used pharmaceuticals to treat inflammation and pain world-wide [4]. NSAIDs act by blocking the action of the COX enzymes, COX-1 and COX-2, resulting in the reduction of pro-

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CONFLICT OF INTEREST
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inflammatory ARA metabolites, and in particular, prostaglandin E_2 (PGE_2) [5]. COX-1 is reportedly involved in maintaining the homeostatic balance of COX metabolites and is responsible, in part, for normal gastrointestinal (GI) function [6]. Prostacyclin (PGI_2) and PGE_2 produced in the GI tract play important roles in modulating GI mucosal defense and repair [7]. Therefore, the use of NSAIDs is often limited by side effects emanating from disrupting the levels of these protective COX metabolites. Following the discovery of a second form of COX enzyme (COX-2) which is inducible by cytokines and growth factors [8–10], COX-2 selective inhibitors (coxibs) such as celecoxib (Celebrex®) and rofecoxib (Vioxx®) were subsequently developed in an effort to circumvent these problems [8, 11]. Coxibs are effective against inflammation and pain, with less risk of severe GI toxicity associated with conventional NSAIDs [12]. However, there are safety concerns with coxib use due to an increase in the risk of cardiovascular events associated with the imbalance of the PGI_2 and thromboxane (TXA_2) metabolite levels [13]. Inhibition of COX-1 reduces platelet-derived TXA_2, an eicosanoid which functions as a vasoconstrictor and facilitates platelet aggregation [14]. PGI_2 is a major metabolite of ARA in the heart and is associated with vasodilation and the prevention of platelet aggregation [15]. Selective inhibition of COX-2 affects the PGI_2/TXA_2 ratio to favor TXA_2, increasing the risk of mortality from ischemic heart disease [16–18]. These findings were supported by several clinical trials involving coxibs including APPROVE, VIGOR, CLASS, and TARGET [17]. Recently, a metabolomic approach to understand the relationship between adverse cardiovascular events and the use of rofecoxib suggested that this drug acts, in part, through accumulation of 20-hydroxyeicosatetraenoic acid (20-HETE) which is a potent vasoconstrictor among ARA metabolites [19]. In addition, it is reported that chronic use of coxibs also increases GI side effects, albeit the risk is lower than NSAIDs [20, 21]. Treatment of inflammation and pain constitutes significant medical needs because more people are afflicted with these conditions than any other disease state. Thus, there is a growing demand for safer but efficacious NSAIDs or coxibs [4]. Emerging concepts and approaches for the treatment of inflammation and pain have moved towards simultaneously targeting multiple enzymes in the ARA cascade through combination therapy and multitarget inhibitors such as dual inhibitors with the aim of overcoming the risks involved in single enzyme or pathway inhibition.

2. THE ARA CASCADE

ARA, a twenty-carbon fatty acid, is one of the most abundant polyunsaturated fatty acids found in the phospholipid bilayer of cells involved in inflammatory responses [22]. Activation of phospholipase A_2 (PLA_2, generally cytoplasmic PLA_2) [23] and other lipases in response to various stimuli, leads to release of ARA into the cellular milieu. ARA is then metabolized by three major enzymatic pathways, the COX, LOX, and CYP pathways [24]. The ARA metabolites of the COX enzymes, or prostanoids, are primarily pro-inflammatory mediators [25]. The eicosanoid metabolites of the LOX pathway include both pro-inflammatory leukotrienes (LTs) [26] and hydroxyeicosatetraenoic acids (HETEs) [27], as well as mediators of inflammation resolution, the lipoxins (LXs) [28]. The CYP hydroxylases generate the vasoconstrictor 20-HETE [29], and the CYP epoxygenases generate anti-inflammatory epoxyeicosatrienoic acids (EETs) [30]. The EETs are further metabolized by soluble epoxide hydrolase (sEH) to their corresponding diols,

dihydroxyeicosatrienoic acids (DHETs), which are reported to have less biological activity [31] (Fig. 1).

Detailed discussions on each of these metabolic pathways are beyond the scope of this review. Therefore, the focus is on the major enzymes that are targeted in combination therapy by dual inhibitors or multitarget agents, and crosstalk among the ARA metabolizing pathways.

2.1. The COX Pathway

The COX pathway is well studied and has recently been summarized in several reviews [32–34]. It is well established that there are two isoforms of COX, COX-1 and COX-2 in mammals. While COX-1 is constitutively expressed in most mammalian cells under physiological conditions [35], the expression of COX-2 can be induced by pro-inflammatory stimuli such as cytokines, bacterial lipopolysaccharide (LPS), growth factors, and tumor-promoting agents [36, 37]. Recent evidence has shown that COX-2 is constitutively expressed in several tissues including spinal cord [38], brain [39] and kidney [40], and although the roles of constitutively expressed COX-2 are not fully understood, this expression pattern creates challenges in selectively targeting COX-2 for therapeutic purposes [41, 42]. The COX enzymes are dual function enzymes that initially transform ARA into unstable prostaglandin G_2 (PGG_2) via the COX function, and then into the more stable prostaglandin H_2 (PGH_2) via the peroxidase (POX) function. PGH_2 is then converted to various bioactive prostanoids such as prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2) and TXA_2 by cell- and tissue-selective prostanoid synthases [41–43] (Fig. 2). Among these prostanoid synthases, PGE_2 synthases (microsomal PGE synthase-1 (mPGES-1), membrane PGES-2 (mPGES-2), and cytosolic PGES (cPGES)) [44], have recently attracted a great deal of attention as potential targets in the treatment of inflammation and pain [45]. These enzymes are downstream of COX and therefore may not produce the same side-effects as the COX mediated disruption of PGI_2 formation. Both mPGES-2 and cPGES are constitutively expressed enzymes, while mPGES-1 is inducible by inflammatory stimuli and primarily coupled with COX-2 [46, 47]. These findings have made mPGES-1 attractive as a potential target for inflammation and pain [45].

Biological Effects of Prostanoids—Prostanoids are autocrine and paracrine lipid mediators, which are widely distributed and involved in a broad range of physiological responses (reviewed in [6]). Typically these metabolites are high-affinity agonists of their respective receptors all of which are G protein-coupled receptors that modulate second messenger levels (e.g., Ca^{2+}, cAMP and inositol phosphates) [48]. Since the initial studies of von Euler describing prostanoids in late 1936 [49], nine human prostanoid receptors have been isolated and cloned including two isoforms activated by PGD_2 (DP_1, DP_2), four by PGE_2 (EP_1, EP_2, EP_3, EP_4) and three by $PGF_{2\alpha}$, PGI_2, and TXA_2 (FP, IP and TP respectively). These receptors and their diverse functions explain much of the multiplicity of the biological effects of prostanoids [50]. The roles and functions of prostanoids vary in a context dependent manner in the body [25]. They have important roles in GI function, with PGE_2, $PGF_{2\alpha}$ and PGI_2 protecting the gastric mucosa blood flow and stimulating mucus formation as well as bicarbonate secretion [51]. In the cardiovascular system, PGD_2, PGE_2

and PGI_2 mediate vascular tone by eliciting vasodilation, whereas TXA_2 is a potent vasoconstrictor [52]. In the airways, $PGF_{2\alpha}$ acts as a bronchoconstrictor and PGE_2 acts as a bronchodilator [53]. The role of PGs in inflammatory pain is well established [54]. Specifically PGE_2 is one of the most painful endogenous substances known to man and mediates a number of inflammatory processes including redness, swelling, and pain [55]. Binding of PGs to prostanoid receptors sensitizes pain specific neurons in several distinct ways to stimulate pain. For example PGE_2 acts on EP_1 and EP_2 receptors in the peripheral and central nociceptive systems respectively, and PGI_2 acts on IP receptors in the peripheral nociceptive system [56]. PGE_2 also acts on neurons and contributes to the systemic responses to inflammation such as fever, fatigue, and pain hypersensitivity [57]. These diverse and differential roles of prostanoids obviously require an intricate balance to maintain homeostasis and to avoid overacting inflammatory responses [58].

2.2. The LOX Pathway

There are three LOX isozymes, 5-, 12-, and 15-LOX, that have been identified in human which convert ARA into their respective pro-inflammatory hydroperoxyeicosatetraenoic acids (HPETEs), 5-, 12-, and 15-HPETE respectively [59]. In contrast to other LOX enzymes, 15-LOX also initiates the synthesis of lipoxins (LXs) which are involved in the resolution phase of inflammation [60]. Although 12-LOX and 15-LOX have been implicated in inflammatory diseases such as psoriasis [61] and arthrosclerosis [62], this review will focus on the role of 5-LOX and synthesis of LTs as targets for treating inflammation. 5-LOX is a unique LOX isozyme which utilizes two co-factors, Ca^{2+} and adenosine triphosphate (ATP) [63], and protein-protein interactions with five-LOX activating protein (FLAP) for catalytic activity [64]. The metabolism of ARA by 5-LOX first generates 5(S)-HPETE [65], and then this intermediate is either reduced to the corresponding alcohol, 5-HETE, or to the very short-lived epoxy-leukotriene, LTA_4 [66]. Further systematic metabolism of LTA_4 results in a series of LTs starting with the hydrolysis of its epoxide by LTA_4 hydrolase (LTA_4H) [67] to the corresponding diol, LTB_4, or its metabolism to the cysteine-adduct LTC_4 [68]. Other cysteinyl-LTs (Cys-LTs), LTD_4 and LTE_4, are sequentially formed from LTC_3 (Fig. 3). Recently a detailed description of the synthesis and biological action of the LOX metabolites has been reviewed [69].

Biological Effects of LTs—LTs are paracrine lipid mediators that play various roles in inflammation and immunological function through specific rhodopsin-like G protein-coupled receptors (reviewed in [70]). Four LT receptors including two cysteinyl LTs receptors ($CysLT_1$ and $CysLT_2$) and two LTs B_4 receptors (BLT_1 and BLT_2) have been isolated and cloned [71–73]. LTB_4 is a potent chemotactic agent for inflammatory cells including neutrophils, macrophages and eosinophils [74, 75]. These LTs increase leukocyte tissue infiltration and play an important role in immune reactions by enhancing the release of pro-inflammatory cytokines by macrophages and lymphocytes [76]. Cys-LTs such as LTC_4, LTD_4, and LTE_4, are involved in immediate hypersensitivity reactions and are potent constrictors of airway smooth muscles leading to bronchoconstriction [26, 77]. Cys-LTs also increase vascular permeability leading to edema by contracting endothelial cells (EC) in the microvasculature. LTs also play an important role in GI mucus protection [78, 79]. In addition, they can interact with sensory nerve fibers, leading to changes in their excitability

and enhanced release of tachykinins. Therefore LTs are important mediators of various inflammatory diseases and allergic disorders including asthma, rheumatoid arthritis, inflammatory bowel disease, ulcerative colitis, psoriasis and allergic rhinitis [80].

2.3. The Cytochrome P450/sEH Pathway

The role of mammalian CYPs in the oxidative metabolism of ARA was not uncovered until 1981 [81–85]. The CYP enzymes relevant to ARA metabolism include two distinct pathways: the ω-hydroxylases (CYP4A) which produce HETEs such as 20-HETE, and the epoxygenases (CYP2C and CYP2J) which produce the EETs [86]. Research into the biological activity of 20-HETE has shown this metabolite possesses potent vasoconstrictor activity [87]. The EETs have been suggested to be endothelium-derived hyperpolarizing factors (EDHFs) with vasodilatory properties [88], and have various biological activities in several inflammatory diseases [89, 90] and pain [91]. EETs are primarily synthesized in endothelial cells [92–96], and are further metabolized by sEH, to their corresponding and less active diols, DHETs (Fig. 4) [97, 98]. EETs not only decrease inflammation, but also decrease platelet aggregation in order to maintain vascular homeostasis [99]. Importantly, other fatty acids such as the ω-6 linoleic acid (LA) and the ω-3 α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are oxidized by CYPs through mechanisms similar to those for ARA [100]. Recent work by Morisseau *et al.* showed these various epoxy-fatty acids (EpFAs) are efficiently metabolized by sEH to the corresponding diols. The epoxides of DHA have demonstrated to be potently anti-hyperalgesic, although their other biological actions remain unclear [101]. Potential roles of EpFAs in pain are well-described in recent review [102].

Biological Effects of Epoxyeicosatrienoic Acids—The CYP epoxygenases convert ARA to four EET regioisomers, 5,6-, 8,9-, 11,12-, and 14,15-EETs, that function as autocrine and paracrine mediators [103]. The presence of a receptor(s) for EETs has been suggested [48], but membrane receptor(s) or binding protein(s) have yet to be reported. EETs have been shown to exhibit anti-inflammatory effects on the endothelium by inhibiting activity of IKK and TNF-α, thus attenuating cytokine-induced NF-κB activation [93]. This, in turn, suppresses the expression of adhesion molecules such as vascular cell-adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E-selectin [93, 104–106]. Inhibition of sEH by small molecules has been shown to reduce inflammatory responses by stabilizing the blood levels of EpFAs including EETs, and indirectly reduce the expression level of COX-2, 5-LOX, iNOS, and VCAM-1 in LPS-induced mouse models of systemic inflammation [107, 108]. These findings are consistent with the opinion that EETs prohibit amplification of an inflammatory event by preventing nuclear translocation of NF-κB, and thus its subsequent transcriptional activity [93, 106]. Animal models of inflammatory pain have shown that exogenous administration of EETs or treatment with sEH inhibitors, results in antihyperalgesic responses [91, 109]. While some types of pain respond well to treatment with opioids, NSAIDs and coxibs, treatment of neuropathic pain including diabetic neuropathy remains a challenge because of side effects and low efficacy of current treatments [110, 111]. Therefore the evidence of sEH inhibitor mediated antihyperalgesia in a diabetic neuropathy model offers an important new approach in meeting this challenge. The biological roles of EETs in inflammatory disease and pain is

extensive [112] and in the following articles further information about EETs and specific disease can be found: vascular inflammation [113], pulmonary hypertension [114, 115], metabolic syndrome [116, 117], renal inflammation [118, 119], cardiovascular diseases [120–122], cancer [123], pain [112, 124]. In addition, mono-epoxides of EPA and DHA also possess antihyperalgesic properties [101] and their roles in pain relief have been reviewed [112].

3. CROSSTALK BETWEEN PATHWAYS IN THE ARA CASCADE

It is clear that bioactive lipid mediators from ARA metabolism evoke potent inflammatory and anti-inflammatory responses. Therefore, an intricate communication between the COX, LOX, and CYP pathways would be required to regulate inflammation and inflammation resolution. This communication, or crosstalk, between pathways is far from being well understood, but recent technologies such as lipid profile analysis has enabled the ability to examine changes in the ARA metabolome when inhibiting one or more of the ARA pathways. Coupling this type of technology with a better understanding of the role of certain metabolites in human disease will greatly increase the ability to predict potential risks from inhibiting enzymes in the ARA cascade. The next section will discuss recent findings in the interactions between the multiple pathways.

3.1. Crosstalk Between COX and LOX Pathways

It has been theorized that inhibition of a single pathway in the ARA cascade may lead to the shunting of ARA into the other untargeted pathways within the cascade. For example, inhibiting COX pathway can shunt the metabolism of ARA towards LOX pathway, resulting in an increase synthesis of LTs, and thus potentially diminishing the beneficial effects of reducing prostanoid synthesis [125, 126]. While this theory is currently viewed as an overly simplistic contributor to biological effects, such an event was observed in the case of aspirin-induced asthma, where the inhibition of the COX pathway resulted in a significant increase in the 5-LOX product, LTE_4 [127]. Recently, using an LPS-challenged murine model, Liu *et al.* demonstrated that inhibition of 5-LOX not only reduced both 5-HETE and 15-HETE, but also significantly decreased both PGE_2 and TXB_2 in the COX metabolites [128]. Cumulatively, these data demonstrate that communication exists not only within the LOX pathway, but between LOX and COX pathways, and this crosstalk is bidirectional. It is unclear if these responses are due to effects on the transcriptional regulation of the associated enzymes, or if this is a result from direct effects on enzyme activity. However, it has been shown that LOX metabolites of ARA and LA can differentially affect the substrate selectivity [129] and reaction mechanism [130] of the LOX enzymes through allosteric regulation. This suggests a direct protein/metabolite interaction may regulate crosstalk within the ARA cascade, and that metabolite interactions may extend into other pathways such LA and ω-3 fatty acids metabolizing cascades.

3.2. Crosstalk Between COX and CYP/sEH Pathways

In addition to the COX/LOX pathway crosstalk, Kozak *et al.* showed that 11,12-EET, a CYP monooxygenase-derived ARA metabolite, suppresses production of PGE_2 in monocytes through modulating COX-2 activity [131], and Schmelzer *et al.* demonstrated *in*

vivo that co-inhibition of both the COX pathway and CYP pathway (via sEH inhibition) elicited an additive response in alleviating pain [132]. In the latter experiment, there was an observed reduction in the COX-2 protein expression levels and a dramatic shift in the oxylipin metabolomic profile [107]. This work demonstrated that crosstalk within the ARA cascade can, in part, result from effects on transcriptional regulation of proteins involved in ARA metabolism. It was proposed in this work, that increasing the anti-inflammatory EETs level by sEH inhibition, while simultaneously decreasing the pro-inflammatory PGE_2 level by the reduction in COX-2 expression, synergistically affected the inflammatory signaling and the associated pain response. It should be noted that this synergistic effect was obtained by a combination of sEH inhibitor and a low-dose of NSAIDs or coxibs that are not effective doses when used alone. These findings are significant in that incidences for the cardiovascular adverse effects of NSAIDs or coxibs are typically higher with chronic high-dose treatment [133]. In addition, the inhibition of sEH alone or in combination with NSAIDs or coxibs did not disrupt the relative ratio of PGI_2 to TXA_2 in the plasma of LPS-challenged mice. These data indicated that a combination therapy involving sEH and COX-2 inhibition may result in the desired decrease in inflammatory eicosanoids such as PGD_2 and PGE_2 without the potential cardiovascular risks from the imbalance of the TXA_2-to-PGI_2 ratio.

3.3. Crosstalk Between LOX and CYP/sEH Pathways

Recent evidence now links the LOX and CYP pathway [134], closing the ARA cascade-feedback loop. In this work, Liu *et al.* demonstrated that inhibition of sEH in a LPS-challenged murine model significantly decreased the plasma DHETs and TXB_2, while unexpectedly reducing two LOX metabolites, 5-HETE and 15-HETE, in plasma [128]. These results suggest that the CYP-generated EETs and their metabolizing enzyme, sEH, are intricately connected to both the LOX and COX pathways. Interestingly, oral administration of acetylsalicylic acid significantly lowered plasma levels of metabolites from all three pathways of the ARA cascade. It is clear from these results that there is a complex mechanism of interaction between the distinct pathways of the ARA cascade, and therefore, disturbances in any one of these pathways may lead to undesirable consequences from the over-production or under-production of critical lipid signaling molecules [115].

4. RATIONALLY DESIGNED MULTITARGET AGENTS

Because selectively blocking one of the pathways in the ARA cascade in most cases also causes the production of undesirable or unexpected metabolites, new approaches have been developed to combine therapies to modulate multiple pathway targets simultaneously [135–137]. However, administering two drugs in combination therapy raises safety concerns and presents unique challenges for optimizing dosage. For example, it is not safe to assume that two drugs with relatively high individual safety profiles will be safe when co-administered [138]. Co-administration treatment requires extensive investigations into optimal dose regiments including safety studies, a complex dosage ranging investigation, and drug-drug interaction analysis, all of which may significantly raise the practical cost and complexity of developing combination therapies [139]. During the drug development process, predicting pharmacodynamic and pharmacokinetic relationships is substantially less complex if

multitarget inhibition is obtained from a single agent rather than from combination therapies (co-administration). In addition, improvement of patient compliance is expected with these multitarget inhibitors as fixed-dose drug combinations [140]. For these reasons, interest has grown in designed multiple ligands (also known as DMLs) [135, 141] with the following aims: 1) to enhance drug efficacy, 2) to improve drug safety by acting specifically on multiple targets, 3) to amend patient compliance, and 4) to eliminate the challenges of formulation problems and drug-drug interactions in coadministrative therapies [142]. This review article will focus on current research on DMLs as dual inhibitors which target two or more enzymes in the pathways in ARA cascade.

4.1. COX/5-LOX Dual Inhibitors

Dual inhibition of COX and LOX pathways by a single entity has been attracting attention in academia and industry. Many of the COX/LOX dual inhibitors developed have focused on inhibition of COX and specifically 5-LOX. They can be classified into three classes based on the type of 5-LOX inhibition: redox inhibitors, iron chelators, and nonredox/ non-chelators.

4.1.1. Redox Inhibitors—Benoxaprofen which is a radical scavenger is the first commercially available COX-2/5-LOX inhibitor. However, it has been discontinued due to reports of causing photosensitivity, effects on nail and hair growth, and liver and kidney failure. These side-effects were not proposed to be associated with the inhibition of their target enzymes but remain significant obstacles to their clinical use [143, 144].

Di-tert-Butylphenols: This class of di-*tert*-butylphenol derivatives is the most extensively studied group of dual inhibitors of COX and 5-LOX enzymes. It was found that naturally occurring diarylheptanoids such as curcumin or yakuchinones exhibited COX and LOX inhibition *in vitro* [145], but these compounds failed to demonstrate inhibition of LTB_4 biosynthesis or anti-inflammatory activity *in vivo* [146]. Therefore, in an effort to improve both the *in vitro* and *in vivo* activities of these diarylheptanoids, various analogues containing similar structural 2,6-di-*tert*-butylphenols have been developed as dual inhibitors (Fig. 5). Structure-activity relationship (SAR) studies have indicated that 2,6-di-*tert*-butyl-1-hydroxybenzene substituted in the fourth position is optimum for dual activity. The substituents are either five- or six-membered heterocycles or chains. The phenol moiety possesses the antioxidant and radical scavenging properties which is considered to be responsible for anti-inflammatory potency and low ulcerogenic potential. The therapeutic index of these compounds (ratio of anti-inflammatory efficacy to GI safety profile) has uniformly been shown to be superior to that of conventional NSAIDs [147]. For these reasons, a variety of COX/5-LOX dual inhibitors containing such a phenol have been developed including darbufelone (CI-1004) [148], S-2474 [149], and tebufelone [150]. Among them, S-2474 also displays cytokine-modulating properties and is currently being evaluated in clinical trials for arthritis [147–149, 151–153].

Pyrazoline Derivatives: BW-755C and phenidone are COX/5-LOX dual inhibitors functioning as one-electron reducing agents (see Fig. 6). These compounds were originally developed as antioxidant 5-LOX inhibitors and later demonstrated to possess inhibitory

activity towards COX isozymes [143, 154, 155]. Phenidone significantly decreased eosinophils (ED_{50} = 15 mg/kg) in bronchoalveolar lavage (BAL) fluid of guinea pigs sensitized to ovalbumin and those challenged with aerosoilized antigen, while indomethacin had no effect in eosinophils accumulation [156].

4.1.2. Iron Chelators

Hydroxamic Acids: Tepoxalin (Zubrin®) is a COX-2/5-LOX dual inhibitor which exhibited potent anti-inflammatory activity with excellent gastric protection (Fig. 7). It is approved for veterinary use only (in canines) [157]. Oral administration in humans revealed that tepoxalin inhibits whole blood COX, LOX, and platelet function [158] and its further development has been stopped due to its hepatotoxicity [159].

Thiophene Derivatives: RWJ 63556 (Fig. 8), was developed as 5-LOX inhibitor [160] that was subsequently found to inhibit COX-2 selectively over COX-1 [161]. This compound displayed significant anti-inflammatory activity in carrageenan-induced inflammation in canines [160] and inhibited the development of neurogenic edema induced by saphenous nerve stimulation [162]. L-652,343 was demonstrated to inhibit COX and 5-LOX *in vitro* and was efficacious in acute and chronic inflammation models *in vivo* [163, 164]. However, L-652,343 failed to show inhibition of 5-LOX *in vivo* in human skin [165] or LTB_4 production *ex vivo* using stimulated human whole blood [166].

Pyridone Derivatives: Chowdhury *et al*. developed a COX-2/5-LOX dual inhibitor such as 1 in (Fig. 9) by replacing the tolyl group in celecoxib with the *N*-difluoromethyl-1,2-dihydropyrid-2-one moiety, which exhibited good anti-inflammatory activity (ED_{50} = 27.7 mg/kg p.o.) and compared favorably with reference drugs such as celecoxib (ED_{50} = 10.8 mg/kg p.o.) and ibuprofen (ED_{50} = 67.4 mg/kg p.o.) [167]. The same strategy has also been applied to NSAIDs [168, 169].

4.1.3. Non-Redox, Non-Chelators—Due to the non-selective action of redox and chelating inhibitors which cause undesirable side effects, there was an effort to design a new class of small molecule inhibitors without these actions. Therefore the following compounds are discovered or developed as new class of COX/LOX dual inhibitors.

DHDMBFs: 7-*tert*-Butyl-2,3-dihydro-3,3-dimethylbenzofurans (DHDMBF) was originally found as a metabolite of tebufelone. Interestingly, this compound lacks the anti-oxidant phenolic moiety, but selectively inhibits both 5-LOX and COX-2 enzymes and displays anti-inflammatory activity equivalent to tebufelone in the rat carrageenan paw edema assay [147]. Among DHMBF derivatives, PGV-20229 is of particular interest because it showed excellent gastric safety and was active in a rat arthritis model with an ED_{50} of 6.6 mg/kg [147]. The acute ulcerogenic dose (UD_{50}) was much higher, with no GI lesions observed at oral doses up to 1000 mg/kg. In addition, in a refed rat antral damage model, the UD_{50} for PGV-20229 was greater than naproxen and nabumetone and no GI damage was seen in 13-day studies in dogs and rats at oral doses up to 200 mg/kg/day. It has been demonstrated that a large variety of substituents (R) are compatible at the 5 position in DHDMBF (see the

general structure in Fig. 10) retaining the anti-inflammatory and analgesic activities [152, 153].

Structural Modification of NSAIDs: In the attempt to find potent COX/LOX dual inhibitors, structural modifications have been performed on conventional NSAIDs (Fig. 11). Tenidap, a COX/5-LOX inhibitor and cytokine modulator, is more effective in the clinical treatment of rheumatic arthritis than diclofenac [170]. However, this compound has been withdrawn from the market due to liver and kidney toxicity attributed to its reactive oxidative metabolites with a thiophene moiety [171–173]. To avoid these toxic side effects, a series of analogues including compound **2** have been developed replacing the thiophene ring in tenidap. These compounds showed comparable or stronger anti-inflammatory and analgesic activities, and better gastric tolerance *in vivo* than tenidap [174].

Structural Modification of Coxibs: The combination of a COX-2 pharmacophore and a 5-LOX pharmacophore led to potent selective COX-2/5-LOX inhibitors (Fig. 12). For example, compound **3** is a combination of the pyrazole-based tricyclic moiety found in celecoxib (Pfizer) and the 4-(3-fluoro-5-oxy)phenyl-4-methoxy tetrahydropyran from the 5-LOX inhibitor ZD-2138 (Zeneca) [175]. This dual inhibitor reduced ARA-induced ear edema by i.v. and oral administration. Several others in this class of COX-2/5-LOX dual inhibitors have also developed using similar approach such as compounds **4** (possessing a *p*-SO_2Me COX-2 pharmacophore) [176] and **5** (possessing a 3,4-diaryl-2(5*H*)furanone COX-2 pharmacophore as in Vioxx®) [177].

Pyrrolizine-Based Dual Inhibitors: Licofelone [178, 179] (ML 3000, 2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1*H*-pyrrazolin-5-yl] acetic acid) has shown anti-inflammatory, analgesic and anti-asthmatic effects in several animal models (Fig. 13) [180]. Licofelone is one of the most promising COX/5-LOX inhibitors for human therapy and successfully finished phase III clinical trial for the treatment of osteoarthritis (OA) [181]. Notably, it possesses superior GI protective effect by decreasing gastrotoxic LTs through 5-LOX inhibition [182]. In addition, it exhibits protective anti-thrombotic activity due to its inhibition of COX-1 [183]. This is an excellent example demonstrating that simultaneous inhibition of COX-1, COX-2, and 5-LOX can circumvent the side effects associated with the individual inhibition of these enzymes. Recent study [184] showed that licofelone might suppress PGE_2 formation by inhibiting mPGES-1 [185], and not through COX-2 inhibition.

4.2. COX/sEH DUAL INHIBITORS

Recent work evaluating the co-inhibition of COX-2 and sEH has led to the development of COX-2/sEH dual inhibitors (Fig. 14) [186]. The potencies of these compounds were evaluated using recombinant enzymes assays *in vitro* and the efficacy of the most promising candidate was evaluated using a LPS induced rat pain model *in vivo*. The COX-2/sEH dual inhibitor by s.c. injection exhibited anti-allodynic activity that is more efficacious than the same dose of either a celecoxib or a sEH inhibitor (*t*-AUCB) alone, or the coadministration of sEH inhibitor with celecoxib. This work demonstrated that the use of a dual inhibitor as a DML was more efficacious than combination therapy in a rat pain model. Use of

COX-2/sEH dual inhibitors could be an alternative approach for treating inflammation and pain compared to well-studied COX/5-LOX dual inhibitors.

4.3. LOX/sEH Dual Inhibitors

5-LOX/sEH dual inhibitors have been recently discovered by dual-target virtual screening using an *in silico* approach [187]. Among the eighty compounds selected by the virtual screening, thirty six compounds have been tested with *in vitro* enzyme assays. One compound has inhibited both 5-LOX and sEH enzymes at a marginal concentration (Fig. 15). Although no biology is associated with 5-LOX/sEH dual inhibitors, further development is warranted based on elevation of LOX products 5-HETE and 15-HETE with long-term sEH inhibition [134].

4.4. Dual Inhibitors Targeting Enzymes Downstream in the COX or LOX Pathways

In addition to targeting 5-LOX and COX enzymes, a number of alternative multitarget approaches which focus on enzymes downstream of LOX and COX pathways have also been reported. The limitations of NSAIDs or coxibs use are derived from on-target side-effects such as GI injury, renal irritation, cardiovascular events, and off-target side effects such as an increase of LT formation. Therefore a new approach has been developed that targets enzymes downstream in the COX or LOX pathway to minimize potential on-target side-effects.

5-LOX and mPGES-1 Dual Inhibitors: mPGES-1 is one of the most attractive targets for this new approach. As described in section 2.1, unlike other PGE_2 synthases, mPGES-1 is inducible by various inflammatory stimuli and is primarily coupled to COX-2 expression [46, 47]. This synthase is a PGE_2-forming enzyme downstream of COX in the pathway which has the potential to selectively affect PGE_2 and not other prostaglandins or TXA_2 synthesis. Recent evidence demonstrated that mPGES-1 knockout mice did not display impaired cardiovascular function [188]. In addition, licofelone (section 4.1.3.), the most promising COX/5-LOX dual inhibitor, is likely suppressing the formation of PGE_2 by inhibiting mPGES-1 and not through COX-2 inhibition [184]. Based on these findings several arylpyrrolizines derivatives have been developed as 5-LOX and mPGES-1 dual inhibitors such as **6** (Fig. 16) [189]. In addition, pirinixic acid **7** [190] and 2-mercaptohexanoic acid **8** [191] derivatives have been reported as 5-LOX/mPGES-1 dual inhibitors. In comparison with indomethacin (5 mg/kg), YS121 (1.5 mg/kg) was as efficient in reducing exudates formation and leukocyte infiltration with reduced pleural levels of PGE_2 and LTB_4 in the carrageenan-induced rat pleurisy model [192]. Recently virtual screening using a comparative model of the human 5-LOX led to the discovery of new 5-LOX/mPGES-1 dual inhibitors **9** and **10** possessing novel scaffolds [193].

In addition to targeting mPGES-1, downstream enzymes in the LOX or COX pathways such as LTA_4H and TXA_2 synthase are also being investigated as new approaches for developing dual inhibitors acting in the ARA cascade.

COX-2 and LTA_4H Dual Inhibitors: Recently Chen *et al.* reported dual inhibitors that target COX and the downstream leukotriene metabolizing enzyme, LTA_4H, an epoxide

hydrolase that catalyzes the conversion of LTA_4 into LTB_4 (Fig. 17) [194]. These dual inhibitors were developed by combining a COX-2 selective inhibitor nimesulide with an LTA_4H inhibitor, 1-(2-(4-phenoxy phenoxy) ethyl)pyrrolidine, however no *in vivo* efficacy was reported [195].

5-LOX and TXA_2 Synthase Dual Inhibitors: Hibi *et al.* reported dual inhibitors such as E3040 targeting both 5-LOX and TXA_2 synthase (Fig. 17). E3040 inhibited the production of LTB_4 and TXB_2, but not PGE_2, and was efficacious at a dose of 100 mg/kg p.o. in the induced chronic colitis model compared to sulfasalazine (500 mg/kg) [196]. E6700, having better absorption compared to E3040, was under clinical trial for asthma treatment [197].

4.5. Dual Inhibitors Targeting Enzymes Downstream in the CYP Pathway and Enzymes Outside ARA Cascade

Extensive research on the benefits of therapeutic application of EETs has triggered development of dual inhibitors that include sEH inhibition. This approach is not limited to the enzymes in the arachidonic cascade. Examples here outline the future direction of valuable dual inhibitors for inflammation and pain.

sEH/11β-HSD1 Dual Inhibitors: GlaxoSmithKline (GSK) reported dual inhibitors for sEH and 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzymes. 11β-HSD1 converts the inactive glucocorticoids (e.g. cortisone) to the active glucocorticoids (e.g. cortisol) (Fig. 18) [198]. Cortisone injections have been a proven solution for short-term relief of pain associated with swelling from inflammation of a joint, tendon, or bursa. However, their use is restricted due to adverse side effects. Thus, inhibition of sEH in concert with 11β-HSD1 is predicted to yield favorable results for the treatment of diseases mediated by the sEH or 11β-HSD1 [199].

Sorafenib: In a recent study which examined the structural similarities of the FDA-approved anti-cancer drug sorafenib (Nexavar ®) and sEH inhibitors, Liu *et al.* observed that sorafenib possesses potent *in vitro* and *in vivo* sEH inhibitory activity (IC_{50} = 12 nM) in addition to multi-kinase inhibition (Fig. 18) [200]. Therefore, it has been suggested that sEH inhibition might ameliorate the toxicity of sorafenib during treatment through demonstrated anti-inflammatory and antihypertensive effects. However, the contribution of sEH inhibition on the efficacy of sorafenib has yet to be fully described.

5. CONCLUSION

Inflammation and pain are extremely complex biological processes that involve a great number of lipid mediators providing a theoretically wide range of possible targets for pharmacological treatment. Of the current available drugs that inhibit a single branch of the ARA cascade, inhibition of the COX pathway with NSAIDs is by far the most commonly used approach in the treatment of inflammation and inflammatory pain. This drug class, as well as the second generation coxibs, is not without serious GI and cardiovascular risks which stem from an imbalance in homeostatic eicosanoid metabolite levels. In addition, it is clear that inhibiting a single pathway can affect the metabolite output of the other pathways

and crosstalk. This has led to a significant improvement in our understanding of the on-target adverse effect risks of COX inhibition [201]. However these serious side-effects from inhibiting either of the COX enzymes remain and the risks are not dogmatically separated as were previously thought [202]. While attempts to mitigate side-effects based on this historical dogma drove the exploration of dual COX/LOX inhibition, little success has come from this approach in the past decades. Several of these compounds, especially COX/5-LOX inhibitors, have been extensively studied and exhibited significant increases in efficacy both *in vitro* and *in vivo* without the undesired on-target side-effects associated with single pathway inhibition. However the off-target toxicity and adverse events limited many of these early dual inhibitors. Many of these initial dual inhibitors or multitarget drugs were not rationally designed; rather were subsequently found to have activity towards unintended targets. For example, compounds possessing anti-oxidant or iron-chelating properties originally developed as 5-LOX inhibitors, also possessed COX inhibitory activity due to their non-selective inhibition which eventually resulted in off-target adverse effects. Therefore the need persists for safer and effective multitarget agents by rational design. Recent advances such as the recent X-ray crystal structure of human 5-LOX [203] and computer-aided drug design (CADD), will allow medicinal chemists to rationally design new therapies through virtual screening using molecular docking, ligand-based and structure-based approaches. In addition, emerging roles of CYP metabolites in inflammation, pain, and other diseases will expand the scope of multitarget agents in ARA cascade. Dual inhibitors that target sEH and COX enzymes are an example of such a novel approach that holds great promise for treating pain and inflammation. This approach was fundamentally different from the previously developed COX/LOX dual inhibitors since the object was not to reduce undesirable metabolites, but to increase the lifetime of the CYP pathway metabolites (e.g., EETs) through sEH inhibition. The beneficial effects of the stabilized EETs allow dose-sparing of COX inhibitors, thereby potentially attenuating GI or pro-thrombic side-effects. Although sEH inhibitors share some of the same indications as coxibs, the synergy observed in lowering prostaglandins with combination therapy [132] and in antihyperalgesia with the dual inhibitor [186] merits inhibiting both target enzymes. Another promising approach is the development of dual inhibitors targeting enzymes down-stream of the COX or LOX enzymes to prevent unavoidable on-target side-effects. While these new approaches are novel, recent results from use of these multitarget agents or dual inhibitors indicate they are a viable alternative pharmacological strategy for treatment of inflammation and pain.

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ABBREVIATIONS

11β-HSD1	11β-Hydroxysteroid Dehydroge nase Type 1

20-HETE	20-Hydroxyeicosatetraenoic Acid
5-LOX	5-Lipoxygenase
ALA	α-Linolenic Acid
ARA	Arachidonic Acid
ATP	Adenosine Triphosphate
BAL	Bronchoalveolar Lavage
BLT_1 & BLT_2	Two Leukotriene B_4 Receptors
CADD	Computer-Aided Drug Design
COX	Cyclooxygenase
COX-2	Cyclooxygenase-2
cPGES	Cytosolic Prostaglandin E_2 Synthase
CYP450	Cytochrome P450
$CysLT_1$ / $CysLT_2$	Cysteinyl Leukotrienes Receptors
Cys-LTs	Cysteinyl-Leukotrienes
DHA	Docosahexaenoic Acid
DHDMBF	7-*tert*-Butyl-2,3-dihydro-3,3-dimethylbenzofurans
DHETs	Dihydroxyeicosatrienoic Acids
DMLs	Designed Multiple Ligands
EC	Endothelial Cells
EDHFs	Endothelium-Derived Hyperpolarizing Factors
EETs	Epoxyeicosatrienoic Acids
EPA	Eicosapentaenoic Acid
EpFAs	Epoxy-Fatty Acids
GI	Gastrointestinal
HETEs	Hydroxyeicosatetraenoic Acids
HPETEs	Hydroperoxyeicosatetraenoic Acids
ICAM-1	Intracellular Adhesion Molecule-1
LA	Linoleic Acid
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTA_4H	Leukotriene A_4 Hydrolase
LTs	Leukotrienes

mPGES-1	Microsomal Prostaglandin E_2 Synthase-1
mPGES-2	Membrane Prostaglandin E_2 Synthase-2
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OA	Osteoarthritis
PGE_2	Prostaglandin E_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2 (Prostacyclin)
PLA_2	Phospholipase A_2
POX	Peroxidase
SAR	Structure-Activity Relationship
sEH	Soluble Epoxide Hydrolase
***t*-AUCB**	*trans*-4-[4-(3-Adamantan-1-ylureido)-cyclohexyloxy]-benzoic acid
TXA_2 synthase	Thromboxane A_2 Synthase
VCAM-1	Vascular Cell-Adhesion Molecule-1

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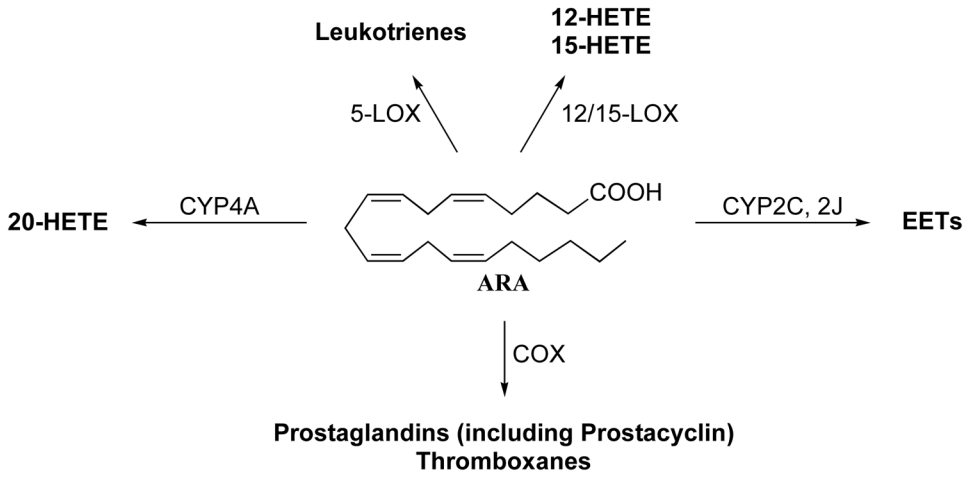


Fig. 1.
ARA cascade.

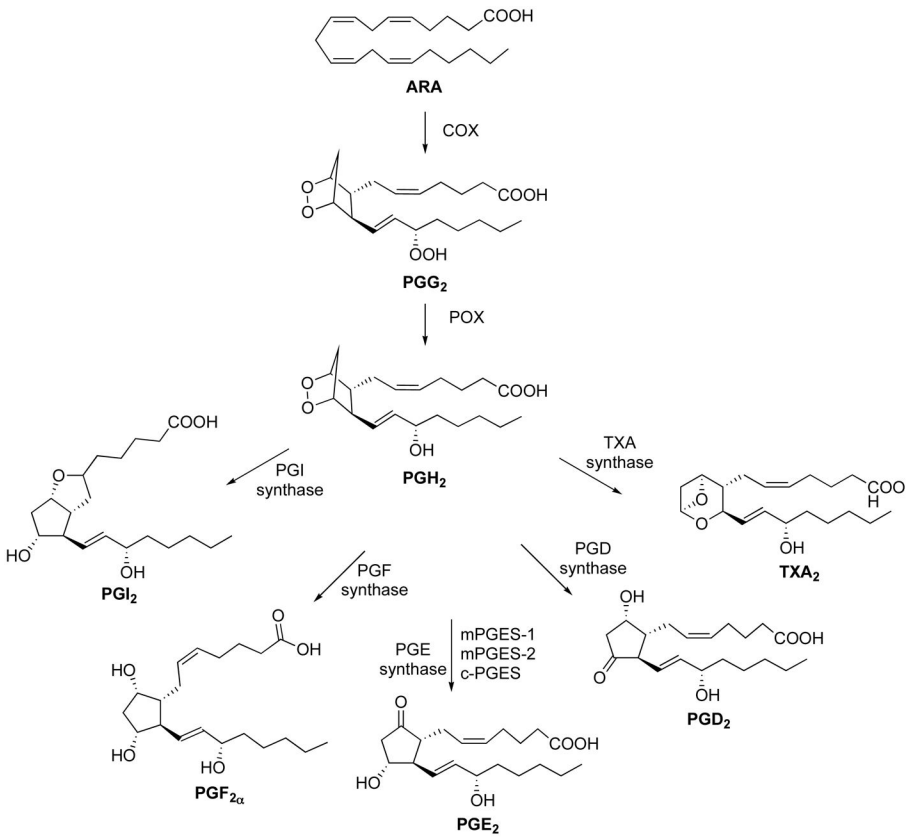


Fig. 2.

Prostaglandins, PGI_2 and TXA_2 from the COX pathway.

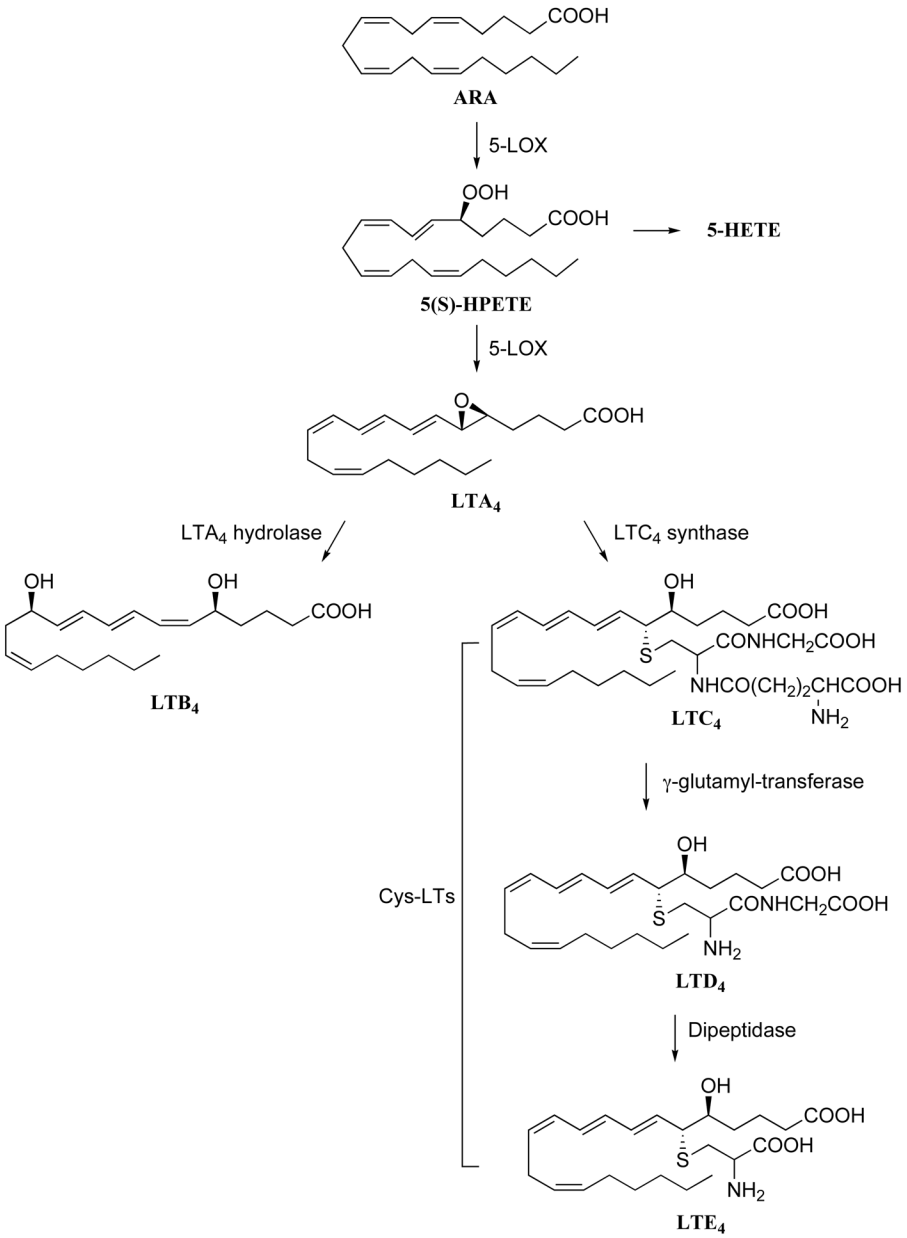


Fig. 3.
LTs derived from the LOX pathway.

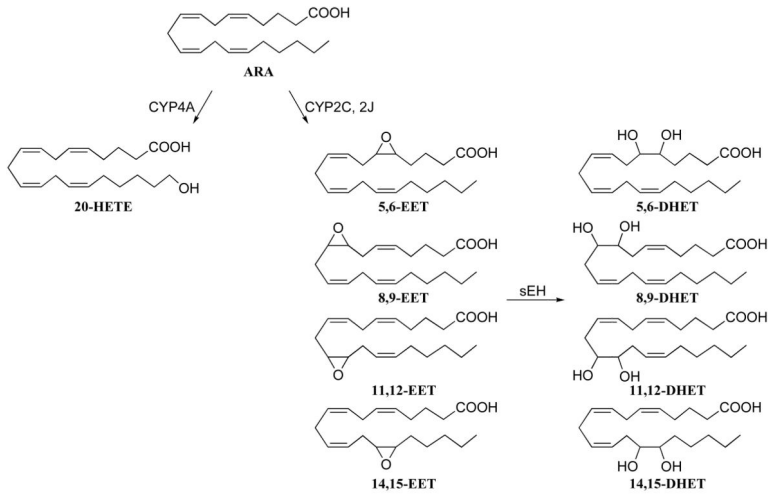


Fig. 4.
Epoxyeicosatrienoic acids and 20-HETE from the CYP450 pathways.

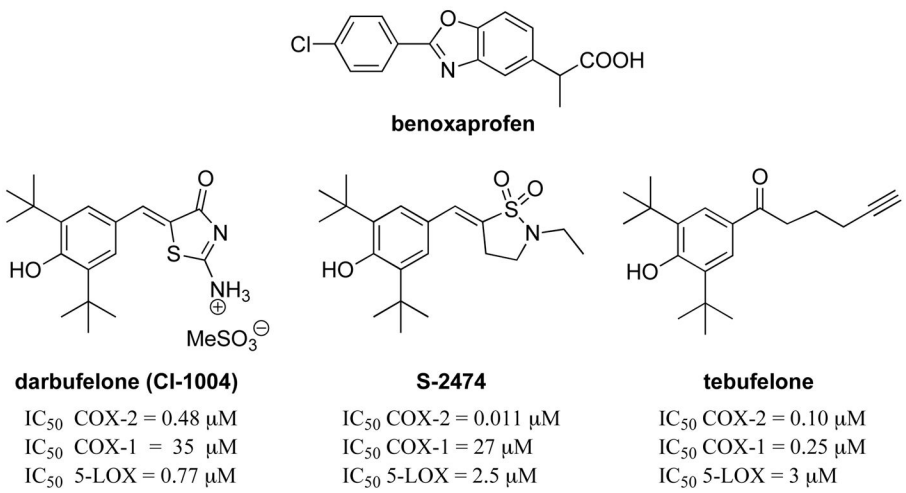
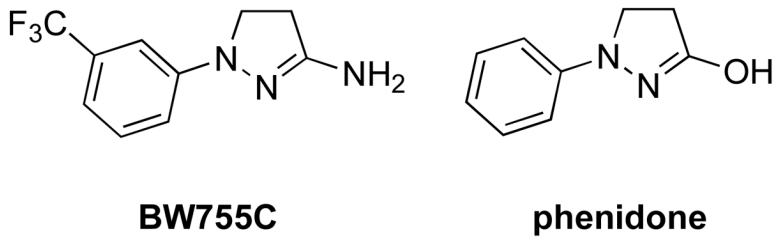


Fig. 5.
Structures of redox inhibitors.



BW755C **phenidone**

Fig. 6.
Structures of pyrazoline derivatives.

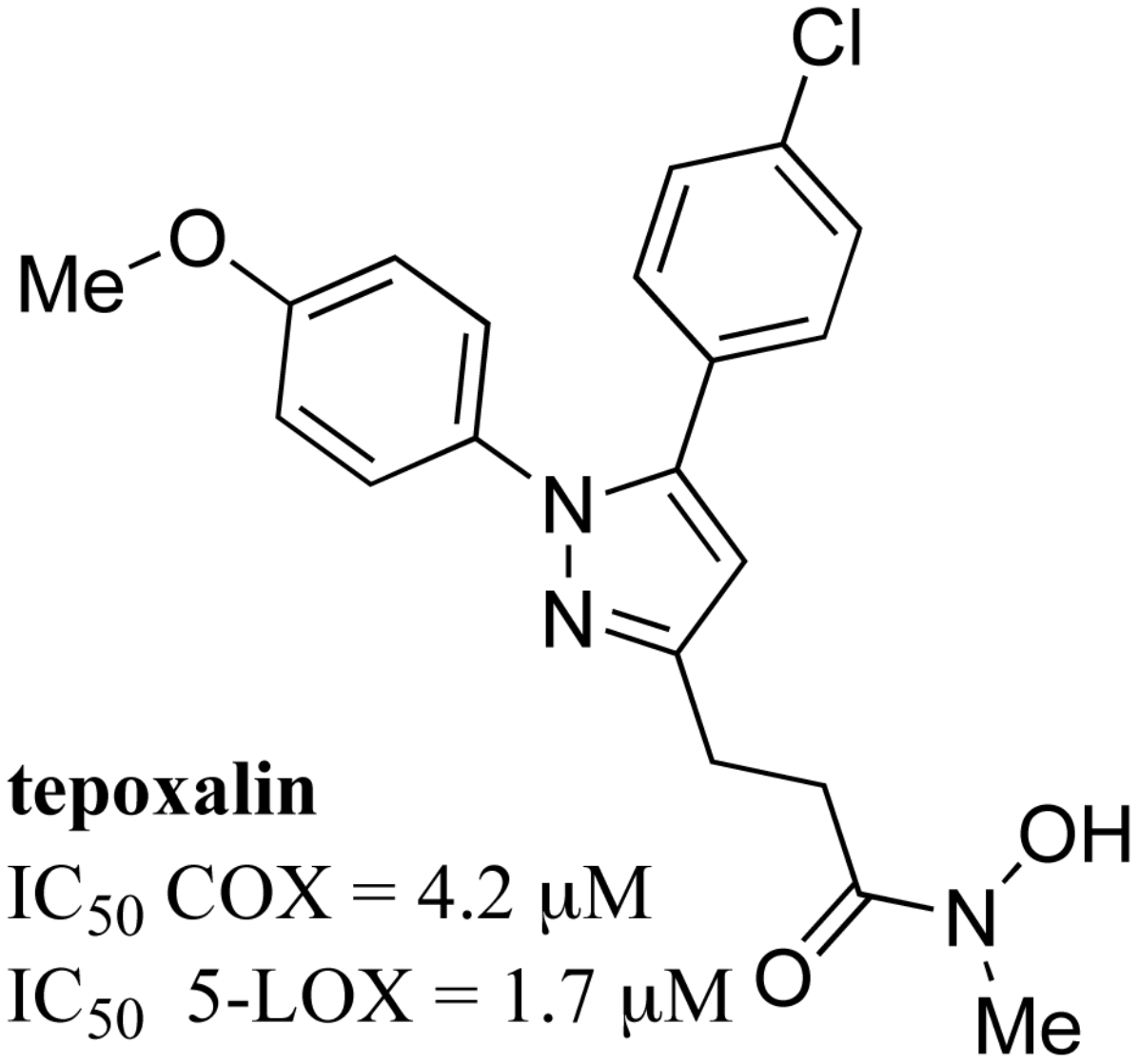
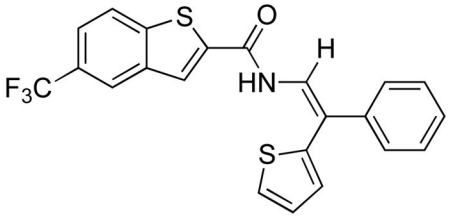
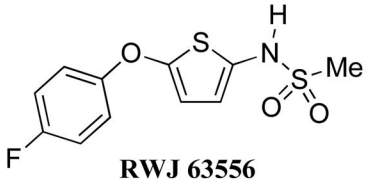


Fig. 7.
Structure of tepoxalin.



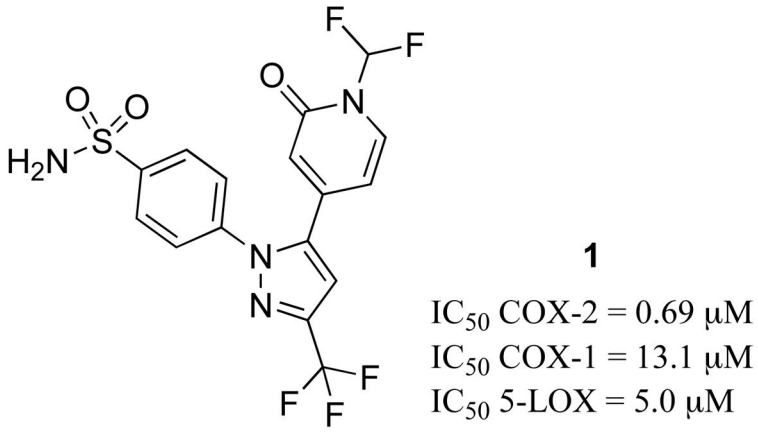
L-652,343



RWJ 63556

IC_{50} COX-2 = 1.86 μM
IC_{50} COX-1 > 10 μM
IC_{50} 5-LOX = 0.13 μM

Fig. 8.
Structures of thiophene based dual inhibitors.



1

IC_{50} COX-2 = 0.69 μM
IC_{50} COX-1 = 13.1 μM
IC_{50} 5-LOX = 5.0 μM

Fig. 9.
Structure of pyridone based dual inhibitor.

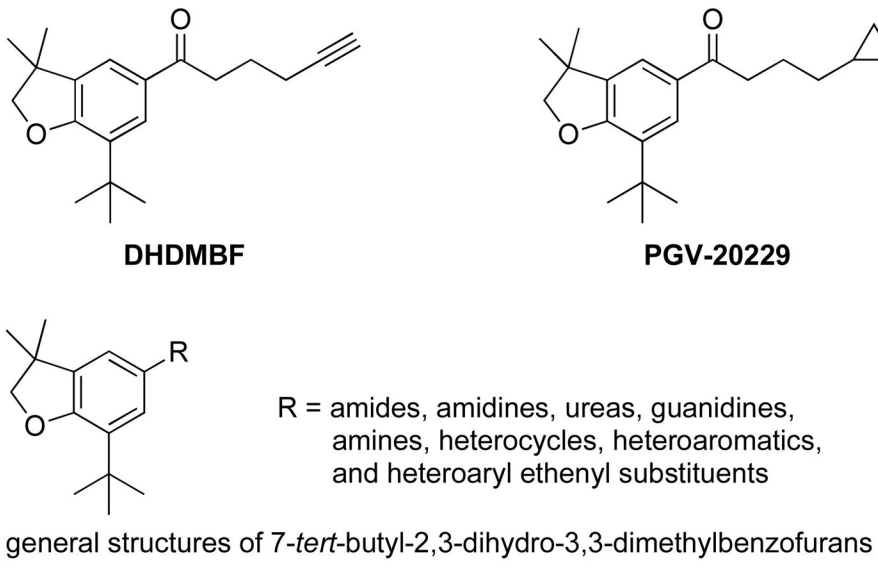
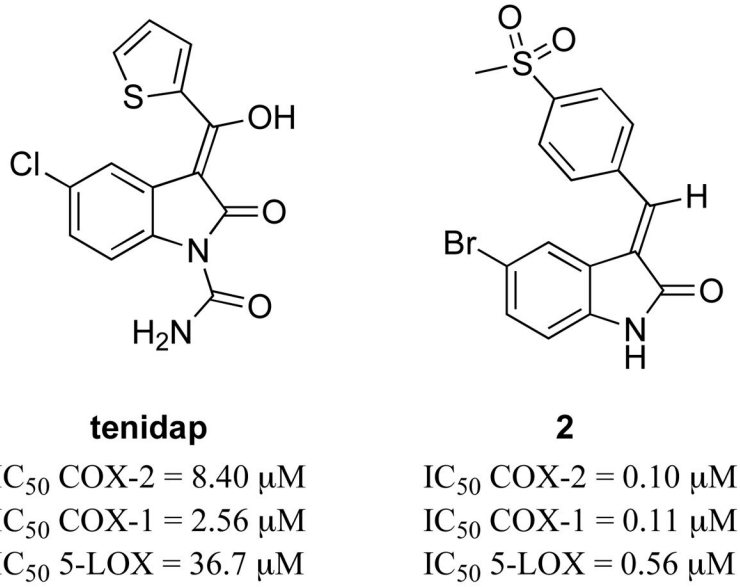


Fig. 10.
Structures of DHDMBFs.



tenidap

IC_{50} COX-2 = 8.40 μM
IC_{50} COX-1 = 2.56 μM
IC_{50} 5-LOX = 36.7 μM

2

IC_{50} COX-2 = 0.10 μM
IC_{50} COX-1 = 0.11 μM
IC_{50} 5-LOX = 0.56 μM

Fig. 11.
Structures of NSAID based COX/5-LOX dual inhibitors.

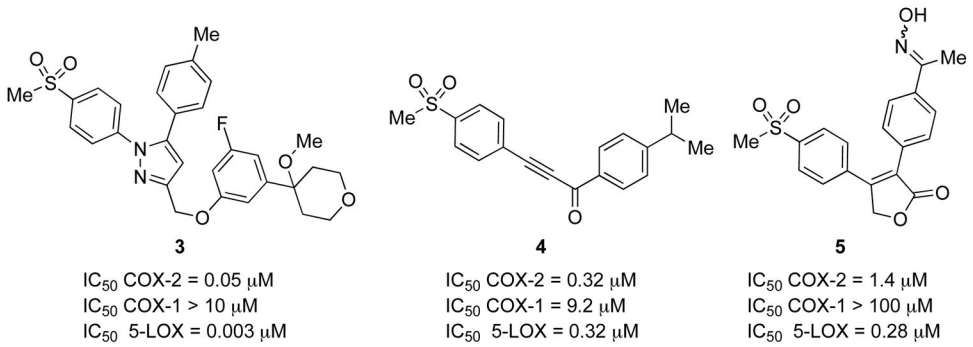


Fig. 12.
Structures of coxib based COX-2/5-LOX dual inhibitors.

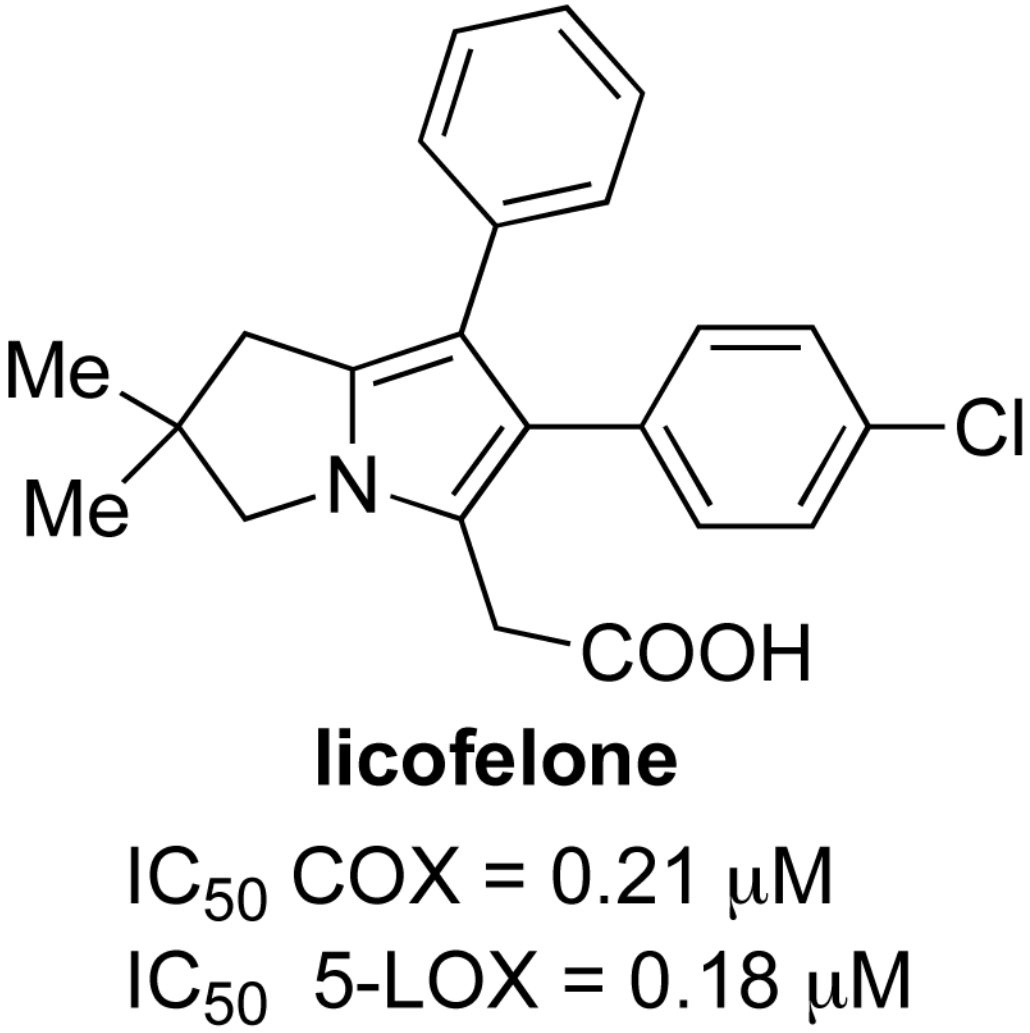


Fig. 13.
Structure of pyrrolizine based COX/5-LOX dual inhibitor.

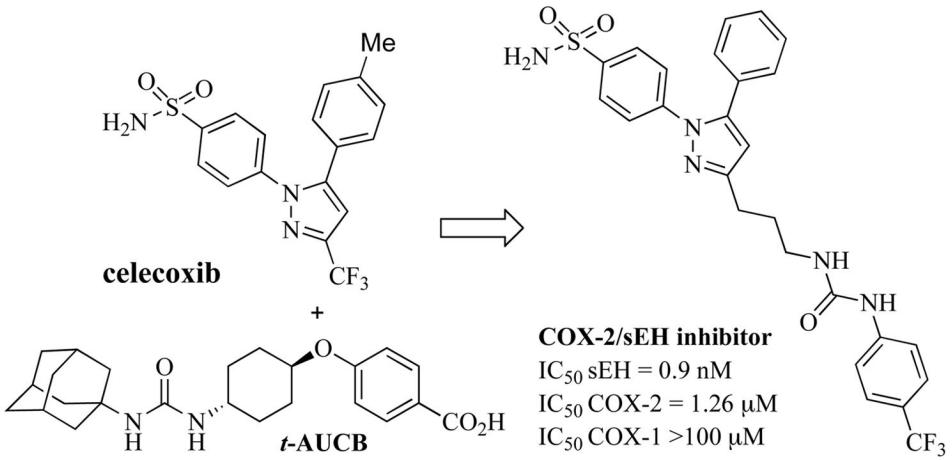


Fig. 14.
Structure of COX-2/sEH dual inhibitor.

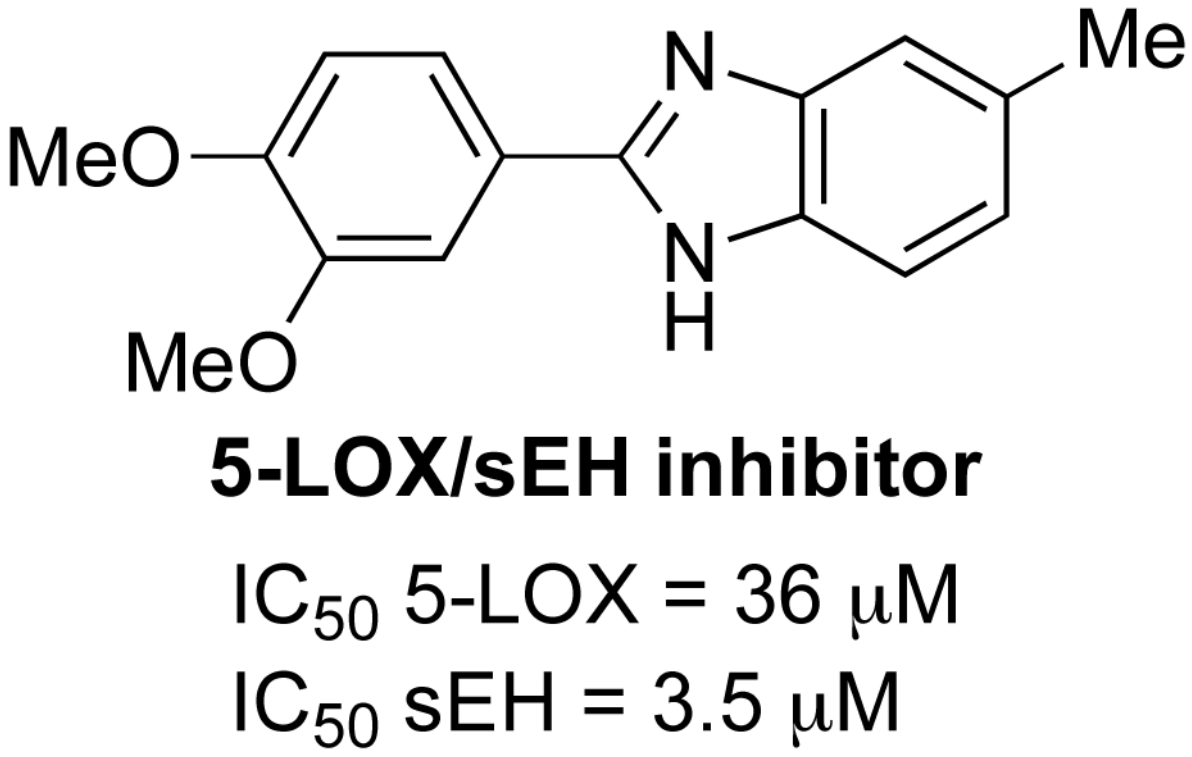


Fig. 15.
Structure of 5-LOX/sEH dual inhibitor.

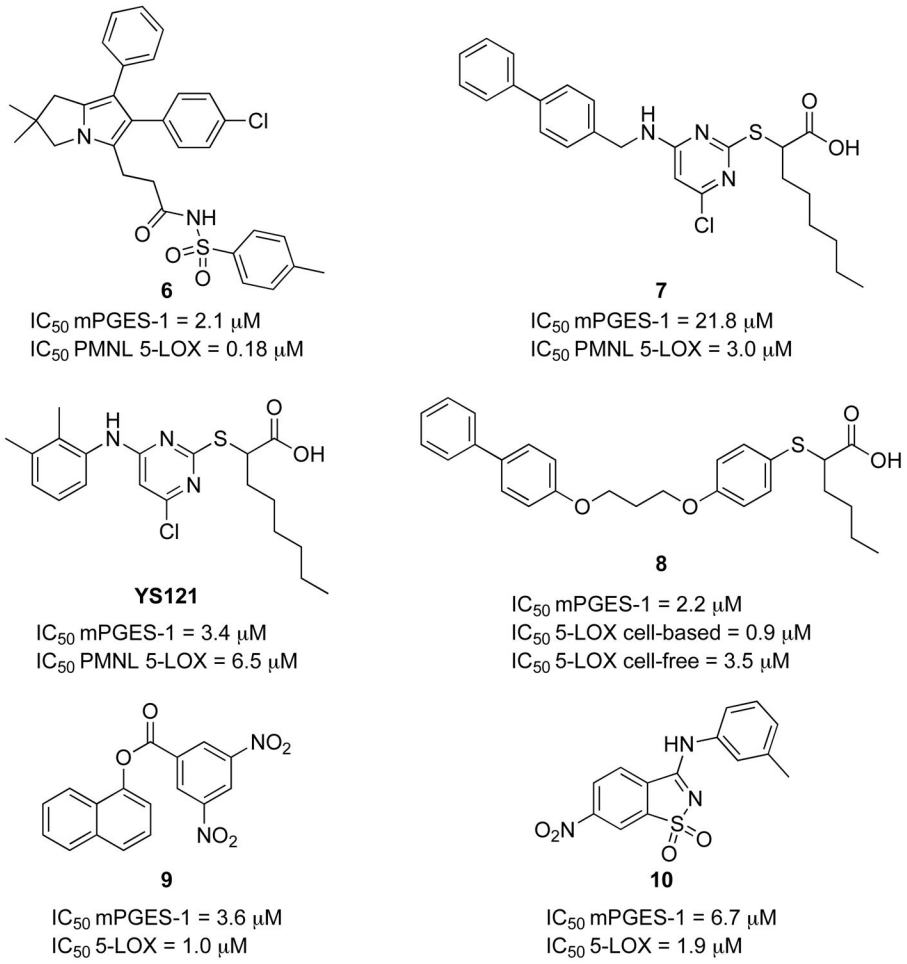


Fig. 16.
Structures of 5-LOX/mPGES-1 dual inhibitors.

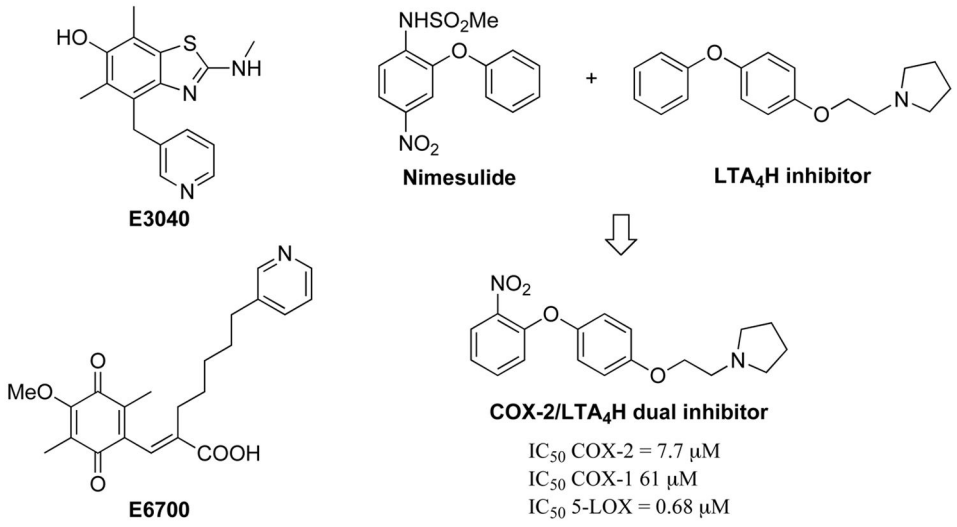


Fig. 17.

Structure of COX-2/LTA_4H and 5-LOX/TXA_2 synthase dual inhibitors.

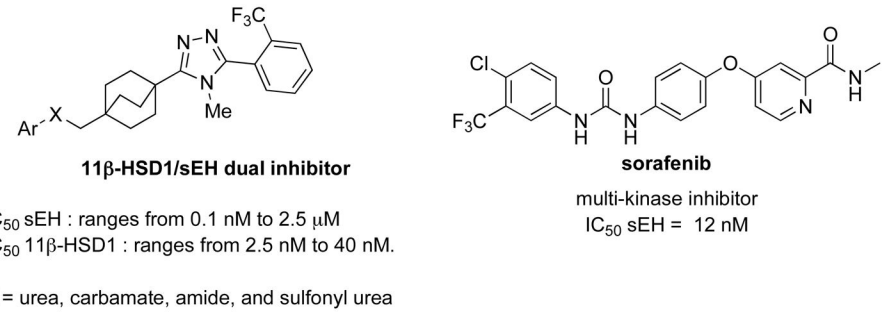


Fig. 18.

Structures of 11β-HSD1/sEH dual inhibitors and sorafenib.