5-Lipoxygenase Metabolic Contributions to NSAID-Induced Organ Toxicity

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ABSTRACT

Cyclooxygenase (COX)-1, COX-2, and 5-lipoxygenase (5-LOX) enzymes produce effectors of pain and inflammation in osteoarthritis (OA) and many other diseases. All three enzymes play a key role in the metabolism of arachidonic acid (AA) to inflammatory fatty acids, which contribute to the deterioration of cartilage. AA is derived from both phospholipase A₂ (PLA₂) conversion of cell membrane phospholipids and dietary consumption of omega-6 fatty acids. Nonsteroidal antiinflammatory drugs (NSAIDs) inhibit the COX enzymes,

but show no anti-5-LOX activity to prevent the formation of leukotrienes (LTs). Cysteinyl LTs, such as LTC₄, LTD₄, LTE₄, and leukoattractive LTB₄ accumulate in several organs of mammals in response to NSAID consumption. Elevated 5-LOX-mediated AA metabolism may contribute to the side-effect profile observed for NSAIDs in OA. Current therapeutics under development, so-called “dual inhibitors” of COX and 5-LOX, show improved side-effect profiles and may represent a new option in the management of OA.

Keywords: arachidonic acid; cyclooxygenase; flavocoxid; leukotrienes; licofelone; 5-lipoxygenase; NSAIDs; prostacyclin; prostaglandin; tepoxalin; thromboxane

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INTRODUCTION

Leukotrienes (LTs) play an important role in normal inflammatory processes. They are also intimately involved in allergic asthma, atopic dermatitis, allergic rhinitis, arthritis, atherosclerosis and ischemia, tumorigenesis, and septic shock [1–7]. LTs are leukoattractive substances as well as active modifiers of vascular dimension, fluid

balance, immunity, and pain responses. LTB_4 is a potent chemoattractive agent, which activates neutrophils at the site of injury by binding to cell receptors, inducing cell adhesion, neutrophil degranulation with release of degradative tissue enzymes, an increased production of cytokines, and pain induction [8]. Cysteinyl LTs induce vasoconstriction, mucus secretion, increased vascular permeability, and act in immunomodulation by binding specific receptors in smooth muscle and endothelial cells. Uncontrolled expression of LTs is instrumental in the pathogenesis of a wide variety of diseases affecting pulmonary, gastrointestinal (GI), cardiovascular (CV), and musculoskeletal function, including osteoarthritis (OA) [9].

Sánchez-Borges et al. [10] have suggested that, second only to β -lactam drugs, nonsteroidal antiinflammatory drugs (NSAIDs), as a class, are the leading cause of acute drug reactions and drug-induced side effects. Since 5-lipoxygenase (5-LOX) contributions to NSAID-induced adverse events are not widely recognized, it is important to understand these mechanisms and develop new types of antiinflammatory compounds that inhibit 5-LOX as well as the cyclooxygenase (COX) enzymes. This review presents available literature evidence published on Medline and PubMed between 1980–2011 that supports the hypothesis that NSAID-induced 5-LOX upregulation of LT production from arachidonic acid (AA) may contribute to NSAID side effects, particularly those affecting the pulmonary, GI, CV, and renal systems. In addition, musculoskeletal effects of LTs, which occur after NSAID administration, will be described. Examples of current “dual inhibitors” of COX and 5-LOX enzymes, both marketed and under development, are provided as they relate to reducing organ-specific adverse events.

METHODS

The authors independently reviewed the Medline and PubMed databases using the search words cyclooxygenase, COX-1, COX-2, lipoxygenase, 5-LOX, nonsteroidal antiinflammatory drugs, NSAID toxicity, NSAID organ toxicity, arachidonic acid metabolism, and combinations thereof. Of more than 1000 scientific papers reviewed, the authors identified and summarized several hundred that were thought to be most relevant. These publications were then reviewed in detail and published papers selected as being most pertinent for the purposes of this review. This review is intended to present the current knowledge in peer-reviewed literature with regard to NSAID-induced increases in LTs through the 5-LOX enzyme, and is not intended as a systematic review.

AA METABOLISM

COX Enzyme Metabolism of AA

Although this review is not intended to compare the mechanisms of AA metabolism by the COX or 5-LOX enzymes in detail, it is important to understand basic fatty acid metabolism through these pathways. AA is an essential fatty acid obtained from the diet as well as from the enzymatic conversion of phospholipids from damaged cell membranes by phospholipase A_2 (PLA_2) [11]. This fatty acid is a necessary substrate for a variety of physiological processes, including those involving cell membrane composition, platelet function, inflammation, and tissue function and repair. During the metabolic conversion of AA by the COX enzymes, two oxygen molecules are added by a cyclooxygenase activity to AA to yield prostaglandin (PG)- G_2 [12]. The PGG_2 intermediate is then converted to PGH_2

by peroxidase activity via a reduction reaction. A variety of cellular and tissue specific isomerases and synthases then convert PGH_2 to various PGs and prostacyclin (PC) as well as thromboxane (Tx).

COX-1 is generally thought of as a housekeeping enzyme, maintaining low-level generation of fatty acid metabolites in various organs, while COX-2 is the inducible form of the enzyme [12]. It was originally hypothesized that differences in AA metabolism between COX-1 and COX-2 could be accounted for due to compartmentalization within the cell. Both enzymes, however, are associated with the endoplasmic reticulum and nuclear membranes in equal proportions [13]. The cyclooxygenase activity of COX-2 requires approximately 10-fold less hydroperoxide for activation than that of COX-1, and metabolizes AA with a greater turnover rate [12]. It has also been shown that both COX-1 and COX-2 produce maintenance levels of PGI_2 , a vasodilator, from human endothelial cells [14]. PGI_2 is, however, produced at a faster rate by COX-2 with a threefold lower K_m and 2.5-fold faster initial rate of velocity compared to COX-1 [15]. In addition, each isozyme appears to be coupled to specific synthases and/or isomerases for the final conversion of the PGH_2 intermediate from each COX enzyme [11]. For example, PGI_2 is specifically produced in the CV system through coupling of COX-2 with PGI_2 synthase, while TxA_2 production is coupled in platelets to COX-1 and TxA_2 synthase. The importance of this coupling of the COX enzymes with specific synthases and isomerases led to the development of cyclooxygenase inhibitors with overlapping, yet specific side-effect profiles. The idealized conversion of phospholipids and dietary omega-6 fatty acids to AA and then to fatty acid metabolites is shown in Fig. 1.

Fig. 1. Metabolism of arachidonic acid by COX-1 and COX-2. COX=cyclooxygenase; PG=prostaglandin; PLA_2 =phospholipase A_2 ; TxA_2 =thromboxane A_2 .

Selective COX-2 inhibitors were conceived and designed to preserve PG production in the stomach and, thus, reduce the incidence of upper GI damage [16,17]. Six-month, well-controlled trials of celecoxib against traditional NSAIDs have shown this selective COX-2 inhibitor to be GI sparing [17,18]. In an analysis by the US Food and Drug Administration (FDA), however, follow-up of subjects in the Celecoxib Long-term Arthritis Safety Study (CLASS) trial for an additional 6 months after the trial ended showed that when total ulcer complications were considered, according to the pre-specified criteria in the trial protocol, there was no appreciable difference in cumulative percentage between celecoxib and traditional NSAIDs [19,20]. Until longer, well-controlled, or phase 4 studies are performed comparing celecoxib to traditional NSAIDs, there will be no definitive answer on this subject. Part of the reason for long-term ulcer complications with selective COX-2 NSAIDs may be that both COX-1 and

COX-2 are required for maintenance of the gastric mucosa. GI toxicity of COX-2 agents is aggravated in the presence of even low-dose aspirin taken for cardioprotective purposes [21]. Therefore, PGs produced by both COX-1 and COX-2 contribute to the healing responses in the stomach.

PGI₂, generated from AA by COX enzymes in arterial endothelial cells, is required for vasodilatation of vessels and is antagonistic to vasoconstrictive TxA₂ produced in platelets [22,23]. Selective COX-2 agents inhibit PGI₂ generation while minimally affecting TxA₂ in renal and cardiac microvasculature [24,25]. Such hemodynamic changes present clinically with reduced urine volume, hypertension, peripheral edema, myocardial ischemia, and may lead to acute CV events, such as myocardial infarction (MI) and stroke, particularly in susceptible individuals with pre-existing atherosclerotic disease. Immediately before MI, the level of vasodilatory PGs and PCs increases dramatically [26,27]. In animal models of MI, the concentrations of PGs and PCs were reduced when rats and mice were administered a selective COX-2 inhibitor compared with a placebo [28].

A meta-analysis of more than 4400 patients taking celecoxib for at least 6 weeks showed that there was an increase in MI over placebo, whereas there was no difference in composite analysis of CV deaths and strokes to placebo [29]. In addition, the same authors showed a significant increased risk of MI for celecoxib compared to placebo, diclofenac, ibuprofen, and paracetamol in a secondary meta-analysis of six studies (12,780 patients), but not other outcome measures. Although celecoxib appears not to elevate risk of overall CV dysfunction in normal patients compared to traditional NSAIDs, the data on rofecoxib are more compelling. Elevated CV adverse events, as shown in the VioxxTM GI Outcomes Research (VIGOR) trial [16],

inaccuracies in reporting that were discovered after publication of the trial for early MIs [30], and an excess of thrombotic events compared to placebo in the Adenoma Polyp Prevention in VioxxTM (APPROVe) study [31] caused Merck to withdrawal rofecoxib from the market in September 2004 [32]. After this point, all NSAIDs carried the same CV warnings based on analysis of adverse events in the literature and in-market side effects. A recent review by Herman [33] summarized CV risk factors for naproxen, ibuprofen, and acetaminophen, with ibuprofen having the highest risk for adverse events. New findings from Danish nationwide registries of 83,677 patients with previous MIs found that even short-term exposure to NSAIDs substantially increased death/recurrent MIs [34].

PGs are potent renal vasodilators and, together with PCs, are required for proper renal perfusion and regulation of salt balance [35]. In the kidney, COX-2 is produced constitutively and upregulated in the presence of salt deprivation [36]. In the presence of reduced circulatory volume, increased production of vasoconstrictive compounds, such as Tx_s, supports blood flow by increasing blood pressure. In hypertensive individuals, selective COX-2 inhibitors were found to further increase systolic pressure [37,38]; thus, contributing to the increased incidence of acute CV events [39]. Chronic NSAID users ($n=882$) with hypertension or coronary artery disease compared to nonchronic NSAID users ($n=21,694$) analyzed from the INternational Verapamil Trandolapril STudy (INVEST) had an increased risk of CV adverse events during long-term follow-up [40]. Based on this analysis and many others, all NSAIDs appear to affect blood pressure to varying degrees. The dynamic process of AA metabolism and inhibition of specific fatty acid metabolites in the induction of NSAID-associated side effects may be further complicated by 5-LOX

pathway metabolite production in the presence of antiinflammatory agents.

5-LOX Metabolism of AA

LTs are normally produced by neutrophils, eosinophils, basophils, monocytes, macrophages, mast cells, and B lymphocytes in response to trauma, infection, and/or inflammation. Lipid peroxidation of AA and linoleic acid results in the production of 4-hydroxynonenal (4-HNE). The fatty acid-derived 4-HNE, together with reactive oxygen species (ROS), act as a stimulus through mitogen-activated protein (MAP) kinase pathways to induce *5-lox* gene expression primarily via nuclear factor- κ B (NF- κ B) and early growth response factor-1 (Egr-1) transcription factors (Fig. 2) [41]. The *5-lox* gene promoter and intronic sequences contain

a number of inducible elements for binding of transcription factors, such as NF- κ B, Sp1, Erg-1, Myb, and vitamin D₃ [42]. A helper protein, 5-LOX activating protein (FLAP), is coactivated and coexpressed with 5-LOX [43]. Lipid peroxidation, as a result of poor oxidative status, also destabilizes cell membranes, leading to induction of calcium-dependent PLA₂ (cPLA₂), which hydrolytically attacks phospholipids, leading to the generation of AA [44]. The 5-LOX enzyme translocates to the nuclear membrane and, along with FLAP, converts AA to LTs in a multistep process. AA, coordinated by FLAP, is converted by 5-LOX to the intermediate 5-hydroperoxyeicosatetraenoic acid (HPETE) (Fig. 3). HPETE is converted to LTA₄ by several NF- κ B-dependent intermediate steps stimulated by cytokine-coupled ROS generation [45–47]. LTB₄ and LTC₄ are then formed from LTA₄ by hydration and enzymatic steps involving

Fig. 2. Induction of 5-LOX and FLAP. AA=arachidonic acid; cPLA₂=calcium-dependent phospholipase A₂; Egr=early growth response factor; FLAP=5-LOX activating protein; HNE=hydroxynonenal; LOX=lipoxygenase; MAPK=mitogen-activated protein kinase; NFκB=nuclear factor-κB; ROS=reactive oxygen species.

Fig. 3. Metabolism of arachidonic acid by 5-LOX.

FLAP=5-lipoxygenase activating protein;

HPETE=hydroperoxyeicosatetraenoic;

LOX=lipoxygenase; LT=leukotriene.

conversion by glutathione S-transferase, respectively. LTD₄ is formed by liberation of glutaric acid from LTC₄, and LTE₄ by conversion of LTD₄ by glutamyltranspeptidase and dipeptidase activity (Fig. 3).

Other Pathways of AA Metabolism

Epoxygenase-derived eicosanoids are produced from AA by cytochrome P (CYP)-450 enzymes in response to vascular endothelial inflammation [48]. In particular, CYP2C and CYP2J family enzymes catalyze epoxidation of AA to epoxyeicosatrienoic acids [49]. Epoxyeicosatrienoic acids are then hydrolyzed to dihydroxyeicosatrienoic acids by soluble epoxide hydrolase and counteract vascular inflammation [50]. Mutations in CYP2J2 have been associated with elevated risk of CV dysfunction [51]. Alternatively, CYP4A and CYP4F family enzymes convert AA to 20-hydroxyeicosatetraenoic acid, which is a

potent vasoconstrictor that, when upregulated, contributes to oxidative stress and endothelial dysfunction in the CV system, and increases peripheral vascular resistance associated with some forms of hypertension [52]. AA is also metabolized by 12-LOX and 15-LOX to generate antiinflammatory lipoxins with and without the involvement of aspirin as a cofactor [53]. Although these enzymes represent alternate AA processing pathways and may produce molecules that counter or augment some of the effects of elevated LT generation and an overabundance of Txs in blood vessels, they are beyond the scope of this review article.

NSAID-ASSOCIATED, LT-MEDIATED ORGAN TOXICITY

Pulmonary

In patients with aspirin intolerance, nasal secretions contain increased levels of LTC₄ and LTD₄ during reactions to aspirin [54]. Aspirin-intolerant syndrome is characterized by urticaria, wheezing, bronchoconstriction, nasal polyps, and in severe instances, laryngeal edema and respiratory insufficiency [3]. Although there are several subtypes of aspirin sensitivity, these reactions have in common increased AA metabolism through the 5-LOX pathway, possibly due to blockage of the COX-1 synthase enzyme and reduction in available PGE₂. As a consequence, an under-opposed increase in LT synthetase activity results in elevated tissue levels of bronchoconstrictive LTC₄ and LTD₄, histamine, and FLAP [55,56]. The incidence of aspirin-intolerant syndrome is about 10% of the US population, with most NSAIDs showing some level of cross-reactivity [57,58]. COX-2 inhibitors also cross-react with aspirin with a much lower incidence and the order of cross-reactivity appears to vary inversely with the

strength and selectivity of COX-2 inhibition, eg, nimesulide > meloxicam > rofecoxib [59]. The data on celecoxib is mixed, with at least two small studies ($n=27$ and $n=21$) where tolerance was established by gradually increasing dose, showing no bronchospasms in asthmatic patients [60,61]. Another small study ($n=59$) and individual patient reports, however, show that celecoxib has a very low, but measurable, incidence of upper respiratory reaction in sensitive populations [62–64]. This suggests there is less cross-reactivity for more selective COX-2 inhibitors with aspirin compared to traditional NSAIDs. Theoretically, antiinflammatory agents with 5-LOX inhibitory activity should be well-tolerated by individuals with aspirin sensitivities despite inhibition of the COX enzymes.

Licofelone (also known as ML3000), a dual inhibitor of COX and 5-LOX currently in phase 3 clinical trials in Europe, has been shown to mitigate the effects of antigen-induced asthma in a sheep model [65]. Another dual inhibiting therapeutic, flavocoxid, a medical food for the management of OA administered under physician supervision, a federal statutory requirement for this category in the US (Orphan Drug Act, 1988, 21 U.S.C. 360ee [b] [3]), exhibited significantly fewer upper respiratory adverse events in a clinical safety study when compared to placebo in OA subjects, possibly due to a reduction of LT expression as well as potential antiinfective properties [66].

GI

A number of factors contribute to GI ulceration, such as infection by *Helicobacter pylori*, production of ROS, and inflammatory responses, including LT generation [67]. Normally PGE_2 , produced by COX-1 in the gut, serves to protect the gastric mucosa by promoting mucous secretion and downregulating ROS production

from neutrophils. Leukoattractive molecules promote neutrophil infiltration, which, in turn, results in high levels of ROS production in the mucosa and submucosa [67]. Although not chemoattractive to leukocytes, cysteinyl LTs cause vasoconstriction and ischemia, thus contributing to tissue damage [68]. Indomethacin and aspirin-induced gastric ulceration, for example, correlates with increased LT levels in the gastric mucosa of rats and pigs [69,70]. Administration of MK-886, a FLAP inhibitor, prior to indomethacin or aspirin treatment reduces the extent of gastric lesions in these animal models and reduces the indomethacin-triggered production of LTB_4 . Even a relatively balanced COX enzyme inhibitor, such as nabumetone, which has a somewhat lower risk of GI side effects than other NSAIDs [71], increases metabolism of AA by 5-LOX. The nabumetone metabolite, 6-methoxy-2-naphthylacetic acid, has COX inhibitory activity but also increases LTB_4 levels in rat mucosa in vitro [72]. The 5-LOX inhibitor, phenidone, and the dual inhibitor of COX and 5-LOX, BW755C (3-amino-1-[trifluoromethylphenyl]-2-pyrazoline hydrochloride), both suppress LTB_4 production in rat mucosal tissue whilst in the presence of nabumetone in this model.

Wallace and Ma [67] suggest that metabolic shunting toward the 5-LOX pathway is a major contributor to gastric injury in humans. When stimulated by calcium ionophore, human gastric mucosa and GI smooth muscle shows a reduction in the release of PGE_2 and increases in production of LTB_4 and cysteinyl LTs [73]. LT generation in this model is specifically reduced by the 5-LOX inhibitor, nordihydroguaiaretic acid (NDGA), and the COX/5-LOX dual inhibitor, BW755C. In human subjects, indomethacin (150 mg/day) produces endoscopically verified gastric damage that has been linked to LTB_4 -mediated leukocyte adherence in blood vessels

of the gut [74]. In the same study, when subjects were coadministered indomethacin and a somatostatin analog that reduced leukocyte adherence, gastric lesions were ameliorated. Other studies also support the hypothesis that LTB₄ attracts both eosinophils and neutrophils to the gastric mucosa endothelium when NSAIDs reduce PGE₂, thereby causing damage to the submucosa [75–77]. In rheumatoid arthritis (RA) and OA subjects taking NSAIDs, a study showed statistically elevated levels of LTB₄ and reduced PGE₂ content in gastric biopsies compared with those taking placebo [78]. The evidence cited above suggests LT involvement in NSAID-induced GI toxicity.

Tepoxalin, a combined cyclooxygenase/ peroxidase inhibitor of COX-1, COX-2, and 5-LOX, decreases LTB₄ levels, neutrophil adhesion, gastric inflammation, and mucosal damage in rats, and is approved for use in canines for control of pain and inflammation associated with OA [79]. In human subjects, no ulcerations are found with doses of 200 or 400 mg twice daily (b.i.d.) of licofelone, whereas 500 mg of naproxen b.i.d. produces gastric lesions in 20% of subjects over a 4-week period when assessed by endoscopy [80]. Flavocoxid (500 mg b.i.d.) showed statistically fewer upper GI adverse events compared to naproxen (500 mg b.i.d.) over a 12-week period [81]. Flavocoxid also had better overall tolerability in previously NSAID GI-intolerant patients over an 8-week administration and a reduction or cessation in gastroprotective medication use in these same patients [82].

CV

Mice with heterogeneous knockouts of the *5-lox* gene maintained on high fat and cholesterol diets show a substantial decrease in atherosclerotic aortic lesions compared to a control wild-type strain [83].

5-LOX-expressing macrophages localize to aortic aneurysms and arterial lesions in both mice and humans [84,85], and mutations in the *5-lox*, *LTA4 hydrolase*, and *arachidonate 5-LOX-activating protein (ALOX5AP)* genes, coding for FLAP, upregulate the production of LTB₄ and are associated with an increased incidence of atherosclerosis, MI, and stroke in humans, especially in patients with high AA intakes due to poor diet [86–89]. Elevated serum LT concentrations occur in patients with acute CV events, suggesting involvement of 5-LOX-mediated AA metabolism in CV disease [90].

In the VIGOR trial, rofecoxib showed a fourfold increase in CV-related events compared with naproxen [16]. Additionally, a retrospective study of over 54,000 patients in two different health plans showed a threefold increase in heart attack among elderly adults in the first 90 days of rofecoxib therapy of 25 mg or greater per day [91]. Similarly, in a retrospective cohort study of 610,001 persons in a Medicaid population, acute MI, stroke, and death from coronary heart disease for patients taking celecoxib, rofecoxib, valdecoxib, ibuprofen, naproxen, diclofenac, and indomethacin were studied [92]. An increased risk of CV adverse events among all users of rofecoxib, valdecoxib, and indomethacin was found in patients with no CV disease. In patients with known CV disease, rofecoxib was associated with an increased risk of CV adverse events. The data on celecoxib is conflicting at doses higher than 200 mg per day. Two studies of celecoxib at ≥400 mg per day showed an increase the incidence of CV complications in a dose-dependent manner [93,94], whereas the Arthritis, Diet, and Activity Promotion Trial (ADAPT) showed no statistical increase in CV adverse events compared to placebo for celecoxib administered at 200 mg b.i.d. or 400 mg daily [95]. All NSAIDs increase the risk of CV dysfunction, with naproxen recently

shown to be the safest in a large meta-analysis [96], though selective COX-2 agents are thought to elevate CV risks [97]. An association with an imbalance of COX-1 versus COX-2 activity that may reduce the concentration of vasodilatory PCs compared to vasoconstrictive TxS has been suggested as a possible cause for the elevated CV adverse event profile of these drugs [24]. Increased generation of vasoconstrictive cysteinyl LTs by 5-LOX while on NSAIDs may also play a role.

A variety of cardiopulmonary complications highlight the interplay between the COX enzymes and the 5-LOX pathway [21,98]. Afferent vessel response in ischemic reperfusion is abolished by indomethacin in rats [99]. A specific 5-LOX inhibitor reverses this effect, presumably by suppressing LT production. Vasoconstriction observed in rings of human internal mammary arteries in organ baths exposed to angiotensin II is mediated by the action of TxA_2 [100]. This activity, however, can be abated with a TxA_2 agonist. In the same system, indomethacin caused an increase in angiotensin II-mediated vasoconstriction and LTC_4 production. Specific 5-LOX inhibitors, such as phenidone and baicalein, prevented LTC_4 generation in this same assay system [100]. Animals treated with celecoxib showed increased LT production in vascular tissue in the breast [101]. In humans with lung inflammation, 200 mg per day of celecoxib elevated levels of LTE_4 in urine [102]. Cultured human pulmonary artery endothelial cells exposed to calcium ionophore induce LT generation [103]. Hypoxic CV tissue from animals and humans demonstrates a similar leukocyte attraction mediated by LTB_4 , and vasoconstriction by LTC_4 and LTD_4 [104-106]. Licofelone and the dual COX/5-LOX inhibitors, CI-986 and BW-755C, counteracted the LT-mediated effects in these model systems. In rats with atherosclerotic

lesions, licofelone reduces neo-intimal formation and decreases 5-LOX expression and LTB_4 production in femoral arteries compared to rofecoxib [106]. Conversely, rofecoxib caused a nonstatistically significant increase in 5-LOX expression. Based on the evidence cited above, NSAIDs promote increased generation of LTs and could potentially exacerbate both known and undiagnosed CV disease through 5-LOX-mediated LT generation.

Renal

Oxidative status and inflammation contribute to kidney disease, particularly during renal failure and hemodialysis [107]. Low polyunsaturated fatty acid intake correlates with the development of renal disease [108]. COX-1 and COX-2, as well as metabolites generated from polyunsaturated fatty acids by these enzymes, are important for renal function. In the kidney, COX-2 is produced constitutively [109]. Both PGs and PCs are required for proper renal perfusion and as key regulators of salt balance [35]. PGs, like PCs, are strong renal vasodilators, which maintain urine production by regulating renal blood flow. It has been found that macrophages present in the glomeruli synthesize LTB_4 when exposed to calcium ionophore, suggesting that the 5-LOX pathway is part of the normal regulatory capacity within the kidneys [110]. 5-LOX and FLAP are expressed at higher levels in humans with glomerulonephritis [111].

All NSAIDs have the capacity to cause renal damage. Under these circumstances, renal upregulation of 5-LOX expression and an increase in LTB_4 generation causes influx of activated leukocytes that produce histamine, ROS, and cytokines [112-114]. Specific 5-LOX inhibition of LTB_4 and cysteinyl LT biosynthesis decreases LTC_4 synthase activity and reduces renal impairment in experimental

glomerulonephritis [115,116]. Zileuton, a 5-LOX inhibitor, reduces the inflammatory effects of LTB₄ in wild-type animals in this model [117]. In human clinical trials, although there was a lower incidence of hypertension with licofelone, there was no statistical difference between naproxen and licofelone in edema [118]. Tepoxalin did not affect renal function in canines [119,120]. Flavocoxid showed no statistically significant effect on markers of renal function in three different animal species [121-123] or in humans [82,121,124]. In a long-term, randomized, double-blind study there was a nonstatistically significant trend toward reduction in serum creatinine in the flavocoxid group compared with a similar trend toward an increase in creatinine in the naproxen group [81]. Further long-term studies of dual COX/5-LOX inhibitors are required to clarify their overall renal safety.

Musculoskeletal

The etiology of OA is complex and often initiated by injury or trauma. During subsequent development of the disease, metabolic and inflammatory factors contribute to cellular and cartilage degradation leading to the release of phospholipids from damaged cells, which are then converted by PLA₂ into AA [125]. Other factors, such as a dietary imbalance between omega-6 and omega-3 fatty acid consumption, have also been implicated as a potential contributing factor to OA [126,127]. Treatment with NSAIDs also has the potential to aggravate cartilage damage by increasing intra-articular LT levels.

A greater induction of FLAP in cultured subchondral osteoblasts from patients with more severe OA was seen than from patients with moderate arthritis [128,129]. The combination of celecoxib and a 5-LOX inhibitor reduced the severity of collagen-induced cartilage

damage in mice compared to celecoxib or the 5-LOX inhibitor alone, suggesting the need for inhibition of both PG and LT production to prevent joint damage [130]. In a 1-year study, indomethacin decreased radiographically measured joint space in the knees of patients with OA compared to placebo [131]. One possible explanation for this result is induction of LT-coupled cytokine synthesis and LTB₄ mRNA expression promoted by indomethacin, naproxen, and the COX-2 inhibitor, NS-398, a phenomenon demonstrated in cultured human articular chondrocytes and explants [132-134]. For these reasons, dual inhibitors may represent a therapeutic option to potentially preserve cartilage in OA [135,136].

In canines in which the anterior cruciate ligament is sectioned to induce OA, licofelone reduces gene expression of *5-lox*, matrix metalloproteinases (MMPs), and several aggrecanases in synovial fluid [137]. Licofelone also reduces histological scores and synovial and vascular proliferation, as well as cartilage and bone destruction in rats [138]. Using this model, Boileau et al. [139] showed that licofelone reduces the chondrocyte apoptosis and the amount of aggrecanase, COX-2, and inducible nitric oxide synthase (iNOS) in the joint. Human and porcine synovial joint explants cultured for 1-5 days and exposed to tepoxalin had lower cytokine expression than with other 5-LOX inhibitors, such as MK-886 [140]. Tepoxalin, an inhibitor of both COX and 5-LOX enzymes, also decreased the release of cartilage proteoglycans from canine cartilage explants exposed to interleukin (IL)-1 β [141]. Finally, when subjects with symptomatic knee OA were treated with 200 mg licofelone b.i.d. or 500 mg naproxen b.i.d. for 2 years in a double-blind, randomized clinical trial to evaluate treatment effects on cartilage loss measured by magnetic resonance imaging (MRI), the licofelone group showed

significantly less cartilage volume loss compared to the naproxen group [142]. This result suggests that dual inhibitors have the potential to preserve cartilage volume in patients with OA.

RA, a true inflammatory arthritis, is also treated with NSAIDs along with disease modifying agents, biologics, and other therapies. It has long been known that RA patients have elevated LT both systemically, excreted in urine, and in synovial fluid [143–145]. In RA, synovial fluid contains dramatically elevated numbers of leukocytes comprised predominantly of neutrophils [146]. Although the involvement of LT in RA is not completely understood, LT-deficient mice have been shown to be remarkably resistant to induction of inflammatory joint damage [147]. Significant elevations of LTB_4 and LTC_4 from neutrophils are also present in animal models that mimic RA, suggesting that the 5-LOX pathway is significantly involved in generating inflammatory arthritis [148,149]. Neutrophils derived from RA patients treated with methotrexate show suppression of LTB_4 synthesis in culture [150]. LTB_4 has been shown in murine model of peritonitis to be involved in the amplification tumor necrosis factor (TNF)- α , IL-1 β , and IL-18 to LTB_4 as well [151]. Although these data are compelling, it remains to be seen whether LTs are a causative agent in the induction of RA.

Other Tissues

NSAID-induced LT generation also occurs in other tissues. Hypersensitivity to aspirin and other NSAIDs may produce urticaria and angioedema mediated by LTs, histamine, immunoglobulin (Ig)-E, and other inflammatory factors [152]. NSAIDs may also be etiologically associated with atopic dermatitis, especially in canines [153]. As with urticarial reactions [154],

reduction of LT activity is essential for control of this condition.

The lower digestive tract is particularly sensitive to the effects of antiinflammatory drugs, both from de novo-induced damage and from exacerbation of pre-existing inflammatory bowel diseases (IBD) [155]. IBDs are, in part, mediated by LTs [156]. NSAIDs aggravate these diseases by increasing production of ROS and LTs [157]. Although specific 5-LOX inhibitors, such as zileuton, have been used to treat conditions related to IBD [158], licofelone and tepoxalin have not been tested in these clinical settings. Flavonoids, like those in flavocoxid, reduce inflammation related to lower bowel diseases [159]. In an animal model of acute pancreatitis, flavocoxid reduces 5-LOX levels, blunts the induction of LTB_4 , reduces enzyme elevations, and preserves pancreatic histology [160].

COX/5-LOX “DUAL INHIBITORS” AND THEIR MECHANISMS OF ACTION

There are three COX/5-LOX dual inhibitors that are in the advanced stage of clinical development (licofelone) or on the market for OA as prescription therapeutics (tepoxalin and flavocoxid). Licofelone is a drug developed for humans, tepoxalin is approved for use in canines, and flavocoxid is a medical food indicated for OA in humans. Each of these agents has a distinct mechanism of action that may affect safety and efficacy. Licofelone strongly and specifically inhibits the cyclooxygenase moieties of the COX enzymes as well as 5-LOX induction [161].

The inhibition of LT generation by licofelone is due not to a direct interaction with 5-LOX, but rather to a modulation or interference with FLAP [162]. Licofelone has been shown to work by also downregulating specific inflammatory factors such as IL-1 β ,

MMP-13, cathepsin K, and aggrecanases via modulation of p38 kinase [137,163]. Tepoxalin inhibits both cyclooxygenase and peroxidase moieties of the COX-1 and COX-2 enzymes, as well as 5-LOX [164]. In addition, tepoxalin possesses an antioxidant activity, which inactivates NF- κ B [165], the controlling factor for inducible inflammatory molecules such as cytokines, COX-2, 5-LOX, and iNOS. Flavocoxid, though similar to both licofelone and tepoxalin, is unique in its antiinflammatory mechanism of action. Flavocoxid modulates NF- κ B activation in both macrophages and mice, decreasing expression of COX-2 and 5-LOX [166,167]. In addition, flavocoxid upregulates I- κ B α , the cytoplasmic control factor of NF- κ B, thus, downregulating gene expression for multiple cytokines, including iNOS, COX-2, and 5-LOX [166-168]. Unlike licofelone and tepoxalin, however, flavocoxid shows balanced inhibition of the peroxidase moieties of COX-1 and COX-2 enzymes, as well as having 5-LOX inhibitory activity [168]. Recent work employing oxygen-sensing cyclooxygenase assays, however, has shown flavocoxid to have very limited cyclooxygenase moiety inhibition of COX-1 and no detectable cyclooxygenase inhibitory activity of COX-2 compared to indomethacin and NS-398, respectively [168]. In addition, flavocoxid has been shown to downregulate both p38 and JunK [166]. Finally, flavocoxid has potent, direct antioxidant activity and prevents the oxidative generation of malondialdehyde [166,168].

CONCLUSION

The history of NSAIDs for the treatment of OA, while replete with examples of symptomatic efficacy, has been tainted by organ-specific toxicities. These side effects tend to overlap for both classes of NSAIDs and vary somewhat from agent to agent. The in-market frequency and

severity of some of these side effects in clinical trials has prompted “black box” warnings advising both physicians and patients about their potential risks on the GI tract and CV system. Two COX-2 inhibitors, rofecoxib and valdecoxib, were removed from the market, further contributing to an environment of confusion and caution regarding these agents. To date, clinicians have not fully appreciated the contributions of the 5-LOX pathway to NSAID-linked side effects as a result of induction of LTs.

This article reviews the putative contributions of the 5-LOX pathway of AA metabolism in the exacerbation of NSAID-induced injury to multiple organ systems and suggests the pervasiveness of this phenomenon. Although the extent and complete role of LTs induced by COX-inhibiting NSAIDs in the production of organ toxicity is not fully defined, there are numerous examples of LT upregulation in cell and animal models as well as humans to suggest that these fatty acids may complicate therapy. Because inhibition of COX enzymes increases the conversion of AA by 5-LOX to leukoattractive and vasoconstrictive LTs, specific dual inhibitors of both COX and 5-LOX have been developed and are currently being prescribed to manage OA in humans and canines. These dual inhibitors, due to their inhibition of both COX and 5-LOX enzymes and other unique properties have, on balance, a superior adverse event profile in clinical trials and in-market experience. Further clinical studies and extended post-market surveillance are required to fully elucidate their safety and efficacy.

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REFERENCES

- Hallstrand TS, Henderson WR. An update on the role of leukotrienes in asthma. *Curr Opin Allergy Clin Immunol*. 2010;10:60–66.
- Chari S, Clark-Loeser L, Shupack J, Washenik K. A role for leukotriene antagonists in atopic dermatitis? *Am J Clin Dermatol*. 2001;2:1–6.
- Peters-Golden M, Gleason MM, Togias A. Cysteinyl leukotrienes: multi-functional mediators in allergic rhinitis. *Clin Exp Allergy*. 2006;36:689–703.
- Mathis S, Jala VR, Haribabu B. Role of leukotriene B₄ receptors in rheumatoid arthritis. *Autoimmun Rev*. 2007;7:12–17.
- Back M. Leukotriene signaling in atherosclerosis and ischemia. *Cardiovasc Drugs Ther*. 2009;23:41–48.
- Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer*. 2010;10:181–193.
- Rossi A, Cuzzocrea S, Sautebin L. Involvement of leukotriene pathway in the pathogenesis of ischemia-reperfusion injury and septic and non-septic shock. *Curr Vasc Pharmacol*. 2009;7:185–197.
- Henderson WR. The role of leukotrienes in inflammation. *Ann Intern Med*. 1994;121:684–697.
- Peters-Golden M, Henderson WR Jr. Leukotrienes. *N Engl J Med*. 2007;357:1841–1854.
- Sánchez-Borges M, Caballero-Fonseca F, Capriles-Hulett A, Gonzalez-Aveledo L. Hypersensitivity reactions to nonsteroidal anti-inflammatory drugs: an update. *Pharmaceuticals*. 2010;3:10–18.
- Brash AR. Arachidonic acid as a bioactive molecule. *J Clin Invest*. 2001;107:1339–1345.
- Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem*. 2000;69:145–182.
- Spencer AG, Woods JW, Arakawa T, Singer II, Smith WL. Subcellular localization of prostaglandin endoperoxide H synthases-1 and -2 by immunoelectron microscopy. *J Biol Chem*. 1998;273:9886–9893.
- Bolego C, Buccellati C, Prada A, Gaion RM, Folco G, Sala A. Critical role of COX-1 in prostacyclin production by human endothelial cells under modification of hydroperoxide tone. *FASEB J*. 2009;23:605–612.
- Ruan CH, So SP, Ruan KH. Inducible COX-2 dominates over COX-1 in prostacyclin biosynthesis: mechanisms of COX-2 inhibitor risk to heart disease. *Life Sci*. 2011;88:24–30.
- Bombardier C, Laine L, Reicin A, et al. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med*. 2000;343:1520–1528.
- Silverstein FE, Faich G, Goldstein JL, et al. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: a randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA*. 2000;284:1247–1255.
- Chan FK, Lan A, Scheiman J, Berger MF, Nguyen H, Goldstein JL. Celecoxib versus omeprazole and diclofenac in patients with osteoarthritis and rheumatoid arthritis (CONDOR): a randomized trial. *Lancet*. 2010;376:173–179.
- Chaiamnuay S, Allison JJ, Curtis JR. Risks versus benefits of cyclooxygenase-2-selective nonsteroidal antiinflammatory drugs. *Am J Health Syst Pharm*. 2006;63:1837–1851.
- Jüni P, Dieppe P. Older people should NOT be prescribed “coxibs” in place of conventional NSAIDs. *Age Ageing*. 2004;33:100–104.
- Fiorucci S, Distrutti E, de Lima OM, et al. Relative contribution of acetylated cyclo-oxygenase (COX)-2 and 5-lipoxygenase (LOX) in regulating gastric mucosal integrity and adaptation to aspirin. *FASEB J*. 2003;17:1171–1173.

22. Bunting S, Moncada S, Vane JR. The prostacyclin-thromboxane A₂ balance: pathophysiological and therapeutic implications. *Br Med Bull*. 1983;39:271-276.
23. Davidge ST. Prostaglandin H synthase and vascular function. *Circ Res*. 2001;89:650-660.
24. Grosser T, Fries S, Fitzgerald GA. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest*. 2006;116:4-15.
25. Antman EM, DeMets D, Loscalzo J. Cyclooxygenase inhibition and cardiovascular risk. *Circulation*. 2005;112:759-770.
26. Bolli R, Shinmura K, Tang XL, et al. Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. *Cardiovasc Res*. 2002;55:506-519.
27. Dai W, Kloner RA. Relationship between cyclooxygenase-2 inhibition and thrombogenesis. *J Cardiovasc Pharmacol Ther*. 2004;9:51-59.
28. Guo Y, Bao W, Wu WJ, Shinmura K, Tang XL, Bolli R. Evidence for an essential role of cyclooxygenase-2 as a mediator of the late phase of ischemic preconditioning in mice. *Basic Res Cardiol*. 2000;95:479-484.
29. Caldwell B, Aldington S, Weatherall M, Shirtcliffe P, Beasley R. Risk of cardiovascular events and celecoxib: a systematic review and meta-analysis. *J R Soc Med*. 2006;99:132-140.
30. Targum SL. Consultation NDA 21-042, S-007. Review of cardiovascular safety database. Rofecoxib (MK-0966). US Food and Drug Administration. Available at: http://www.fda.gov/ohrms/dockets/ac/01/briefing/3677b2_06_cardio.pdf. Accessed October 4, 2011.
31. Bresalier RS, Sandler RS, Quan H, et al. Adenomatous Polyp Prevention on Vioxx (APPROVe) Trial Investigators. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med*. 2005;352:1092-1102. Published correction appears in *N Engl J Med*. 2006;355:221.
32. Merck and Co. Inc. Merck announces voluntary worldwide withdrawal of Vioxx (news release; September 30, 2004). Available at: http://www.merck.com/newsroom/vioxx/pdf/vioxx_press_release_final.pdf. Accessed November 2, 2006.
33. Herman M. Cardiovascular risk associate with nonsteroidal anti-inflammatory drugs. *Curr Rheumatol Reports*. 2009;11:31-35.
34. Schjerning Olsen AM, Fosbøl EL, Lindhardsen J, et al. Duration of treatment with nonsteroidal anti-inflammatory drugs and impact on risk of death and recurrent myocardial infarction in patients with prior myocardial infarction: a nationwide cohort study. *Circulation*. 2011;123:2226-2235.
35. Harris RC. Cyclooxygenase-2 in the kidney. *J Am Soc Nephrol*. 2000;11:2387-2394.
36. Whelton A, Hamilton CW. Nonsteroidal anti-inflammatory drugs: effects on kidney function. *J Clin Pharmacol*. 1991;31:588-598.
37. Whelton A, Schulman G, Wallemark C, et al. Effects of celecoxib and naproxen on renal function in the elderly. *Arch Intern Med*. 2000;160:1465-1470.
38. Whelton A, Fort JG, SUCCESS VI Study Group, et al. Cyclooxygenase-2-specific inhibitors and cardiorenal function: a randomized, controlled trial of celecoxib and rofecoxib in older hypertensive osteoarthritis patients. *Am J Ther*. 2001;8:85-95.
39. Deray G. Renal and cardiovascular effects of non-steroidal anti-inflammatories and selective cox 2 inhibitors. *Presse Med*. 2004;33:483-489.
40. Bavry AA, Khaliq A, Gong Y, Handberg EM, Cooper-Dehoff RM, Pepine CJ. Harmful effects of NSAIDs among patients with hypertension and coronary artery disease. *Am J Med*. 2011;124:614-620.
41. Chen SH, Fahmi H, Shi Q, Benderdour M. Regulation of microsomal prostaglandin E₂ synthase-1 and 5-lipoxygenase-activating protein/5-lipoxygenase by 4-hydroxynonenal in human osteoarthritic chondrocytes. *Arthritis Res Ther*. 2010;12:R21.
42. Seuter S, Vaisanen S, Radmark O, Carlberg C, Steinhilber D. Functional characterization of vitamin D responding regions in the human 5-lipoxygenase gene. *Biochim Biophys Acta*. 2007;1771:864-872.
43. Peters-Golden M, Brock TG. 5-Lipoxygenase and FLAP. *Prostaglandins Leukot Essent Fatty Acids*. 2003;69:99-109.
44. Suzuki YJ, Forman HJ, Sevanian A. Oxidants as stimulators of signal transduction. *Free Radical Biol Med*. 1996;22:269-285.

45. Bonizzi G, Piette J, Schoonbroodt S, et al. Reactive oxygen intermediate-dependent NF- κ B activation by interleukin-1 β requires 5-lipoxygenase or NADPH oxidase activity. *Mol Cell Biol*. 1999;19:1950–1960.
46. Woo CH, Eom YW, Yoo MH, et al. Tumor necrosis factor- α generates reactive oxygen species via a cytosolic phospholipase A2-linked cascade. *J Biol Chem*. 2000;275:32357–32362.
47. Rola-Pleszczynski M. LTB4 and PAF in the cytokine network. *Adv Exp Med Biol*. 1991;314:205–221.
48. Node K, Huo Y, Ruan X, et al. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science*. 1999;285:1276–1290.
49. Spector AA, Norris AW. Action of epoxyeicosatrienoic acids on cellular function. *Am J Physiol Cell Physiol*. 2007;292:C996–C1012.
50. Nithipatikom K, Gross GJ. Review article: epoxyeicosatrienoic acids: novel mediators of cardioprotection. *J Cardiovasc Pharmacol Ther*. 2010;15:112–119.
51. Spiecker M, Liao J. Cytochrome P450 epoxygenase CYP2J2 and the risk of coronary artery disease. *Trends Cardiovasc Med*. 2006;16:204–208.
52. Williams JM, Murphy S, Burke M, Roman RJ. 20-Hydroxyeicosatetraenoic acid: a new target for the treatment of hypertension. *J Cardiovasc Pharmacol*. 2010;56:336–344.
53. Serhan CN, Olin E. Unorthodox routes to prostanoid formation: new twists in cyclooxygenase-initiated pathways. *J Clin Invest*. 2001;107:1481–1489.
54. Ferreri NR, Howland WC, Stevenson DD, Spiegelberg HL. Release of leukotrienes, prostaglandins, and histamine into nasal secretions of aspirin-sensitive asthmatics during reaction to aspirin. *Am Rev Respir Dis*. 1988;137:847–854.
55. Picado C. Mechanisms of aspirin sensitivity. *Curr Allergy Asthma Rep*. 2006;6:198–202.
56. Cowburn AS, Sladek K, Soja J, et al. Overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest*. 1998;101:834–846.
57. Hedman J, Kaprio J, Poussa T, Nieminen MM. Prevalence of asthma, aspirin intolerance, nasal polyposis and chronic obstructive pulmonary disease in a population-based study. *Int J Epidemiol*. 1999;28:717–722.
58. Babu KS, Salvi SS. Aspirin and asthma. *Chest*. 2000;118:1470–1476.
59. Bavbek S, Celik G, Ozer F, Mungan D, Misirligil Z. Safety of selective COX-2 inhibitors in aspirin/nonsteroidal anti-inflammatory drug-intolerant patients: comparison of nimesulide, meloxicam, and rofecoxib. *J Asthma*. 2004;41:67–75.
60. Dahlen B, Szczeklik A, Murray JJ. Celecoxib in patients with asthma and aspirin intolerance. The Celecoxib in Aspirin-Intolerant Asthma Study Group. *N Engl J Med*. 2001;344:142.
61. Celik G, Pasaoglu G, Bavbek S, et al. Tolerability of selective cyclooxygenase inhibitor, celecoxib, in patients with analgesic intolerance. *J Asthma*. 2005;42:127–131.
62. Valero A, Enrique E, Baltasar M, Cisteró A, Martí E, Picado C. Celecoxib in NSAID-induced cutaneous and respiratory adverse reactions. *Med Clin (Barc)*. 2003;121:695–696.
63. Mastalerz L, Sanak M, Gawlewicz A, Gielicz A, Faber J, Szczeklik A. Different eicosanoid profile of the hypersensitivity reactions triggered by aspirin and celecoxib in a patient with sinusitis, asthma, and urticaria. *J Allergy Clin Immunol*. 2006;118:957–958.
64. Muñoz-Cano R, Bartra J, Vennera MC, Valero A, Picado C. Asthmatic reaction induced by celecoxib in a patient with aspirin-induced asthma. *J Investig Allergol Clin Immunol*. 2009;19:64–79.
65. Abraham WM, Laufer S, Tries S. The effects of ML 3000 on antigen-induced responses in sheep. *Pulm Pharmacol Ther*. 1997;10:167–173.
66. Morgan SL, Baggott JE, Moreland L, Desomond R, Kendrach A. The safety of flavocoxid, a medical food, in the dietary management of knee osteoarthritis. *J Med Food*. 2009;12:1143–1148.
67. Wallace JL, Ma L. Inflammatory mediators in gastrointestinal defense and injury. *Exp Biol Med (Maywood)*. 2001;226:1003–1015.
68. Peskar BM. Role of leukotriene C4 in mucosal damage caused by necrotizing agents and indomethacin in the rat stomach. *Gastroenterology*. 1991;100:619–626.
69. Rainsford KD. Leukotrienes in the pathogenesis of NSAID-induced gastric and intestinal mucosal damage. *Agents Actions*. 1993;39:C24–C26.
70. Rainsford KD. Inhibition by leukotriene inhibitors, and calcium and platelet-activating factor antagonists, of acute gastric and intestinal damage in arthritic rats and in cholinomimetic-treated mice. *J Pharm Pharmacol*. 1999;51:331–339.

71. Laine L, Sloane R, Ferretti M, Cominelli FA. Randomized, double-blind comparison of placebo, etodolac and naproxen on gastrointestinal injury and prostaglandin production. *Gastrointest Endosc.* 1995;42:428-433.
72. Melarange R, Spangler R, Hoult JR. The in vitro effects of 6-methoxy-2-naphthylacetic acid, the active metabolite of nabumetone, on rat gastric mucosal eicosanoid synthesis and metabolism. *Prostaglandins Leukot Essent Fatty Acids.* 1996;55:195-200.
73. Dreyling KW, Hoppe U, Peskar BA, Morgenroth K, Kozuschek W, Peskar BM. Leukotriene synthesis by human gastrointestinal tissues. *Biochim Biophys Acta.* 1986;878:184-193.
74. Scheiman JM, Tillner A, Pohl T, et al. Reduction of non-steroidal anti-inflammatory drug induced gastric injury and leucocyte endothelial adhesion by octreotide. *Gut.* 1997;40:720-725.
75. Vieth M, Müller H, Stolte M. Can the diagnosis of NSAID-induced or Hp-associated gastric ulceration be predicted from histology? *Z Gastroenterol.* 2002;40:783-788.
76. Taha AS, Dahill S, Morran C, et al. Neutrophils, *Helicobacter pylori*, and nonsteroidal anti-inflammatory drug ulcers. *Gastroenterology.* 1999;116:254-258.
77. Konturek JW, Dembinski A, Stoll R, Domschke W, Konturek SJ. Mucosal adaptation to aspirin induced gastric damage in humans. Studies on blood flow, gastric mucosal growth, and neutrophil activation. *Gut.* 1994;35:1197-1204.
78. Hudson N, Balsitis M, Everitt S, Hawkey CJ. Enhanced gastric mucosal leukotriene B₄ synthesis in patients taking non-steroidal anti-inflammatory drugs. *Gut.* 1993;34:742-747.
79. Kirchner T, Aparicio B, Argentieri DC, Lau CY, Ritchie DM. Effects of tepoxalin, a dual inhibitor of cyclooxygenase/5-lipoxygenase, on events associated with NSAID-induced gastrointestinal inflammation. *Prostaglandins Leukot Essent Fatty Acids.* 1997;56:417-423.
80. Bias P, Buchner A, Klessner B, Laufer S. The gastrointestinal tolerability of the LOX/COX inhibitor, licofelone, is similar to placebo and superior to naproxen therapy in healthy volunteers: results from a randomized, controlled trial. *Am J Gastroenterol.* 2004;99:611-618.
81. Levy RM, Khokhlov A, Kopenkin S, et al. Efficacy and safety of flavocoxid, a novel therapeutic, compared with naproxen, in subjects with osteoarthritis of the knee. *Adv Ther.* 2010;27:731-742.
82. Pillai L, Burnett BP, Levy RM. GOAL: multicenter, open-label, post-marketing study of flavocoxid, a novel dual pathway inhibitor anti-inflammatory agent of botanical origin. *Curr Med Res Opin.* 2010;26:1055-1063.
83. Mehrabian M, Allayee H, Wong J, et al. Identification of 5-lipoxygenase as a major gene contributing to atherosclerosis susceptibility in mice. *Circ Res.* 2002;91:120-126.
84. Zhao L, Moos MP, Grabner R, et al. The 5-lipoxygenase pathway promotes pathogenesis of hyperlipidemia-dependent aortic aneurysm. *Nat Med.* 2004;10:966-973.
85. Spanbroek R, Grabner R, Lotzer K, et al. Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis. *Proc Natl Acad Sci USA.* 2003;100:1238-1243.
86. Helgadottir A, Gretarsdottir S, St Clair D, et al. Association between the gene encoding 5-lipoxygenase-activating protein and stroke replicated in a Scottish population. *Am J Hum Genet.* 2005;76:505-509.
87. Maznyczka A, Braund P, Mangino M, Samani NJ. Arachidonate 5-lipoxygenase (5-LO) promoter genotype and risk of myocardial infarction: a case-control study. *Atherosclerosis.* 2008;199:328-332.
88. Dwyer JH, Allayee H, Dwyer KM, et al. Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. *N Engl J Med.* 2004;350:29-37.
89. Allayee H, Baylin A, Hartiala J, et al. Nutrigenetic association of the 5-lipoxygenase gene with myocardial infarction. *Am J Clin Nutr.* 2008;88:934-940.
90. Carry M, Korley V, Willerson JT, Weigelt L, Ford-Hutchinson AW, Tagari P. Increased urinary leukotriene excretion in patients with cardiac ischemia. In vivo evidence for 5-lipoxygenase activation. *Circulation.* 1992;85:230-236.
91. Solomon DH, Schneeweiss S, Glynn RJ, et al. Relationship between selective cyclooxygenase-2 inhibitors and acute myocardial infarction in older adults. *Circulation.* 2004;109:2068-2073.

92. Roumie CL, Choma NN, Kaltenbach L, Mitchel EF Jr., Arbogast PG, Griffin MR. Non-aspirin NSAIDs, cyclooxygenase-2 inhibitors and risk for cardiovascular events – stroke, acute myocardial infarction, and death from coronary heart disease. *Pharmacoepidemiol Drug Saf.* 2009;18:1053–1063.
93. Solomon SD, McMurray JJ, Pfeffer MA, et al. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med.* 2005;352:1071–1080.
94. Solomon SD, Pfeffer MA, for the APC and PreSAP Trial Investigators, et al. Effect of celecoxib on cardiovascular events and blood pressure in two trials for the prevention of colorectal adenomas. *Circulation.* 2006;114:1028–1035.
95. ADAPT Research Group. Cardiovascular and cerebrovascular events in the randomized, controlled Alzheimer's Disease Anti-inflammatory Prevention Trial (ADAPT). *PLoS Clin Trials.* 2006;1:e33.
96. Trelle S, Reichenbach S, Wandel S, et al. Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. *BMJ.* 2011;342:c7086.
97. Kearney PM, Baigent C, Godwin J, Halls H, Emberson JR, Patrono C. Do selective cyclooxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomized trials. *BMJ.* 2006;332:1302–1308.
98. Solomon DH. Selective cyclooxygenase 2 inhibitors and cardiovascular events. *Arthritis Rheum.* 2005;52:1968–1978.
99. Sun SY, Wang W, Schultz HD. Activation of cardiac afferents by arachidonic acid: relative contributions of metabolic pathways. *Am J Physiol Heart Circ Physiol.* 2001;281:H93–H104.
100. Stanke-Labesque F, Devillier P, Bedouch P, Cracowski JL, Chavanon O, Bessard G. Angiotensin II-induced contractions in human internal mammary artery: effects of cyclooxygenase and lipoyxygenase inhibition. *Cardiovasc Res.* 2000;47:376–383.
101. Brown PH, Subbaramaiah K, Salmon AP, et al. Combination chemoprevention of HER2/neu-induced breast cancer using a cyclooxygenase-2 inhibitor and a retinoid X receptor-selective retinoid. *Cancer Prev Res (Phila).* 2008;1:208–214.
102. Duffield-Lillico AJ, Boyle JO, Zhou XK, et al. Levels of prostaglandin E metabolite and leukotriene E(4) are increased in the urine of smokers: evidence that celecoxib shunts arachidonic acid into the 5-lipoxygenase pathway. *Cancer Prev Res (Phila).* 2009;2:322–329.
103. Zhang YY, Walker JL, Huang A, et al. Expression of 5-lipoxygenase in pulmonary artery endothelial cells. *Biochem J.* 2002;361:267–276.
104. Allen S, Dashwood M, Morrison K, Yacoub M. Differential leukotriene constrictor responses in human atherosclerotic coronary arteries. *Circulation.* 1998;97:2406–2413.
105. Sala A, Aliev GM, Rossoni G, et al. Morphological and functional changes of coronary vasculature caused by transcellular biosynthesis of sulfidopeptide leukotrienes in isolated heart of rabbit. *Blood.* 1996;87:1824–1832.
106. Vidal C, Gomez-Hernandez A, Sanchez-Galan E, et al. Licofelone, a balanced inhibitor of cyclooxygenase and 5-lipoxygenase, reduces inflammation in a rabbit model of atherosclerosis. *J Pharmacol Exp Ther.* 2007;320:108–116.
107. Vlassara H, Torreggiani M, Post JB, Zheng F, Uribarri J, Striker GE. Role of oxidants/inflammation in declining renal function in chronic kidney disease and normal aging. *Kidney Int Suppl.* 2009;76:S3–11.
108. Lauretani F, Semba RD, Bandinelli S, et al. Plasma polyunsaturated fatty acids and the decline of renal function. *Clin Chem.* 2008;54:475–481.
109. Komers R, Epstein M. Cyclooxygenase-2 expression and function in renal pathophysiology. *J Hypertens Suppl.* 2002;20:S11–15.
110. Lefkowitz JB. Essential fatty acid deficiency inhibits the in vivo generation of leukotriene B4 and suppresses levels of resident and elicited leukocytes in acute inflammation. *J Immunol.* 1988;140:228–233.
111. Rifai A, Sakai H, Yagame M. Expression of 5-lipoxygenase and 5-lipoxygenase activation protein in glomerulonephritis. *Kidney Int Suppl.* 1993;39:S95–99.
112. Rahman MA, Nakazawa M, Emancipator SN, Dunn MJ. Increased leukotriene B4 synthesis in immune injured rat glomeruli. *J Clin Invest.* 1988;81:1945–1952.
113. Maccarrone M, Taccone-Gallucci M, Meloni C, et al. Activation of 5-lipoxygenase and related cell membrane lipoperoxidation in hemodialysis patients. *J Am Soc Nephrol.* 1999;10:1991–1996.

114. Noiri E, Yokomizo T, Nakao A, et al. An in vivo approach showing the chemotactic activity of leukotriene B(4) in acute renal ischemic-reperfusion injury. *Proc Natl Acad Sci USA*. 2000;97:823-828.
115. Badr KF. Five-lipoxygenase products in glomerular immune injury. *J Am Soc Nephrol*. 1992;3:907-915.
116. Petric R, Ford-Hutchinson A. Inhibition of leukotriene biosynthesis improves renal function in experimental glomerulonephritis. *J Lipid Mediat Cell Signal*. 1995;11:231-240.
117. Albrightson CR, Short B, Dytko G, et al. Selective inhibition of 5-lipoxygenase attenuates glomerulonephritis in the rat. *Kidney Int*. 1994;45:1301-1310.
118. Raynauld JP, Martel-Pelletier J, Bias P, et al. Protective effects of licofelone, a 5-lipoxygenase and cyclo-oxygenase inhibitor, versus naproxen on cartilage loss in knee osteoarthritis: a first multicentre clinical trial using quantitative MRI. *Ann Rheum Dis*. 2009;68:938-947.
119. Fusellier M, Desfontis JC, Madec S, et al. Effect of tepoxalin on renal function in healthy dogs receiving an angiotensin-converting enzyme inhibitor. *J Vet Pharmacol Ther*. 2005;28:581-586.
120. Kay-Mugford PA, Grimm KA, Weingarten AJ, Brianceau P, Lockwood P, Cao J. Effect of preoperative administration of tepoxalin on hemostasis and hepatic and renal function in dogs. *Vet Ther*. 2004;5:120-127.
121. Burnett BP, Silva S, Mesches MH, Jia Q. Safety evaluation of a combination, defined extract of *Scutellaria baicalensis* and *Acacia catechu*. *J Food Biochem*. 2007;31:797-825.
122. Yimam M, Zhao Y, Ma W, Jia Q, Do SG, Shin JH. 90-Day oral toxicity study of UP446, a combination of defined extracts of *Scutellaria baicalensis* and *Acacia catechu*, in rats. *Food Chem Toxicol*. 2010;48:1202-1209.
123. Burnett BP, Stenstrom KK, Baarsch MJ, Swafford WS, Ehrenzweig J, Levy RM. A flavonoid mixture, dual inhibitor of cyclooxygenase and 5-lipoxygenase enzymes, shows superiority to glucosamine/chondroitin for pain management in moderate osteoarthritic dogs. *Intern J Appl Vet Med*. 2009;7:1-12.
124. Levy R, Saikovskiy R, Shmidt E, Khokhlov A, Burnett BP. Flavocoxid is as effective as naproxen for managing the signs and symptoms of osteoarthritis of the knee in humans: a short-term randomized, double-blind pilot study. *Nutr Res*. 2009;29:298-304.
125. Shimizu T, Wolfe LS. Arachidonic acid cascade and signal transduction. *J Neurochem*. 1990;55:1-15.
126. Zhou BF, Stamler J, Dennis B, et al. Nutrient intakes of middle-aged men and women in China, Japan, United Kingdom, and United States in the late 1990s: the INTERMAP study. *J Hum Hypertens*. 2003;17:623-630.
127. Plumb MS, Aspden RM. High levels of fat and (n-6) fatty acids in cancellous bone in osteoarthritis. *Lipids Health Dis*. 2004;3:12.
128. Paredes Y, Massicotte F, Pelletier JP, Martel-Pelletier J, Laufer S, Lajeunesse D. Study of the role of leukotriene B4 in abnormal function of human subchondral osteoarthritis osteoblasts: effects of cyclooxygenase and/or 5-lipoxygenase inhibition. *Arthritis Rheum*. 2002;46:1804-1812.
129. Maxis K, Delalandre A, Martel-Pelletier J, Pelletier JP, Duval N, Lajeunesse D. The shunt from the cyclooxygenase to lipoxygenase pathway in human osteoarthritic subchondral osteoblasts is linked with a variable expression of the 5-lipoxygenase-activating protein. *Arthritis Res Ther*. 2006;8:R181.
130. Anderson GD, Keys KL, De Ciechi PA, Masferrer JL. Combination therapies that inhibit cyclooxygenase-2 and leukotriene synthesis prevent disease in murine collagen induced arthritis. *Inflamm Res*. 2009;58:109-117.
131. Huskisson EC, Berry H, Gishen P, Jubb RW, Whitehead J. Effects of antiinflammatory drugs on the progression of osteoarthritis of the knee. LINK Study Group. Longitudinal Investigation of Nonsteroidal Antiinflammatory Drugs in Knee Osteoarthritis. *J Rheumatol*. 1995;22:1941-1946.
132. Clausen PA, Flechtenmacher J, Haeuselmann HJ, Kuettner KE, Aydelotte MB, Iyer AP. Evidence of an eicosanoid contribution to IL-1 induction of IL-6 in human articular chondrocytes. *Am J Ther*. 1996;3:101-108.
133. Martel-Pelletier J, Mineau F, Fahmi H, et al. Regulation of the expression of 5-lipoxygenase-activating protein/5-lipoxygenase and the synthesis of leukotriene B(4) in osteoarthritic chondrocytes: role of transforming growth factor β and eicosanoids. *Arthritis Rheum*. 2004;50:3925-3933.
134. Marcouiller P, Pelletier JP, Guévremont M, et al. Leukotriene and prostaglandin synthesis pathways in osteoarthritic synovial membranes: regulating factors for interleukin 1 β synthesis. *J Rheumatol*. 2005;32:704-712.

135. Charlier C, Michaux C. Dual inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs. *Eur J Med Chem*. 2003;38:645-659.
136. Martel-Pelletier J, Lajeunesse D, Reboul P, Pelletier JP. Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. *Ann Rheum Dis*. 2003;62:501-509.
137. Pelletier JP, Boileau C, Boily M, et al. The protective effect of licofelone on experimental osteoarthritis is correlated with the downregulation of gene expression and protein synthesis of several major cartilage catabolic factors: MMP-13, cathepsin K and aggrecanases. *Arthritis Res Ther*. 2005;7:R1091-R1102.
138. Gay RE, Neidhart M, Pataky F, Tries S, Laufer S, Gay S. Dual inhibition of 5-lipoxygenase and cyclooxygenases 1 and 2 by ML3000 reduces joint destruction in adjuvant arthritis. *J Rheumatol*. 2001;28:2060-2065.
139. Boileau C, Martel-Pelletier J, Jouzeau JY, et al. Licofelone (ML-3000), a dual inhibitor of 5-lipoxygenase and cyclooxygenase, reduces the level of cartilage chondrocyte death in vivo in experimental dog osteoarthritis: inhibition of pro-apoptotic factors. *J Rheumatol*. 2002;29:1446-1453.
140. Rainsford KD, Ying C, Smith F. Effects of 5-lipoxygenase inhibitors on interleukin production by human synovial tissues in organ culture: comparison with interleukin-1-synthesis inhibitors. *J Pharm Pharmacol*. 1996;48:46-52.
141. Macrory L, Vaughan-Thomas A, Clegg PD, Innes JF. An exploration of the ability of tepoxalin to ameliorate the degradation of articular cartilage in a canine in vitro model. *BMC Vet Res*. 2009;5:25.
142. Raynauld JP, Martel-Pelletier J, Abram F, et al. Analysis of the precision and sensitivity to change of different approaches to assess cartilage loss by quantitative MRI in a longitudinal multicentre clinical trial in patients with knee osteoarthritis. *Arthritis Res Ther*. 2008;10:R129.
143. Klickstein LB, Shapleigh C, Goetzl EJ. Lipoxygenation of arachidonic acid as a source of polymorphonuclear leukocyte chemotactic factors in synovial fluid and tissue in rheumatoid arthritis and spondyloarthritis. *J Clin Invest*. 1980;66:1166-1170.
144. Koshihara, Y, Isono T, Oda H, Karube S, Hayashi Y. Measurement of sulfidopeptide leukotrienes and their metabolism in human synovial fluid of patients with rheumatoid arthritis. *Prostaglandins Leukot Essent Fatty Acids*. 1988;32:113-119.
145. Nakamura H, Hishinuma T, Suzuki N, et al. Difference in urinary 11-dehydro TXB₂ and LTE₄ excretion in patients with rheumatoid arthritis. *Prostaglandins Leukot Essent Fatty Acids*. 2001;65:301-306.
146. Edwards SW, Hallett MB. Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. *Immunol Today*. 1997;18:320-324.
147. Chen M, Lam BK, Kanaoka L, et al. Neutrophil-derived leukotriene B₄ is required for inflammatory arthritis. *J Exp Med*. 2006;203:837-842.
148. Griffiths RJ, Pettipher ER, Koch K, et al. Leukotriene B₄ plays a critical role in the progression of collagen-induced arthritis. *Proc Natl Acad Sci. USA*. 1995;92:517-521.
149. Griffiths RJ, Smith MA, Roach ML, et al. Collagen-induced arthritis is reduced in 5-lipoxygenase-activating protein-deficient mice. *J Exp Med*. 1997;185:1123-1129.
150. Sperling RI, Benincaso AI, Anderson RJ, Coblyn JS, Austen KE, Weinblatt ME. Acute and chronic suppression of leukotriene B₄ synthesis ex vivo in neutrophils from patients with rheumatoid arthritis beginning treatment with methotrexate. *Arthritis Rheum*. 1992;35:376-384.
151. Allendorf DJ, Yan J, Ross GD, et al. C5a-mediated leukotriene B₄-amplified neutrophil chemotaxis is essential in tumor immunotherapy facilitated by anti-tumor monoclonal antibody and β -glucan. *J Immunol*. 2005;174:7050-7056.
152. de Weck AL, Gamboa PM, Esparza R, Sanz ML. Hypersensitivity to aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs). *Curr Pharm Des*. 2006;12:3347-3358.
153. Goodman L, Coles TB, Budsberg S. Leukotriene inhibition in small animal medicine. *J Vet Pharmacol Ther*. 2008;31:387-398.
154. Asero R. Leukotriene receptor antagonists may prevent NSAID-induced exacerbations in patients with chronic urticaria. *Ann Allergy Asthma Immunol*. 2000;85:156-157.

155. Faucheron JL, Parc R. Non-steroidal anti-inflammatory drug-induced colitis. *Int J Colorectal Dis.* 1996;11:99-101.
156. Wallace JL, Keenan CM. Leukotriene B4 potentiates colonic ulceration in the rat. *Dig Dis Sci.* 1990;35:622-629.
157. Lanas A. Nonsteroidal antiinflammatory drugs and cyclooxygenase inhibition in the gastrointestinal tract: a trip from peptic ulcer to colon cancer. *Am J Med Sci.* 2009;338:96-106.
158. Rask-Madsen J, Bukhave K, Laursen LS, Lauritsen K. 5-Lipoxygenase inhibitors for the treatment of inflammatory bowel disease. *Agents Actions.* 1992;Spec No:C37-46.
159. Shapiro H, Singer P, Halpern Z, Bruck R. Polyphenols in the treatment of inflammatory bowel disease and acute pancreatitis. *Gut.* 2007;56:426-435.
160. Polito F, Bitto A, Irrera N, et al. Flavocoxid, a dual inhibitor of COX-2 and 5-LOX, reduces pancreatic damage in an experimental model of acute pancreatitis. *Brit J Pharmacol.* 2010;161:1002-1011.
161. Fischer L, Hornig M, Pergola C, et al. The molecular mechanism of the inhibition by licofelone of the biosynthesis of 5-lipoxygenase products. *Br J Pharmacol.* 2007;152:471-480.
162. Tries S, Neupert W, Laufer S. The mechanism of action of the new antiinflammatory compound ML3000: inhibition of 5-LOX and COX-1/2. *Inflamm Res.* 2002;51:135-143.
163. Bau B, Gebhard PM, Haag J, Knorr T, Bartnik E, Aigner T. Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. *Arthritis Rheum.* 2002;46:2648-2657.
164. Tam SS, Lee DH, Wang EY, Munroe DG, Lau CY. Tepoxalin, a novel dual inhibitor of the prostaglandin-H synthase cyclooxygenase and peroxidase activities. *J Biol Chem.* 1995;270:13948-13955.
165. Lee JI, Burckart GJ. Nuclear factor κ B: important transcription factor and therapeutic target. *J Clin Pharmacol.* 1998;38:981-993.
166. Altavilla D, Squadrito F, Bitto A, et al. Flavocoxid, a dual inhibitor of cyclooxygenase and 5-lipoxygenase, blunts pro-inflammatory phenotype activation in endotoxin stimulated macrophages. *Brit J Pharmacol.* 2009;157:1410-1418.
167. Messina S, Bitto A, Aguenouz M, et al. Flavocoxid inhibits NF- κ B, MAPKs and COX/5-LOX pathways and improves muscle function and morphology in mdx mice: a comparison study with methylprednisolone. *Exp Neurol.* 2009;220:349-358.
168. Burnett BP, Bitto A, Squadrito F, Levy RM, Pillai L. Flavocoxid inhibits phospholipase A2, peroxidase moieties of the cyclooxygenases (COX), 5-lipoxygenase, modifies COX-2 gene expression and acts as an antioxidant. *Mediators Inflamm.* 2011;385780.