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Leukotriene biosynthetic enzymes as therapeutic targets

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Leukotrienes are powerful immune-regulating lipid mediators with established pathogenic roles in inflammatory allergic diseases of the respiratory tract — in particular, asthma and hay fever. More recent work indicates that these lipids also contribute to low-grade inflammation, a hallmark of cardiovascular, neurodegenerative, and metabolic diseases as well as cancer. Biosynthesis of leukotrienes involves oxidative metabolism of arachidonic acid and proceeds via a set of soluble and membrane enzymes that are primarily expressed by cells of myeloid origin. In activated immune cells, these enzymes assemble at the endoplasmic and perinuclear membrane, constituting a biosynthetic complex. This Review describes recent advances in our understanding of the components of the leukotriene-synthesizing enzyme machinery, emerging opportunities for pharmacological intervention, and the development of new medicines exploiting both antiinflammatory and pro-resolving mechanisms.

Introduction

Leukotrienes are, as the name indicates, mainly biosynthesized by *leuko*cytes from the myeloblastic (neutrophils, eosinophils, and mast cells) and monoblastic lineages (monocytes/macrophages). These lipid mediators act in a paracrine manner, exerting their functions at nanomolar concentrations and causing different responses according to the target cell type (1). There are two classes of leukotrienes: the dihydroxy fatty acid leukotriene LTB_4, and the so-called cysteinyl-leukotrienes (cys-LTs), comprising the lipid-peptide conjugate LTC_4 and its metabolites LTD_4 and LTE_4, which are formed by sequential peptidolytic cleavage. Leukotrienes signal through two sets of GPCRs: BLT1 and BLT2 for LTB_4, and CysLT1, CysLT2, and CysLTE, also known as gpr99, for cys-LTs (2). Classical bioactions of LTB_4 include chemotaxis, endothelial adherence, and activation of leukocytes, while cys-LTs — in particular, LTD_4 — contract smooth muscles in the microcirculation and respiratory tract. In addition, a spectrum of other bioactions exerted by leukotrienes have been reported, all of which support proinflammatory and immune-regulating functions of these mediators, and which have qualified the biosynthetic enzymes as potential drug targets (1, 3, 4).

Leukotrienes are synthesized from the omega-6 polyunsaturated fatty acid arachidonic acid (AA), which is liberated intracellularly from membrane phospholipids by several phospholipase A_2 enzymes (PLA_2s), especially cytosolic PLA2α (cPLA2α) (5). The central enzyme in cellular leukotriene biosynthesis, 5-lipoxygenase (5-LOX), requires a set of stimulatory factors for full activity and is supported by two accessory proteins, 5-lipoxygenase–activating protein (FLAP) and coactosin-like protein (CLP) (6). The catalytic iron center of activated 5-LOX converts AA in a two-step concerted reaction: first a dioxygenation into 5(S)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid [5(S)-HpETE], followed by dehydration to yield the transient epoxide intermediate LTA_4, a step that is referred to as the enzyme's LTA_4 synthase activity (7). Depending on the presence and functional coupling of 5-LOX to downstream enzymes, LTA_4 is further converted into the dihydroxy acid LTB_4 by LTA_4 hydrolase (LTA4H), while LTC_4 synthase (LTC4S) conjugates LTA_4 with glutathione (GSH) to form LTC_4, the parent compound of the cys-LTs LTC_4, LTD_4, and LTE_4. 5-LOX subcellular localization and association with FLAP and CLP play a central role in the regulation of leukotriene production (Figure 1). Before activation, 5-LOX can be located either in the cytosol or in the nucleus of the cells, depending on the cell type and the cellular environment (reviewed in ref. 8). Both cytosolic and nucleoplasmic 5-LOX moves to the nuclear envelope upon cell stimulation by various agonists, and this process is accompanied by leukotriene generation (9).

The enzymes in leukotriene biosynthesis are biochemically diverse; some are cytosolic monomeric enzymes (cPLA2α, 5-LOX, and LTA4H), while others are trimeric integral membrane proteins (FLAP, LTC4S, and MGST2). Together they encompass three distinct enzyme classes: hydrolases (cPLA2α and LTA4H), oxidoreductases (5-LOX), and transferases (LTC4S and MGST2). In this Review, work deciphering the molecular mechanisms of leukotriene biosynthesis will be described along with recent reports on new potential therapeutic applications of drugs interfering with the production of these lipid mediators. Moreover, benefits and drawbacks with various pharmacological strategies will be discussed.

5-LOX at the center stage of leukotriene biosynthesis

5-LOX is primarily found in various myeloid cells, including polymorphonuclear leukocytes (PMNs), monocytes/macrophages, dendritic cells, mast cells, and B lymphocytes (6). Aberrant expression of 5-LOX has been detected in many tumor cells of nonmyeloid origin (10–13). Moreover, CMV infection can induce 5-LOX expression and LTB_4 production in vascular smooth muscle cells (14).

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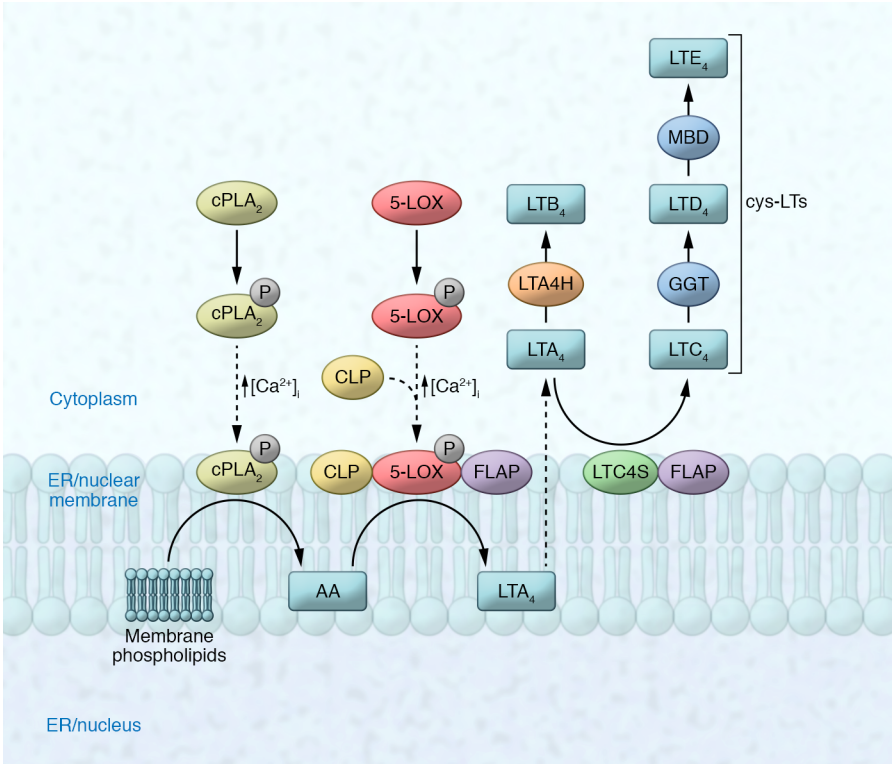

Figure 1. Translocation and assembly of the leukotriene biosynthetic complex. Schematic model for translocation of $cPLA_2$ and 5-LOX, together with CLP, to the ER and perinuclear membrane, where they meet up with FLAP and LTC4S. LTA4H stays in the cytosolic compartment. Ser phosphorylations of $cPLA_2$ and 5-LOX are indicated. GGT, γ glutamyltransferase; MBD, membrane-bound dipeptidase.

Regulation of 5-LOX expression. The human *5-LOX* gene is located on chromosome 10 and consists of 14 exons (15). The promoter region lacks a typical TATA or CCAT box but contains eight GC boxes, five of which are arranged in tandem and bind the transcription factors SP1 and EGR-1 (16, 17). Several natural mutations occur within the functional promoter regions, which have been suggested to play a role in asthma (17, 18). TGF-β and vitamin D_3 strongly increase 5-LOX expression and enzyme activity during differentiation of HL-60 and MonoMac6 human myeloid cells (19). In addition, granulocyte-macrophage CSF (GM-CSF) augments 5-LOX expression in mature human neutrophils, monocytes, and monocytic THP-1 cells (19). 5-LOX expression is also regulated by miR-19a-3p and miR-125b-5p in a cell type– and stimulus-specific manner (20).

A multicomponent system regulates 5-LOX enzyme activity. 5-LOX is a non-heme dioxygenase whose activity is regulated by several soluble and membrane-associated factors acting as allosteric regulators or enzyme scaffolds. Thus, Ca^{2+}, ATP, phosphatidylcholine (PC), cell membrane and diacylglycerols, lipid hydroperoxides, and CLP have all been shown to impact the turnover of the substrate (6).

The functional role of Ca^{2+} is not fully understood but requires the presence of PC or CLP, a 16-kDa F-actin–binding protein that promotes the LTA_4 synthase activity of 5-LOX (21). 5-LOX is also stimulated by ATP, which binds to the protein without any apparent hydrolysis of phosphodiester bonds, apparently acting as an allosteric activator (22).

5-LOX is also subjected to posttranslational modification. Thus, the enzyme is phosphorylated in vitro at three sites: Ser271 by MAPKAP kinase 2, Ser663 by ERK2, and Ser523 by PKA (23–25). Phosphorylation at Ser271 and Ser663 is facilitated by the presence of unsaturated fatty acids, including AA, but seems to influence enzyme activity indirectly via 5-LOX compartmentalization (26, 27). On the other hand, phosphorylation at Ser523 by PKA directly suppresses 5-LOX activity and its trafficking to the nucleus (25, 28), which may explain the inhibitory actions of adenosine and increased cAMP on cellular leukotriene synthesis (29). Interestingly, neutrophils and monocytes from males seem to have a significantly lower capacity to synthesize leukotrienes (30, 31). This effect is due to androgen-induced ERK activation, which paradoxically results in reduced leukotriene synthesis.

The 5-LOX protein and crystal structure. Human 5-LOX is a 78-kDa soluble enzyme that is generally regarded as monomeric, although recent data suggest that it can also exist as a homodimer (32). The enzyme is notoriously unstable and sensitive to oxidative damage, which made its initial purification from isolated human leukocytes a formidable challenge, finally overcome by Rouzer and Samuelsson in 1985 (33). For the same reasons, it took another 25 years until an engineered, stable variant of 5-LOX could be successfully crystallized and structurally characterized at 2.4 Å resolution (34). 5-LOX consists of an N-terminal β-sandwich and an iron-containing C-terminal catalytic domain (Figure 2). The N-terminal domain is composed of two 4-stranded antiparallel β-sheets and is one of the defining members of the PLAT (polycystin-1, lipoxygenase, α-toxin) domain family (35). The 5-LOX N-terminal domain has been shown to bind several regulatory factors, such as Ca^{2+}, PC, and CLP, suggesting that this domain facilitates 5-LOX's association with membranes during catalysis (6). The catalytic domain is composed of several α-helices, and iron is coordinated by three conserved His residues (367, 372, 550) and the carboxylate moiety of the C-terminal Ile673. Unexpectedly, the structure of stable 5-LOX revealed a fully encapsulated catalytic machinery, i.e., the side chains of two aromatic amino acids at the active center (Phe177 and Tyr181) form a cork (termed "FY cork") that seals off the active site and closes the cavity for substrate entry (34). Further studies suggest that the "corking" amino acid Phe177 plays an important role for a fully functional active site, and His600 appears to be required to position the substrate for catalysis (36). Apparently, the concealed FY cork also plays a role in 5-LOX association with the nuclear membrane and its scaffold protein FLAP (37).

FLAP, a critical 5-LOX accessory protein

In activated immune cells, 5-LOX translocates to the ER and perinuclear membranes in response to Ca^{2+}, a process accompa-

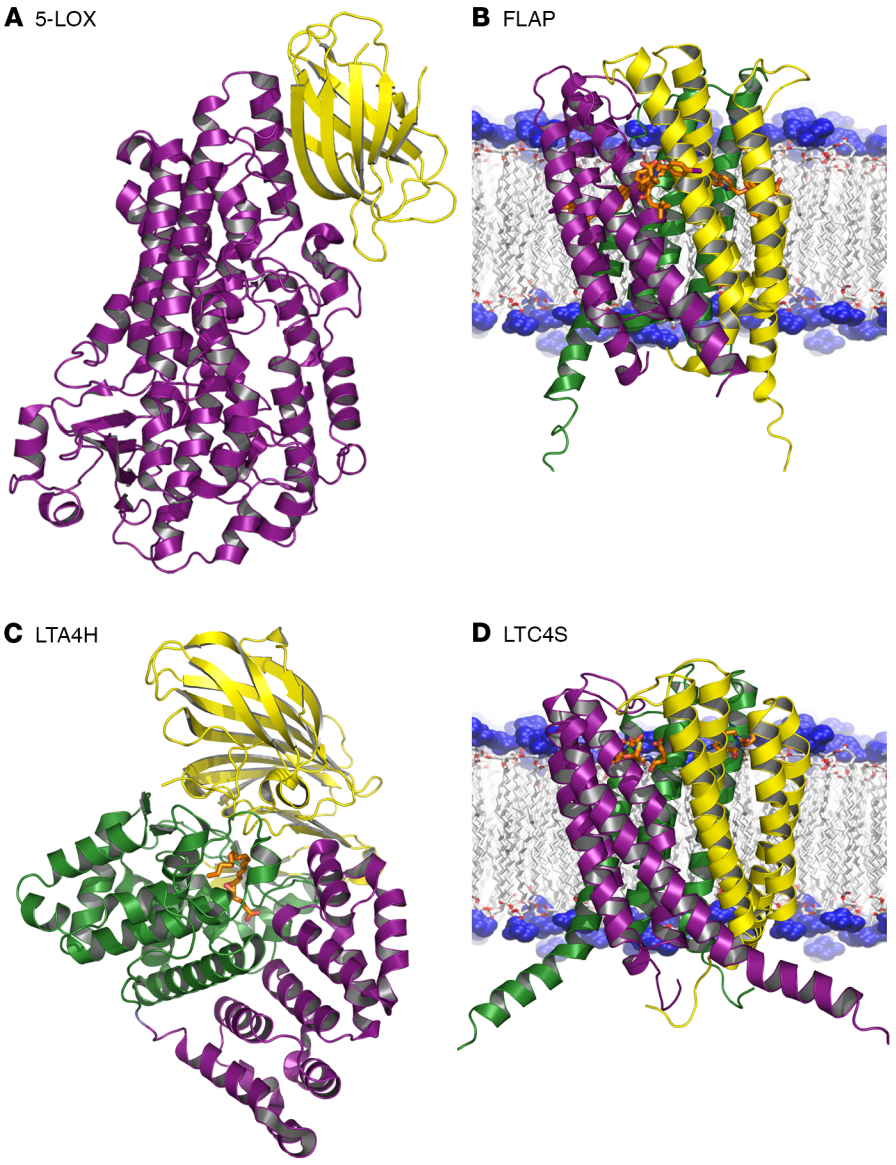

Figure 2. Crystal structure of the key enzymes and proteins in leukotriene biosynthesis. (**A**) Structure of 5-LOX at 2.4 Å resolution depicting the N-terminal β-barrel domain (yellow) and the catalytic domain (magenta). (**B**) Structure of FLAP at 4 Å resolution. It is a trimer with each monomer (yellow, green, and magenta) composed of four transmembrane α-helices. FLAP inhibitors are bound in the intermonomeric cleft. (**C**) Structure of LTA4H at 1.9 Å resolution. The protein is folded into three domains, an N-terminal (yellow), a catalytic (green), and a C-terminal (magenta), between which the catalytic center is located. A molecule of LTA_4 is indicated in the center of the interdomain cavity. (**D**) Structure of LTC4S. It is a trimer with each monomer (yellow, green, and magenta) composed of 4 transmembrane α-helices. Three molecules of the cosubstrate GSH are bound between two monomers toward the cytosolic side of the protein. Figures were derived from the following Protein Data Bank IDs: 5-LOX 3O8Y; FLAP 2Q7R; LTA4H 5NI6; LTC4S 2UUH.

nied by enzyme catalysis followed by enzyme inactivation. At the membrane, 5-LOX associates with FLAP, which was originally discovered through the inhibitory action of a drug, MK-886, on leukotriene biosynthesis in intact cells (38, 39). MK-886 binds to FLAP, interferes with 5-LOX/FLAP interactions, and inhibits 5-LOX activity.

Role of FLAP in cellular leukotriene biosynthesis. Association between FLAP and 5-LOX has been demonstrated in vitro and in whole cells (40–43), involving four cysteines (159, 300, 416, and 418) in 5-LOX (42). The role of FLAP is to present AA to 5-LOX (39, 44), and it is assumed that FLAP acts as a scaffold that governs the distribution of 5-LOX to the perinuclear region, stimulates AA utilization by 5-LOX, and increases the efficiency with which 5-LOX converts 5-HpETE into LTA_4 (45, 46). A recent study indicates that FLAP regulates 5-LOX activity in two ways: by inducing an initial flexible and loose association with 5-LOX for efficient 5-LOX product synthesis, and by subsequent formation of a tight 5-LOX/FLAP complex that terminates 5-LOX activity (43).

Regulation of FLAP expression. The *FLAP* gene is located on chromosome 13 and comprises five exons (47). The promoter contains a possible TATA box, AP-2, NF-κB, and glucocorticoid response elements. The 5′-UTR region contains a polymorphism that is associated with asthma (48). Transcription of *FLAP* appears to be induced in inflammatory cells by IL-3 as well as GM-CSF, dexamethasone, IL-5, LPS, and TNF-α (49). Another study showed that hypoxia induces FLAP expression in endothelial cells via enhancement of HIF-1α and NF-κB binding to the *FLAP* promoter, while expression is downregulated by miR-135a and miR-199a-5p targeting the 3′-UTR of *FLAP* mRNA (50).

FLAP protein and structure. Human FLAP is a 161–amino acid integral membrane protein (51). Notably, FLAP displays no enzyme activity (39). A low-resolution (4 Å) structure of FLAP in complex with inhibitors revealed a trimeric structure with four transmembrane helices in each monomer connected by two cytosolic loops and one lumenal loop (52). The inhibitors bind to membrane-embedded pockets in FLAP, suggesting how they might prevent binding of AA (Figure 2).

LTA4H, an epoxide hydrolase dedicated to LTB_4 synthesis

LTA4H catalyzes the final critical step in the biosynthesis of the proinflammatory compound LTB_4, recently identified as a key signal-relay molecule during neutrophil chemotaxis and swarming (53, 54). Unlike other epoxide hydrolases, LTA4H is highly selective for its substrate, LTA_4, and undergoes suicide inactivation during catalysis with covalent binding of LTA_4 to the protein (55). It is presently unclear how LTA_4, a labile and chemically reactive allylic epoxide, is transferred between 5-LOX and LTA4H and how the product LTB_4 is transported through cytosol to the plasma membrane for export (Figure 1).

LTA4H is ubiquitously expressed. Unlike 5-LOX, LTA4H is widely expressed in almost all mammalian cells, organs, and tissues, albeit at different levels (56). Human *LTA4H* exists as a single copy on chromosome 12q22 divided into 19 exons (57). LTA4H expression is believed to be stable, although it has been reported that IL-4 and IL-13 may upregulate its expression in human PMNs (56). High levels of LTA4H have been observed in several human tumors of the gastrointestinal tract, lung, and thyroid, suggesting a role in cancer (58).

LTA4H is a bifunctional zinc aminopeptidase. LTA4H is a 69-kDa cytosolic protein that converts LTA_4 into LTB_4, a reaction referred to as the enzyme's epoxide hydrolase activity (Figure 1). LTA4H is also a tripeptidase with high affinity for N-terminal arginine (55, 59). Both enzyme activities of LTA4H depend on a catalytic zinc ion that is bound within the signature HEXXH-$(X)_{18}$-E, typical of M1 metallopeptidases (60). LTA4H's crystal structure has been determined (61). The enzyme folds into an N-terminal domain, a catalytic domain, and a C-terminal domain (Figure 2). The interface of the domains forms an active site cavity, which narrows at the zinc-binding site, forming a tunnel into the catalytic domain. The opening and wider parts of the cavity are highly polar; the tunnel is more hydrophobic. LTA4H's two enzyme activities are exerted via distinct yet overlapping active sites. Thus, Glu296 and Tyr383 are specifically required for the aminopeptidase activity, whereas Asp375 is critical only for the epoxide hydrolase reaction. Glu271, Arg563, and the zinc ion are necessary for both catalyses. Interestingly, LTA4H utilizes a single water molecule that is differentially activated by Glu296 or Glu271 to take part in the aminopeptidase or epoxide hydrolase reaction, respectively (62).

LTA4H cleaves and inactivates the chemotactic Pro-Gly-Pro. Snelgrove and coworkers serendipitously discovered that the tripeptide Pro-Gly-Pro (PGP) is an endogenous substrate for extracellular LTA4H (63). PGP is generated from the extracellular matrix and is chemotactic for neutrophils, suggesting that LTA4H exhibits dual and opposite functions during an inflammatory response. In the initial phase, the enzyme's epoxide hydrolase activity will generate proinflammatory LTB_4, while the aminopeptidase activity will inactivate the chemotactic PGP during the resolution phase. Notably, conflicting results regarding the properties of PGP were recently reported (64).

LTC4S, a specialized GSH transferase producing asthma mediators

Slow-reacting substance of anaphylaxis (SRS-A) is a classical mediator of asthma. Work awarded the Nobel Prize chemically characterized SRS-A and identified it as a mixture of LTC_4, LTD_4, and LTE_4, i.e., the cys-LTs (65). LTC4S is a specialized membrane GSH *S*-transferase, which catalyzes conjugation of LTA_4 with GSH (Figure 1). High levels of enzyme expression and capacity to synthesize LTC_4 are observed in immune cells such as eosinophils, mast cells, and monocytes (66). Platelets also contain LTC4S, although these corpuscles cannot produce the substrate LTA_4 (67).

LTC4S is a notoriously unstable 18-kDa enzyme that is stimulated by divalent cations and PC and stabilized by GSH. The primary structure contains consensus sequences for PKC phosphorylation, which reduces LTC4S activity (68). Recently, the ribosomal protein S6 kinase (p70S6K) was shown to play a key role in phosphoregulation of LTC4S in human macrophages, and Ser36 was identified as the major phosphorylation site (69, 70).

LTC4S is a member of the MAPEG superfamily. Molecular cloning of LTC4S revealed a surprising 33% identity with FLAP (71, 72), and further work at Merck Frosst identified two additional homologous microsomal GSH transferases: MGST2 and MGST3 (73, 74). MGST2 is 44% identical with LTC4S and accounts for LTC_4 synthesis in nonhematopoietic cells such as endothelium and testis (75–77). Recently, MGST2 was identified as a key enzyme involved in oxidative DNA damage induced by ER stress and anticancer agents (78). Yet another homolog was subsequently found to catalyze isomerization of prostaglandin H_2 (PGH_2) into prostaglandin E_2 (PGE_2) and was denoted microsomal prostaglandin E synthase type 1 (mPGES-1). This enzyme is induced by LPS and cytokines in tandem with COX-2 and appears to be the origin of PGE_2 synthesized during inflammation (79–81). LTC4S, FLAP, MGST1, MGST2, MGST3, and mPGES-1 are now recognized as members of a common superfamily of integral membrane proteins denoted MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) (82). The human *LTC4S* gene is located on chromosome 5q35 and has a structure similar to that of the *FLAP* gene (83). Promoter characterization has shown that an SP1 site and a putative initiator element (Inr) are involved in non–cell-specific expression, whereas a Krüppel-like transcription factor and SP1 are implied in cell-specific regulation of LTC4S (84). LTC4S expression is induced by cytokines and phorbol-12-myristate-13-acetate (PMA) in human erythroleukemia cells as well as in human eosinophils developed from IL-3– and IL-5–treated cord blood progenitors (67, 85). The enzyme is also upregulated in the monocytic cell line THP-1 after TGF-β treatment, apparently via SP1 and SP3 (86, 87). In addition, IL-4 strongly induces expression of LTC4S in cord blood–derived human mast cells and bone marrow–derived mouse mast cells, a response that may be signaled via STAT-6 (88). Finally, intraperitoneal injection of LPS in the rat caused in vivo induction of the enzyme in liver, heart, adrenal gland, and brain (89).

Crystal structure of LTC4S. The crystal structure of human LTC4S has been solved at high resolution (90, 91). The enzyme is a trimer, and each monomer is composed of 5 α-helices, four of which traverse the membrane (Figure 2). The lipid (LTA_4) binding site is believed to be located in a hydrophobic crevice formed at the interface between two adjacent monomers. The GSH bound deeper in the protein below the hydrophobic cleft adopts a peculiar horseshoe-shaped conformation. Residues from two monomers are involved in GSH binding, and mutagenetic analysis

has demonstrated that Arg104 is catalytic and rapidly activates the GSH thiol (92, 93).

Leukotriene biosynthetic complexes at ER and perinuclear membranes

The superordinate enzymes in leukotriene biosynthesis, cPLA2α (which provides AA) and 5-LOX (which converts AA into LTA_4), traffic from cytosol to the ER and perinuclear membranes in response to cell activation (Figure 1). In the target compartment, FLAP and LTC4S are embedded in the membrane, ready to support biosynthesis of LTA_4 and its further conversion into LTC_4 (94, 95). In fact, biophysical evidence indicates that FLAP and LTC4S form functional heterodimers and trimers within the membrane and that FLAP can act as a scaffold protein for association of 5-LOX, thus creating a multiprotein biosynthetic complex on both the outer and inner nuclear membranes (40, 96). It is not clear how AA, liberated by cPLA2α, can first reach its binding site in FLAP, travel further to the active site of 5-LOX, and, after conversion into LTA_4, reach the terminal LTA4H and LTC4S. To synchronize this machinery, it seems necessary that both cPLA2α and 5-LOX are juxtaposed in direct contact with the nuclear membrane and that all biosynthetic components are spatially interconnected. Since LTA4H is soluble and does not translocate to the nuclear membrane, transfer of LTA_4 from 5-LOX to LTA4H seems to require a carrier, and recent data indicate that this process involves substantial conformational changes within LTA4H, accompanied by gated entry of LTA_4 (62).

Therapeutic and pharmacological opportunities and pitfalls

It is not within the scope of this Review to give a comprehensive description of all pathologies potentially involving leukotrienes. Here, I would like to mention four disease areas that are currently attracting considerable attention. Firstly, leukotrienes are strongly implicated in immunometabolic disorders ranging from obesity to type 2 diabetes. It has been demonstrated that enzymes and receptors of the 5-LOX pathway are upregulated in adipose tissue and that mouse and human adipocytes can secrete leukotrienes (97, 98). Importantly, LTB_4 appears to play a critical role in adipose tissue inflammation, and a FLAP antagonist reduced 5-LOX products and macrophage accumulation in adipose tissue in mice with dietary obesity (97). In addition, pharmacological or genetic ablation of the 5-LOX pathway in WT mice on a high-fat diet resulted in a reduction of adipose tissue macrophage and insulin resistance (98). Recent data demonstrate a similar role for LTB_4 in promoting liver steatosis and insulin resistance in muscle and adipose tissue, reinforcing the role of the LTB_4/BLT1 signaling axis as a main driver for the inflammation–insulin resistance syndrome in obesity (99). Similar work on the BLT1 receptor corroborates the notion that inhibition of LTB_4 signaling can be a useful therapeutic strategy in diseases related to insulin resistance (100).

An increasing body of evidence also suggests that 5-LOX and leukotrienes are involved in neurodegenerative disorders such as Alzheimer's disease (AD). The 5-LOX pathway seems to modulate AD pathology at multiple levels (101). Using transgenic mouse models of AD, it was demonstrated that 5-LOX deficiency decreases amyloid β peptides, which translates to fewer amyloid β plaques and reduced total amyloid burden in the brain, apparently through elevation of all components of the γ-secretase complex (102). Furthermore, 5-LOX contributes to tau hyperphosphorylation, compromised synaptic function, and memory deficits. Notably, pharmacological or genetic inhibition of 5-LOX counteracts all these effects and can even restore learning and memory impairments (103–107). A recent study suggests that cys-LTs may play a role in AD, as a selective CysLT1 antagonist reduced neuroinflammation, elevated hippocampal neurogenesis, and improved learning and memory in rats (108).

Leukotrienes have long been linked to cardiovascular diseases, and major efforts have been invested into development of antileukotrienes for prevention of atherosclerosis and treatment of myocardial infarction (49, 109, 110). Here, I would like to highlight two other pathologies of the cardiovascular system, namely, pulmonary hypertension (PH) and abdominal aortic aneurysm (AAA), both of which appear to be associated with leukotrienes and thus possibly amenable to antileukotriene treatments targeting the enzyme machinery.

Early work in rats and mice linked the 5-LOX pathway to hypoxia-induced PH, and 5-LOX expression was increased in pulmonary macrophages and pulmonary artery endothelial cells in patients with idiopathic pulmonary arterial hypertension (111, 112). These findings were specious, because a prominent pathological feature of PH is accumulation of macrophages near the arterioles of the lung. Further investigations pointed to a critical role of LTB_4 in the disease process. Thus, studies in several rat models of PH as well as cells and tissue samples from patients with PH revealed elevated levels of LTB_4, both systemically and locally in lung tissue, and high levels of LTA4H were observed in accumulated macrophages (113–115). At a functional level, macrophage-derived LTB_4 was shown to induce apoptosis of pulmonary artery endothelial cells as well as proliferation and hypertrophy of pulmonary smooth muscle cells (113). Furthermore, LTB_4 can activate pulmonary artery fibroblasts (116).

Leukotrienes are also involved in AAA disease. In early work on mouse models of atherosclerosis, 5-LOX deficiency protected against AAA, results that could not be reproduced in the angiotensin II–induced model of AAA (117, 118). Based on work with human tissue samples, it was proposed that LTB_4 plays a role in AAA as a chemotactic factor released from neutrophils within the intraluminal thrombus; cys-LTs were identified as main 5-LOX products in human AAA wall and could be linked to release of matrix metalloproteinases (119, 120). In agreement with these data, inhibition of 5-LOX by pharmacological or genetic approaches attenuated aneurysm formation in two different AAA mouse models (121). Only very recently, we showed that cys-LTs are involved in AAA and that the common asthma drug montelukast, a selective CysLT1 antagonist, afforded protection in three different mouse models of AAA (122). The therapeutic significance of this study lies in the fact that montelukast is a well-tolerated and safe drug that can be directly tested in a human clinical trial. However, it is possible that an inhibitor of LTC4S, alone or in combination with an inhibitor of LTA4H, could prove more effective (see below).

5-LOX and FLAP bridge proinflammatory and pro-resolving pathways. 5-LOX inhibitors are divided into three classes depending on their mode of action: (a) redox inhibitors, (b) iron-binding inhibitors, and (c) active site–directed inhibitors. The early com-

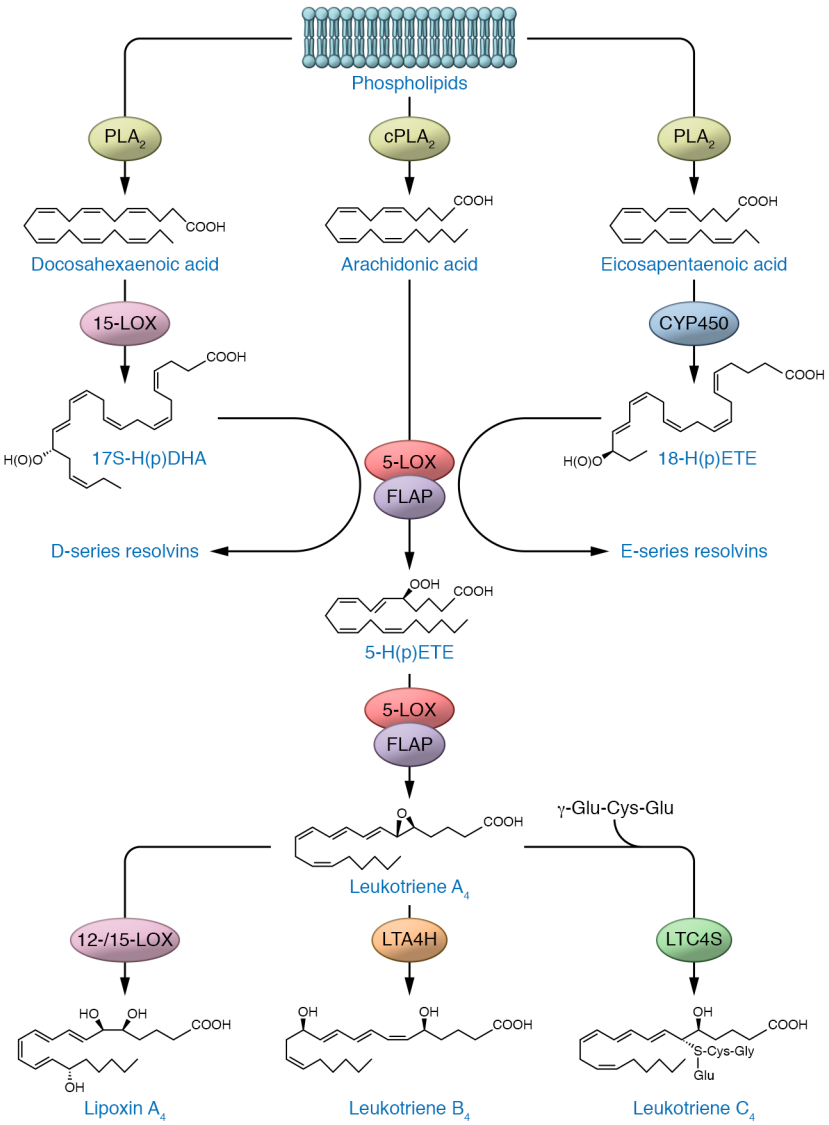

Figure 3. Metabolism of polyunsaturated fatty acids into leukotrienes and resolvins. The scheme illustrates the interconnections between the pathways for synthesis of proinflammatory leukotrienes and antiinflammatory lipoxins and resolvins from arachidonic, eicosapentaenoic, and docosahexaenoic acids. For D- and E-series resolvins, the respective fatty acid substrate is first oxygenated by 15-LOX or cytochrome P450. Inhibition of 5-LOX attenuates leukotriene formation and may interfere with synthesis of pro-resolving molecules. Inhibition of LTA4H and/or LTC4S will block leukotrienes, shunt LTA_4 into lipoxin synthesis, and spare 5-LOX activity for generation of other pro-resolving molecules.

pounds suffered from lack of selectivity, structure-activity relationships, and enantioselectivity; moreover, they elevated methemoglobin levels and displayed poor efficiency and oral availability (1). Nonetheless, in clinical trials, zileuton showed beneficial effects in many inflammatory diseases, and today zileuton is marketed in the United States for treatment of asthma. With the crystal structure of 5-LOX at hand, structure-based approaches have become possible, although a complex between enzyme and an inhibitor has not yet been presented. Nonetheless, a stream of new 5-LOX inhibitors, either synthesized or naturally occurring, have been presented in recent years, but their clinical utility has not been tested (123, 124).

Several inhibitors of FLAP have also been developed over the years: the classical prototype MK-886, the follow-up MK-0591, and Bay-X1005 (1). A second generation of FLAP inhibitors was also developed from the lead molecule denoted AM103, primarily intended for use as an anti-asthma medication (125, 126). Crystal structures of FLAP in complex with inhibitors have been reported (52), but the resolution was rather low, limiting its use in structure-based approaches to drug design (Figure 2).

5-LOX converts AA into LTA_4, which can be lipoxygenated into lipoxin A_4 (LXA_4) and also appears to be involved in the biosynthesis of pro-resolving molecules originating from eicosapentaenoic and docosahexaenoic acids (Figure 3 and ref. 127). This dual role of 5-LOX at the crossroads of both proinflammatory leukotrienes and pro-resolving mediators may weaken the prospects of developing antiphlogistic drugs targeting 5-LOX. However, the roles of 5-LOX and FLAP in biosynthesis of pro-resolving mediators are complex. In one study, lipoxin and resolvin biosynthesis was shown to depend on the presence of FLAP, suggesting that a selective FLAP inhibitor will not allow continued 5-LOX activity for production of pro-resolving mediators (128), while another recent study showed that FLAP inhibition does not affect synthesis of pro-resolving mediators from endogenous docosahexaenoic acid (129). Moreover, it appears that cytosolic 5-LOX, uncoupled to FLAP, favors synthesis of pro-resolving mediators over LTB_4 (130). Interestingly, FLAP and certain 5-LOX inhibitors are more effective in females, and apparently these sex differences are caused by androgens impeding assembly of the leukotriene-biosynthetic 5-LOX/FLAP complex (131).

LTA4H, target for development of resolution-promoting molecules. Development of inhibitors targeting LTA4H began when its zinc content and aminopeptidase activity were discovered, offering a simple model of the active site (55). Thus, academicians identified bestatin and captopril as LTA4H inhibitors (132), which were followed by various transition state mimics (133, 134). Searle/Pharmacia developed the clinical candidate SC-57461A, which is today's benchmark for a potent and selective inhibitor (135). With the crystal structure of LTA4H, programs on rational inhibitor design began within industry. Thus, deCode Genetics developed DG-51 for treatment of myocardial infarction and stroke, while Johnson & Johnson developed JNJ-26993135 for treatment of inflammatory bowel disease and allergic airway inflammation (136–139). Similarly, work at Berlex/Schering led to the development of acebilustat, which is currently in clinical trial for treatment of cystic fibrosis (140). Two additional LTA4H inhibitors, bestatin and tosedostat, are presently entering clinical trials (141). Bestatin is intended for use in pulmonary arterial hypertension and lymphoedema, while tosedostat is an antineoplastic agent for treatment of a variety of severe cancers. However, both bestatin and tosedostat are general aminopeptidase inhibitors, and their pharmacological actions may well be off-target effects (141).

Inhibitors of LTA4H not only block LTB_4 synthesis, they also spare 5-LOX–derived LTA_4 to allow shunting into lipoxin synthesis (Figure 3 and refs. 138, 139). In addition, we recently developed a new class of LTA4H inhibitors, typified by the lead molecule ARM1, that selectively block synthesis of LTB_4 while preserving the peptidolytic inactivation of chemotactic PGP (142). Hence, this type of inhibitor will inhibit LTB_4 synthesis, enhance lipoxin generation, and salvage PGP inactivation, offering a greater therapeutic potential as compared with previously developed small molecules.

LTC4S inhibitors are lukast alternatives with resolution-promoting properties. In spite of its central role in allergic inflammation, very few specific inhibitors of LTC4S have been developed thus far (143–145). This may reflect the success of CysLT1 antagonists, collectively referred to as "lukasts," that are currently used in clinical management of asthma. However, a new generation of lukasts is needed, because a significant proportion of asthma patients (~40%) do not respond to these drugs. In addition, new receptors for cys-LTs have been discovered that are functionally interconnected and cross-regulated (146–149). Hence, the upstream biosynthetic enzyme LTC4S may well prove to be an effective target for pharmacological inhibition of cys-LT signaling, not only for asthma but also for other diseases involving cys-LTs such as AAA, celiac disease, and neurodegenerative disorders (108, 122, 150). It is clear that inhibitors of LTC4S can be made selective among other MAPEG enzymes and effective in vivo, and permit shunting of LTA_4 into LXA_4 synthesis (145).

Antileukotrienes target a metabolic system with multiple levels of complexity. According to the current dogma, leukotrienes are formed within a time frame of seconds to a few minutes following an acute cell stimulus, causing calcium mobilization and activation of 5-LOX. However, synthesis of leukotrienes can occur in response to cell stress without calcium mobilization, and in some instances biosynthesis can proceed over longer periods of time, even hours, in a cell type– and stimulus-dependent manner (27, 129, 151). Moreover, resting monocytes cultured with GM-CSF produce significant amounts of LXA_4, which normally involves 5-LOX activity, without concomitant synthesis of appreciable quantities of leukotrienes (152). Apparently, the activity of 5-LOX and downstream enzymes depends on a multitude of factors leading to differential profiles of mediators in any given cellular context.

It is also important to keep in mind that enzymes of leukotriene biosynthesis interact with other proteins and/or possess secondary catalytic activities, adding additional levels of complexity to the biosynthetic machinery. For instance, 5-LOX is present in the nucleus and interacts with Dicer, suggesting that it may exert effects on micro-RNAs and regulation of other genes (153). 5-LOX also interacts with cytosolic CLP, the functional consequences of which are only beginning to be described (46). Furthermore, LTA4H carries an aminopeptidase activity, and although PGP has been identified as one endogenous substrate, there are probably other substrates and more to learn about this enzyme activity. Similarly, most MAPEG members possess a peroxidase activity toward lipid hydroperoxides and may thus help control the redox milieu at the nuclear membrane with potential consequences for a variety of cellular processes. Hence, caution is warranted in evaluating the effects of pharmacological intervention within the leukotriene cascade, since enzyme inhibitors may give rise to unanticipated effects that may, or may not, be related to attenuated leukotriene synthesis.

Concluding remarks

The powerful bioactivities of leukotrienes have motivated academia and the pharmaceutical industry to develop antileukotriene drugs to treat a range of inflammatory diseases (1, 124). To date, CysLT1 antagonists and one 5-LOX inhibitor have reached the clinic and are used for medical treatment of asthma and allergic rhinitis. The pharmaceutical industry prefers to develop drugs as a single molecule against a single target. However, this classical type of approach has not provided satisfying results when targeting the leukotriene cascade, which may have several explanations. Thus, results from preclinical research on leukotrienes may sometimes poorly reflect the human pathology, and therefore its predictive value for successful drug development may be limited. In addition, leukotrienes are generated by activated leukocytes, and the impact of these mediators will depend on temporal accumulation and composition of immune cells at various stages of the disease process.

Most likely, disappointing outcomes also originate from the fact that branches of the metabolic pathways are interconnected in a complex manner, and a targeted blockade may cause shunting of metabolites to other families of lipid mediators with opposing bioactions, mitigating the effects of the primary intervention.

With increasing knowledge about the flux of metabolites, new "smart" combinations of drugs can be designed. For instance, rather than blocking 5-LOX to eliminate all leukotrienes, one could combine inhibitors of the downstream LTA4H and LTC4S to shunt LTA_4 into lipoxins and to preserve 5-LOX–dependent biosynthesis of other pro-resolving mediators (Figure 3). The spectrum of opportunities increases even further if receptor antagonists and inhibitors of the COX cascade are taken into consideration. Since there is a significant interindividual variation in leukotriene and lipid mediator biosynthesis, it may be necessary and possible in the future to assess lipid

profiles in individual patients to guide tailor-made personalized drug combinations to achieve effective clinical results. We are now experiencing the uncovering of new leukotriene-dependent pathologies, and as the details of the mechanisms regulating the biosynthetic pathways become better understood and controlled, opportunities for development of new drugs, as well as repurposing of already existing drugs, are clearly emerging.

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