

Lipoxygenases – A Challenging Problem in Enzyme Inhibition and Drug Development

Ewa Skrzypczak-Jankun^{*,1}, Joanna Chorostowska-Wynimko², Steven H. Selman¹ and Jerzy Jankun¹

University of Toledo, College of Medicine, Urology Research Center, Toledo, OH 43614, USA; Laboratory of Molecular Diagnostics, National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland

Abstract: Lipoxygenases (LOXs), cytochromes P450 (CYPs) and cyclooxygenases (COXs) catalyze peroxidation of unsaturated fatty acids. In humans they convert arachidonic acid into a variety of eicosanoids, which play a role in all inflammatory responses, cardiovascular and kidney diseases, Alzheimer's, cancer and other ailments. Blocking one pathway can prompt the body to switch to the available alternatives. In contrast to CYP and COX, LOX has a non-heme iron co-factor. Several LOXs are produced or stress-induced in the human body. They share the same mechanism, but differ in sequence causing catalysis on the same substrate to be regio- and stereospecific. The action of 15-LOXs could be pro- or anti-inflammatory, and pro- or anti-carcinogenic. Depending on the dose, LOXs inhibitors can induce or inhibit other oxygenases. Inhibition of these enzymes presents a great challenge in solving the problem of how to control their action and treat diseases, without causing severe side effects and maintaining/restoring a delicate equilibrium between them. Research on CYPs and COXs is more advanced, while studies of LOXs are lagging behind. This article presents a brief review about LOX structures and inhibition, their involvement in human diseases, and their interplay with other oxidoreductases.

Keywords: Lipoxygenase, fatty acids, inhibition, structure, NSAIDs, regulation of oxygenases, eicosanoids in human diseases.

LIPOXYGENASES AND THEIR PLACE AMONG OXIDOREDUCTASES

Polyunsaturated fatty acids (PUFAs) are metabolized in the body *via* three ubiquitous metalloenzymes called oxidoreductases (or oxygenases): epoxyoxygenase, i.e. cytochrome P450 (CYP), cyclooxygenase (COX) and lipoxygenase (LOX). These enzymes catalyze a typical redox reaction in which their metal cofactor changes its oxidation state $Fe^{+2} \leftrightarrow Fe^{+3}$, while incorporating atomic O or molecular O_2 oxygen into the polyunsaturated fatty acid (PUFA) substrate, producing a large variety of products called eicosanoids. In general, the metabolites of FAs oxygenation from these three pathways can be classified as follows: epoxyeicosatrienoic acids (EpETEs) from cytochrome; prostaglandins (PG), prostacyclins, prostamides and thromboxanes (TX) from COX; leukotrienes (LT), lipoxins, hydroperoxy- (HpETEs) and hydroxyeicosanoids (HETEs) from the LOX pathways [1]. CYP and COX are microsomal enzymes, while LOX is mainly cytosolic. However, LOX activity was also observed in the nuclear envelope, mitochondria, microsomes, and in some cases, in intracellular membranes [2]. Oxygenases are present in many cells and organs and their involvement, as well as the role of their metabolites has been well established in inflammatory responses, cardiovascular diseases and cancer. Utilizing the Internet one can find examples of the presence of oxygenase(s) and the relevant eicosanoid(s) for a huge variety of human diseases from brain disorder (Alzheimer's disease

[3]) to osteoporosis [4]. In addition to FAs metabolism, all three oxygenases are very potent biocatalysts metabolizing a wide range of endogenous compounds in our body, as well as xenobiotics, including drugs. It is believed that the drug metabolism occurs mainly (but not exclusively) *via* CYP in liver, *via* LOX in kidney and *via* COX in stomach. In many systems, all three enzymes could be represented and their regulation by nonspecific inhibitors might lead to inhibition or induction depending on the dose, and up-regulation of one enzyme accompanied by down-regulation of another. Therefore, regulation of PUFA metabolizing enzymes is a great challenge with far reaching consequences.

Structurally, these three enzymes are very different (Fig. (1)). Both COX and CYP have their metal cofactor (Fe) incorporated in the heme moiety. CYP has an active site in the immediate vicinity of heme and an additional FA binding site at the molecular surface, while in COX, both the FA substrate and NSAIDs, bind into a channel separated from heme by ~ 20 Å and with no passage to the heme site. In terms of the structure and function, LOXs are unique, because their metal cofactor is a single ion bound by the side chains of the surrounding amino acids and the carboxylic group of the C-terminus, and their inhibitors bind to or near the Fe co-factor.

The microsomal, membrane associated human CYP has several isozymes that participate in the oxidative metabolism of drugs. Three of them [5-7], CYP450 2C8, CYP450 2C9 and CYP450 3A4, have been characterized by X-ray analyses in a native form and/or in complexes with drugs [8, 9]. These structural studies provided evidence of the intricate subtleties of CYP molecular architecture. While two human CYP450 isozymes, 2C8 and 2C9 are 79% identical in sequence, 3A4 has the same fold, but shares only 24%

*Address correspondence to this author at the University of Toledo – Health Science Campus, College of Medicine, Urology Research Center, 3000 Arlington Ave., Toledo, OH 43614, USA; Tel: 419-383-5414; Fax: 419-383-3785; E-mail: ewa.skrzypczak-jankun@utoledo.edu

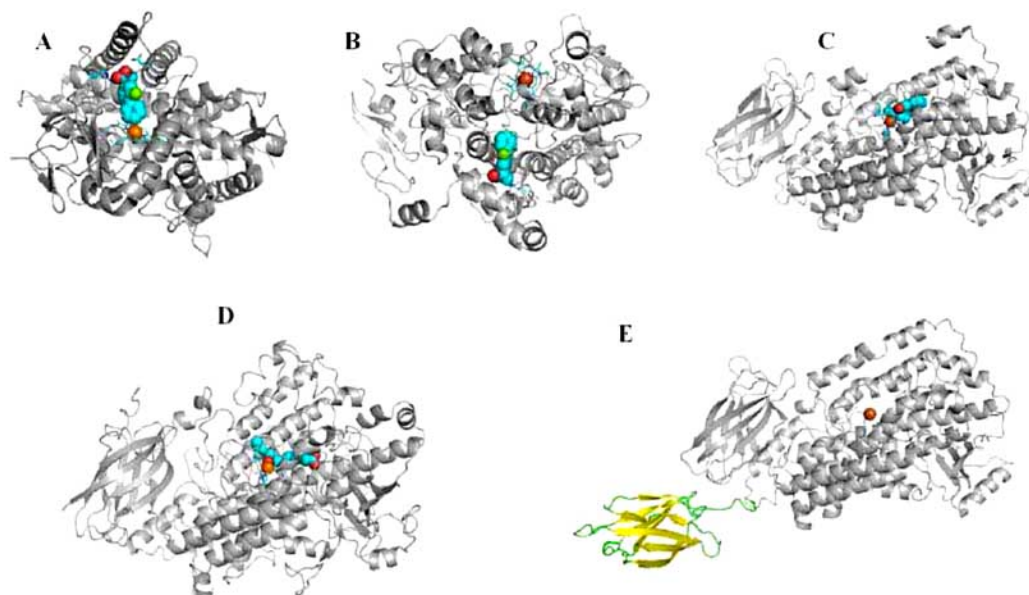


Fig. (1). A schematic representation of three types of fatty acids metabolizing oxygenases, protein as a gray ribbon, iron as an orange ball, heme or selected residues as sticks, inhibitor as a space filling model with colors C-cyan, N-blue, O-red, Cl- green. **A:** Human cytochrome P450 2C9 in complex with flurbiprofen (PDB: 1r9o), the drug binds close to the heme moiety, makes hydrogen bonds to Arg 108 and Asn 204. **B:** Sheep COX-1 complexed with flurbiprofen (PDB: 1eqh), the drug binds in the channel with the entrance at the opposite site of the molecule in relation to the heme, making a hydrogen bond to Tyr 355. **C:** Rabbit 15-LOX-1 complexed with the inhibitor 3-(2-octylphenyl)propanoic acid (PDB:1lox), a non-covalent complex, the ligands to iron are His 361,366,541,545, Ile 663. Residues 178-188, 209-210, 598-599 near the active site were not determined in this structure. **D:** Soybean LOX-3 covalent complex with 13S-HpODE (PDB: 1ik3), the 'purple LOX' with peroxide as the iron ligand together with His 518,523,709, Ile 857, the product's carboxylic group buried deeply in the channel near Arg 726, Ser 510. **E:** A homology model of human 12-LOX demonstrating the flexibility of the N_T -sandwich domain. According to theoretical predictions, the upper part was modeled as a helix with a coil fragment at Gly181-Leu182-Ala183.

sequential identity (with 2C9), differs in the details of the secondary and tertiary structure, and shows a striking differences in the volume (520 \AA^3 vs. 600 \AA^3) and shape of the active site cavity (Fig. (1) in [9]). Human COX has 3 isozymes: normally secreted COX-1, stimuli induced COX-2, and COX-3, which is not yet well understood, differs from the others mainly in its N-terminus, and is most abundant in the cerebral cortex and heart [10]. The structure of human COX-2 in complex with non-steroidal anti-inflammatory drugs (NSAID) has been described by the scientists from the Inflammatory Disease Unit of Roche Bioscience [11], but has not been deposited in the Protein Data Bank (PDB). Hence, our understanding of COX-1 and COX-2 architecture is based on structural data for sheep COX-1 [12, 13], or mouse COX-2 enzymes [14, 15], which share ~90% sequence identity with their human counterparts. The structures of the COX complexes have been studied not only for complexes with drugs, but also with arachidonic acid (AA), giving opposite results as to the AA orientation in the active site. Considering that sheep COX-1 and mouse COX-2 share only 64% identity, the former had heme with Co instead of Fe, and the latter was an apo enzyme, this should not be taken as the conflicting results, but rather as an illustration of how different the outcome can be for topologically similar, yet chemically different molecules.

STRUCTURE OF LIPOXYGENASE

LOXs are classified into 3 major categories [16], as 5-, 12- and 15-LOX, accordingly to the outcome of the

conducted catalysis, i.e. upon which C-atom in the AA chain they act, giving respectively 5-, 12-, 15- and sometimes 8-, 9-, 11-, 13- eicosanoids, that can be of the 'S' or 'R' chirality.

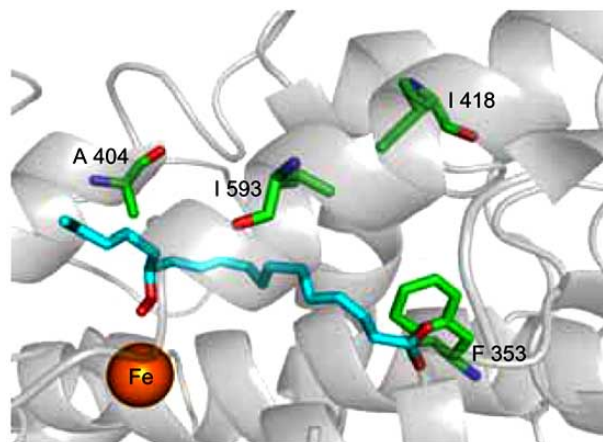
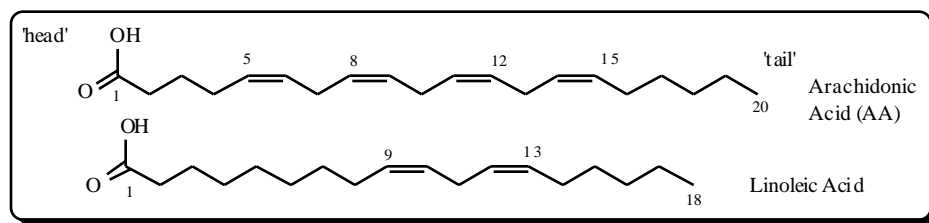


Fig. (2). 13(S)HpODE from PDB: 1ik3 (as in Fig. (1D)) superimpose with r15-LOX (Fig. (1C)) showing positional determinants in the structure of rabbit enzyme. 13-HpODE as product of linoleic acid oxidation is C18 with two double bonds 9Z,11E; while 15- or 12-HpETE would be C20 with four double bonds; 5Z,8Z,11Z,13E for 15-HpETE, or 5Z,8Z,10E,14Z for 12-HpETE. Position of peroxidation, S/R configuration and Z/E of double bonds are regulated by LOX's specificity.



Since the leucocyte-type 12-LOXs are very similar to the reticulocyte-type 15-LOXs, these enzymes are often described as 12/15-LOXs. Comparison of sequences of 6 human lipoxygenases listed in the Swiss-Prot database (5-LOX, 12-LOX 'S' platelet-type and 'R' epidermis-type, 15-LOX-1 and -2, and epidermis eLOX-3) shows a ~50% similarity for enzymes that range from 662 to 711 in content of amino acids (Table 1). Contrary to human COX-1 and COX-2, which share an 89% similarity among ~600 residues, different LOX isozymes are less homologous. This means that although they may share the same type of folding and topology, they might be very different in their molecular properties, for instance: pI (Table 1), the size and shape of cavities, channels, the charge distribution, etc., all factors which have an impact on molecular recognition, interactions and outcome of catalysis. Therefore, regulating LOX activities could be more complicated and a much greater challenge than in the COX case, requiring an intimate knowledge about their three-dimensional structure. Unfortunately, the only available structural data for LOX (from a 'single crystal' X-ray analysis) consider ~30% bigger plant enzymes (soybean LOX-1, soybean LOX-3) or similar in size (to human isozymes), but incomplete structures of 15-LOX from rabbit reticulocytes [17] and 8R-LOX from coral [18]. The structure of the rabbit enzyme lacks the regions near the active site (Fig. (1c)) and has some residues reported without side chains, which is also true for the coral enzyme reported at only 3 Å resolution.

Table 1. Human Isozymes of LOX, COX, CYP450 - Calculated pI

	Swiss-Prot	no. aa	pI
5-LOX	P09917	673	5.51
12-LOX	S-type P18054	662	5.82
12-LOX	R-type O75342	701	7.57
e-LOX-3	Q9BYJ1	711	6.53
15-LOX-1	P16050	671	6.14
15-LOX-2	O15296	676	5.79
COX-1	P23219	599	7.00
COX-2	P35354	604	7.02
COX-3	Q3HY28	630	6.81
CYP_2C8	P10632	490	8.80
CYP_2C9	P11712	490	8.13
CYP_3A4	P08684	502	8.30

Structural Domains and Membrane Binding

Despite their different sizes, plant and mammalian LOXs share a similar topology and consist of two major domains, the N_T -sandwich domain of regulatory function and a much larger catalytic domain, which can be subdivided into 4 subdomains [19]. The N_T -part is classified as a typical PLAT domain that might mediate protein-protein and protein-lipid interactions [20]. All three oxygenases interact with the phospholipid membrane. CYPs have a transmembrane domain of only ~25 residues and its structure has not been determined in the available structural data, which concern molecules where this domain has been truncated. COX attaches to the membrane by a crown of residues from the A-D helices around the entrance to the FA binding channel [21]. In LOXs, the attachment involves both domains (in soybean LOX), the mediation of another protein like FLAP for 5-LOX, or in the other cases, it is not quite clear if that interaction requires a singular or dual action of domains. As for activity, soybean LOX-1 gains activity upon truncation to the catalytic domain, which might be related to easier access to iron, upon removal of the regulatory domain. In r15-LOX, a decrease of activity was observed for the truncated enzyme [22], but one might suspect that it was caused by some decrease in the iron content, rather than a real change in the activity itself (see remarks on this topic in the next paragraph). All LOX crystal structures show the regulatory and catalytic domains adjacent to one another. Soy enzymes (N_T -domain ~170 amino acids) have a much larger interface and their domains are tightly connected by multiple hydrogen bonds, both in crystal as well as in the solution (based on SAXS studies, Hammel *et al.*, 2004 in [22]). In the rabbit enzyme the interface surface is 40% smaller, has only a handful of strong hydrogen bonds and shows unusually high B-factors (average B for N_T - and catalytic domain respectively 63 and 46 Å² for cryogenic data obtained at -190°C). It was proven by mutation studies that Trp100 buried between the domains in the crystal structure is essential for the membrane binding. SAXS results indicate that in solution the domains are separated, the PLAT domain is either tilted (~40° from the catalytic domain) or fully extended expanding the molecular length to ~135 Å in contrast to ~100 Å while packed in the crystal. Our own SAXS studies of hp-12-LOX show an even greater maximum length (~150 Å, Dr. L. Guo, ANL APS BioCAT 18ID, unpublished results) pointing out that this regulatory domain of ~110 residues in mammalian LOXs might have great flexibility utilizing ~14 amino acids long inter-domain linker (from -sandwich to the first helix) to exercise its freedom of movement. The other four subdomains comprise the catalytic domain with the iron co-factor attached covalently to the residues from the largest, fifth subdomain comprising a bundle of up-down helices. These four subdomains define channels leading to the active site and

lining of the central cavity, thus their sequential composition and relative orientation control the size and shape of the passages and cavities determining the enzyme's selectivity and specificity, while together with the first domain regulate access to iron. Even our limited knowledge from modeling of human LOXs allows us to hypothesize that the iron site is more exposed, and the 'traffic control' there could be different in mammalian LOXs than in plant LOXs.

Calcium cations are known to mediate binding between proteins and phospholipids membrane. Oxygenases are no exception and Ca^{+2} has been found to stimulate binding to membrane and in coral 8R-LOX and sLOX-1 also increase the enzymatic activity [18, 23].

Active Site, Iron Content and Specificity of Catalysis

A comparison of sequences shows that ligands to iron are highly conserved among plant and mammalian species and in known structures they are arranged around iron in a pseudo octahedral geometry with one empty site (sometimes reported as occupied by H_2O or OH^-). However, the size, shape and lipophilicity of the cavity lining differ among species and isozymes accordingly to their sequence. Theoretically, the protein to iron stoichiometry is 1:1. For samples produced in the laboratory, the iron content in native LOXs has been reported as 0.35 to 0.7 per enzyme molecule (for mammalian [25, 26], higher for plant [27, 28]), and our own observations fall into that range as well. Its determination requires large quantities of pure protein to be accurate, hence it is seldom examined. An occupancy of iron cannot be refined in X-ray structure unless the data extend to atomic resolution and so it is not surprising that this variable was not refined even for soybean LOX-1 determined at 1.4 Å. Since occupancy and B factor are correlated overestimation of occupancy (1.0 instead of something more realistic) causes elevated B for the iron ion and that is what we commonly see in the PDB entries. A comparison of soybean and rabbit LOX complexes with small molecules [29] shows that the inhibitors bind into the central cavity bound to or in the vicinity of iron, utilizing or blocking 1-2 places of the iron's coordination sphere. The mammalian rabbit 15-LOX has four histidines and C-terminus as ligands for iron co-factor, same as in h-15-LOX, while hp-12-LOX and 5-LOX have asparagine in place of the last histidine like the soy isozymes. Conclusions from studies of soy isozymes suggest that this variable ligand (i.e. His ligand with the highest sequential number or Asn in its place) and His with the lowest sequential number have higher mobility and move during inhibitor (substrate? product?) binding, while the other two histidines stay put, and the terminal isoleucine adapts its position as necessary. The cavity near the active site can substantially expand its size by making changes in the conformations of its residues, responding to the size of the molecule that it interacts with [30].

Positional Specificity

Based on calculated models of 5-, 12- and 15-LOX Gillmor *et al.* [17] hypothesized that the enzyme's positional (or regio-) specificity depends on the depth/volume of the cavity where AA binds. In that 'dip-

stick' model, it was assumed that AA binds with the carboxylic end at the cavity entrance. The structure of the 'purple' form of the soybean LOX-3, of its complex with the product of catalysis (soy LOX catalyses peroxidation of the linoleic acid), is the only glimpse that we have for any lipoxygenase in action [31]. The purple color is typical for $\text{R}_1\text{-Fe}^{+3}\text{-O-O-R}_2$ chromophore in the iron complexes. The soy LOX-3 complex, Enz-Fe-O-O-R , shows 13-HpODE bound covalently to Fe and having its carboxylic end deeply buried inside the catalytic domain (Fig. (1D)). Making far reaching conclusions based on modeling could be risky. Models of human 5-, 12- and 15-LOX (PDB: 2abv, 2abu, 2abt respectively) deposited in the PDB by the researchers from Bioinformatic Centre, Pondicherry University, India, show a rather random conformation with an unspecified secondary structure for the parts near the active site. Unfortunately, there is no information on how the authors made their calculations. Models of human 12- and 15-LOX were also prepared by Kenyon *et al.* [32] based on homology modeling and energy minimization, and successfully utilized for *in silico* searches for inhibitors, proving their usefulness. Although, to the authors disappointment, two out of the three best inhibitors showed little preference toward 15-LOX in relation to 12-LOX (with IC_{50} differences within 2), and one (out of 50,000 virtually screened compounds) was 15-LOX specific with $\text{IC}_{50} = 7\mu\text{M}$ vs. $>200\mu\text{M}$ for 12-LOX. This indicates that theoretical models lack subtle structural features crucial for distinguishing an isozyme's specificity. Experiments with LOXs mutants and photoaffinity labeling have proven that *the enzyme's specificity can be easily changed* by single mutation [22, 33, 34] and that FA orientation could be either 'head' or 'tail' first.

Stereo-Specificity

The isozymes LOX-1 and -3 from soybean seeds are the only examples of LOX 3D-architectures, for which we can compare isozymes from the same source (the structures of two isozymes from soybean leaves LOX-B and LOX-D were deposited in June 2006, awaiting release upon publication, PDB: 2iuj, 2iuk). They both produce 9- or 13-HpODE in both S and R configurations [35-37]. While sLOX-1 makes predominantly (but not exclusively) 13- and S stereoisomer, sLOX-3 turns out equal amounts of both, with a preference for the *cis-trans* vs. *trans-trans* dienoic system and R over S (~60:40).

Both positional and stereo-specificity of LOXs can be manipulated by site directed mutagenesis. Based on mutation studies of mammalian enzymes, Ile593, Ile418 and Phe353 (r15-LOX numbering, and relevant residues in aligned sequences of other mammalian LOXs) were listed as the amino acids affecting the enzyme's specificity [22, 34, 38]. Other researchers suggested [33, 39, 40] that a single residue Ala (Ala 404 in r15-LOX numbering) or Gly, controls specificity, and that the position of peroxidation and chirality of this carbon are interrelated. According to this hypothesis, Ala induces peroxidation of the FA pentadiene center on its end deep in the binding site resulting in the S orientation, while enzymes with Gly act on the proximal end of the reactive pentadiene producing the R stereoisomer. A comparison of these regions in the sequences of sLOX-1 and -3 shows that both have Ala (Table 2), although there is a change Ala→Gly in the same turn of the helix. Based on

structures of the soy isozyme, a region at the opposite site of the cavity (Leu 773 in sLOX-3 corresponding to Leu 597 in r15-LOX) was suggested as cooperating in defining the enzyme specificity in these enzymes. 13-HpODE in soy 'purple' LOX-3 was not found at the area defined by Ile593, Ile418, Phe353 in r15-LOX. The His Fe ligand, with the lowest sequential number, and the adjacent Trp (His518, Trp519 in sLOX-3) were proposed as residues able to remove hydrogen from the active pentadiene and impose chirality of resulting product of oxidation in plant LOXs (Fig. 6 in [31]). Mammalian LOXs have Leu in place of Trp, therefore the residues participating in the catalytic mechanism and binding of FA are not exactly in the same location in space in plant and mammalian enzymes. Fig. 2 illustrates the location of the discussed residues showing Ala 404 on one side of the groove, opposite Leu 597 with Ile 593 at the next turn of the same helix on the other side, and Ile 418 at the groove's end to the right. Ala 404 and Leu 597 are near the oxidized carbon, C13 in the soy LOX product, while its 'head' collides with Phe 353 and its 'tail' points to the left. Fig. 2 is a simple superposition of two proteins, done for the sake of this discussion (only 13(S)-HpODE from PDB: 1ik3 is displayed, soy enzyme is omitted from picture for clarity) without any remodeling that would consider changes in conformation to fit 13-HpODE into the space available in r15-LOX. The specific reaction for r15-LOX leads to 15-HpETE, i.e. 15-hydroperoxy-5Z,8Z,11Z,13E-icosatetraenoic acid which is longer and has 4 double bonds, in contrast to shorter soy product, 13-hydroperoxy-9Z,11E-octadecadienoic acid, with only two double bonds.

Table 2. LOXs Sequence Alignment, Fragments which can Determine Specificity

h- 5-LOX	INTK A REQL	WHLGAVWALSQFQ
hp-12-LOX	INTR A RTQL	LQMAISWHLSTRQ
h-15-LOX	INVR A RTGL	LQMSITWQLGRQ
r-15-LOX	INVR A RNGL	LQMSIVWQLGRDQ
	(a)	(b)
sLOX-1	IN A LARQSL	ISLSVIEILSTHA
sLOX-3	IN G LARLSL	IDLSVIEILSRHA

Marked in bold are residues discussed in the text, corresponding sequence numbers upon structure alignment.

(a) **Ala**: hp-12-LOX 402, r15-LOX 404, sLOX-1 542, sLOX-3 561.

(b) **Leu**: hp-12-LOX 596, r15-LOX 597, sLOX-1 754, sLOX-3 773.

INHIBITION OF LIPOXYGENASES

In general, LOX inhibitors can bind covalently to iron or form the molecular complexes blocking access to iron. Referenced publications [41-43] give a good classification of LOX inhibitors. One can notice that 5-LOX inhibitors are very well described, 12-LOX inhibitors lack updated, recent review, and 15-LOXs do not have a representative review of their inhibitors. Among many publications, the one describing the drug ebselen [44], is an excellent example of a mechanism of lipoxygenase inhibition. It illustrates that the same inhibitor could bind in a competitive or

noncompetitive manner depending on the ionization state of iron. In the presence of the FA substrate, it is competitive, while acting on a ferrous, silent ground-state enzyme it binds covalently, causing irreversible inhibition. In both cases the enzyme's performance can be illustrated by a classic Lineweaver-Burk plot. Many inhibitors do not follow such a linear relation between velocity and the inhibitor's concentration showing a hyperbolic curve instead. In general, the kinetic data are seldom reported. We have observed such behavior (hyperbolic curve) for polyphenolic inhibitors (curcumin, quercetin, EGCG, EGC) interacting with sLOX-3 [45]. The X-ray studies of their complexes indicated conversion of these inhibitors into their metabolites [29, 46, 47], which is not surprising considering the co-oxidative activity of LOXs [2]. Xenobiotic oxidation by soy LOX has been investigated and described, while human enzymes lack such thorough studies. Thus the question arises, "What is really inhibiting LOX, a given chemical or its LOX metabolite?" Another area open for investigation is stoichiometry of LOX complexes. Isothermal titration calorimetry allows an accurate assessment of the stoichiometry of complexes. An examination of 4-nitrocatechol complexes with sLOX-1 and -3 have shown a dramatic difference in the ratio between silent and active enzymes (sLOX-1: 1:0.5, 1:0.1, sLOX-3: 1:1.3, 1:0.4 respectively for the active and silent states [46, 48]). In the case of human enzymes, this area is still an uncharted water, awaiting investigation.

LOX IN HUMAN DISEASES

Respiratory System Diseases

5-LOX and FA metabolites – leukotrienes – produced by this pathway have been investigated most intensely and probably for the longest time, due to their involvement in the inflammatory responses accompanying allergic diseases of respiratory, gastrointestinal and dermatological systems [42]. Despite many years of research, treatment of inflammatory responses is still a hot topic due to the lack of knowledge about the involvement of other oxygenases and their interaction. Below is a short review of known facts and recent findings concerning 5-LOX in the respiratory system, 12-LOX in kidney and related cardiovascular diseases, plus remarks on other findings relevant to human diseases with proven participation of oxidoreductases, their interplay and inhibition.

Bronchial Asthma

Asthma is characterized by reversible airway obstruction, airway hyper-reactivity and airway inflammation, often named chronic allergic eosinophilic bronchitis. Available evidence supports a prominent regulatory role for LOXs and their metabolites in the bronchial asthma. However, most studies are focused on the 5-LOX with significantly less information about 12- and 15-LOX enzymes. While COX is found in most mammalian cells, 5-LOX is restricted to only some cell types. Interestingly, the cells responsible for the fundamental processes in asthma, specific allergic response (mast cells, dendritic cells) and the formation of the inflammatory infiltrate in the bronchi (eosinophils,

neutrophils, monocytes, macrophages) are among those few. Moreover, it has been demonstrated that 5-LOX expression and activity is subject to modulation by Th2 type cytokines produced in high quantities in asthma (IL-3, IL-4), as well as by the activation of the high-affinity of FcR for IgE (Fc RI), which might represent its marked up-regulation during initiation and effector phase of the allergic response in the lungs [49]. Several 5-LOX metabolites, so called cysteinyl leukotrienes (Cys-LT) originating from the unstable intermediate leukotriene A₄ (LTA₄), namely LTB₄, LTC₄, LTE₄, are recognized for their crucial role in the development and propagation of chronic allergic bronchitis in asthmatic airways. Their concentration in the induced sputum or bronchoalveolar lavage (BAL) from asthmatics is significantly higher than in healthy controls and increases considerably during disease exacerbation [50]. Cys-LTs are produced both during the early asthmatic response, immediately after the exposure to the specific allergen (mast cells, basophiles), as well as during the late asthmatic response phase a few hours later, being released mostly by the eosinophils recruited to the airways from the peripheral blood. Cys-LT-induced bronchial smooth muscle constriction has been repeatedly demonstrated both in experimentally and naturally occurring airflow obstruction [51]. Moreover, Cys-LTs potent proinflammatory activity has been well documented. It is due to their considerable chemotactic effect resulting in the influx of the eosinophils and to a lesser extent neutrophils into the airways. Also, human monocytes, eosinophils and macrophages express both CysLT1 and CysLT2 receptors [52]. Therefore Cys-LTs induce in inflammatory cells strong cytokine generation (IL-4, IL-5, IL-13 and many others) that in turn primes those cells for the enhanced Cys-LT receptors expression and stronger chemotactic response [53, 54]. In addition, it has been suggested that Cys-LTs are involved in the maturation of dendritic cells and their homing to the regional lymph nodes following contact with a specific allergen [55]. Both processes are critical for the antigen recognition by the T cells that orchestrate allergic reaction. Cys-LTs also perpetuate the acute inflammatory response by increasing microvascular permeability and plasma protein extravasation as well as up-regulating mucus production in the bronchial mucosa [56]. Their fundamental role in the bronchial hyper-reactivity has been also established [57]. Furthermore, several studies indicate that apart from their perpetuating effect on the chronic allergic inflammation in the bronchi, Cys-LT are responsible for the initiation of the airway remodeling inducing smooth muscle hyperplasia and partaking in the collagen deposition and subepithelial fibrosis in the bronchi [58].

5-LOX synthetic inhibitor (zileuton), FLAP inhibitor (MK-886) and leukotrienes receptor antagonists (lucasts) have proven very effective in reducing eosinophil influx (by ~85%) and activation (cytokine generation) in lung tissue or BAL, in blocking mucus release and airway plugging as well as preventing airflow decrease following specific bronchial provocation. Moreover, in the animal model of chronic asthma both groups effectively prevented matrix remodeling, muscle hyperplasia and collagen deposition as determined after 75-76 days of observation [58, 59]. Both, orally administered 5-LOX inhibitor (zileuton (Zyflo)) and CysLT1 receptor-selective antagonists (montelukast

(Singulair), zafirlukast (Accolate), pranlukast (Onon)) were clinically efficacious in the treatment of patients with bronchial asthma and are currently recommended for the standard asthma treatment. Surprisingly, zileuton though a potent and selective 5-LOX inhibitor, did not prove to be more efficacious than CysLT1 receptor blockers in the clinical studies [60]. Moreover, due to its short half-life time (2.5 hours) it needs to be administered four times a day, which often decreases patient's compliance to asthma therapy. On the contrary, montelukast administered once daily in a tablet form has been shown useful in the chronic treatment of asthma in children and adults with a toxicity profile similar to that of placebo [61, 62]. Some studies also demonstrated its clinical value for exercise-induced asthma (acute asthmatic response) and in aspirin-induced bronchoconstriction [63, 64]. It should be pointed out, however, that some of the Cys-LT1 selective inhibitors, like tomelukasts, verlukasts, and cinalukasts, have been withdrawn from development due to their side effects or overall ineffectiveness.

A new class of the 5-LOX inhibitors, targeting 5-LOX activating protein (FLAP) is in the early experimental phases. Their proposed mechanism of action is to prevent/block FLAP from trafficking 5-LOX from the cytosol to the cell membrane for the formation of leukotrienes [65].

The 15-LOX pathway has been much less extensively studied in asthma, but 15(S)-HETE has been recently shown to contribute to disease pathogenesis. Its elevated levels (30-fold) have been demonstrated in BAL after allergen challenge in mild atopic asthmatics [66]. The major cellular source of 15(S)-HETE is likely submucosal eosinophilis, but the bronchial epithelium was also reported to generate it in high amounts. Correspondingly, an increased expression of 15-LOX mRNA has been shown in airway macrophages and in the submucosal cells but not in the bronchial epithelium as compared to healthy controls [67]. Additionally, 15-LOX expression is up-regulated by the TH2 cytokines, IL-4 and IL-13, important in promoting and perpetuating allergic inflammation [68]. In animal studies inhaled 15(S)-HETE has been shown to induce neutrophils influx, mucus secretion and weak contractile activity [69]. Recent studies suggest that 15(S)-HETE functions as well as a pro-fibrotic, anti-inflammatory mediator able to shift matrix proteinases/antiproteinases balance as to up-regulate fibrotic processes, including collagen deposition in the bronchi subepithelium [70, 71].

5-LOX and 15-LOX interaction results in the generation of lipoxins – A₄ (LXA₄) and B₄ (LXB₄) which are known immunomodulatory and anti-inflammatory mediators. LXs are believed to act by antagonizing LT activity. Since LXA₄ and LTD₄ share the same receptor, LXA₄ blocks LTD₄-induced bronchoconstriction though LXA₄ alone induces weak smooth muscle contraction [72, 73]. LXs are produced in the airways mostly by inflammatory (eosinophils, neutrophils, macrophages), but also by structural cells (epithelium). Their synthesis is higher in mild than in severe asthmatics [74]. It was suggested that increased activity of 15-LOX-2 produces the anti-inflammatory effect, while 15-LOX-1 shows up in the pathological processes. Consequently, LXAs deficit in severe asthma is to be

associated with 15-LOX-1 domination and 15-LOX-2 insufficiency, which as a result do not generate a “stop signal” to inflammation [70].

Synthetic LXs analogs have been developed with variable effects, some of them quite promising. Levy *et al.* were able to block airway hyperresponsiveness and attenuate inflammatory reaction in bronchi [75].

Finally, it should be noted that the profound augmentation of allergic inflammation in the airways has been demonstrated in the COX-2-deficient mice with significant Cys-LTs as well as IgE antibody production enhancement. Similarly, the COX-2 inhibitor, nimesulid, has been shown to augment eosinophilic inflammation in the airways of asthmatic animals. However, no data are available that might suggest similar clinical effect of NSAIDs in humans [76].

Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is characterized by persistent bronchitis with a different than asthmatic cellular profile in the lungs distinguished by the neutrophil domination. Enhanced production of Cys-LT, as well as up-regulated expression of 15-LOX-1, but not 15-LOX-2, has been demonstrated in COPD patients. However, the Cys-LT antagonist, montelukast, has been evaluated in patients with COPD, both smokers and current non-smokers, with no apparent clinical effect [77]. Interestingly, it has been demonstrated that while COX-2 selective inhibitors have no effect on the chronic bronchitis activity in COPD, non-selective COX inhibition caused significant increase in LTB₄ production. If confirmed, these data would be the first to present the respiratory complications of NSAIDs [78].

Cystic Fibrosis (CF)

Chronic endobronchial infection with neutrophils as the primary effector cells is characteristic for CF and leads to the progressive pulmonary destruction. Cys-LT, eosinophil inflammation, and viral infections also contribute to those processes. Therefore, several clinical trials have been conducted with different Cys-LT antagonists producing divergent effects. Amelubant, a specific anti-LTB₄ antagonists was withdrawn due to the increased risk of side effects. Montelukast administered for 21 days has proven to decrease eosinophils number and activity in the peripheral blood with no apparent clinical effects (lung function, clinical score). On the other hand, the effects of its more prolonged administration (8 weeks) have been promising. Significant improvement both in the eosinophil activity and chronic inflammation activity markers have been observed together with considerable clinical improvement [79].

Fibrotic Lung Diseases

Fibrosis is a common end-stage of a number of acute and chronic lung diseases. Cys-LTs are believed to promote the pro-fibrotic processes in opposition to 15-LOX derived eicosanoids, lipoxins and prostaglandin E₂ [80]. In BAL fluid from idiopathic lung fibrosis patients, as well as patients with lung fibrosis due to scleroderma, increased levels of 5-LOX-derived leukotrienes have been observed, with considerably lower concentrations of 15-HETE, LXA₄ and PGE₂ in comparison to healthy controls [81].

Lung Cancer

5-LOX involvement in lung cancer development is well documented. Lung cancer cell lines express 5-LOX and FLAP mRNA, while levels of LOX metabolites are significantly higher than in normal tissue [82, 83]. 5(S)HETE and its metabolite 5-oxo-ETE strongly stimulate lung cancer cells proliferation and 5-LOX inhibitors are effective in the reduction of lung tumor growth [84, 85]. 5-LOX and FLAP inhibitors can reduce tumorigenesis induction by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, the well known tobacco carcinogen. Mice exposed to doses comparable to those received by smokers and treated with A-79175 or MK-886 had significantly reduced incidence and multiplicity of lung cancer tumors [82, 84]. Both compounds reduced cell proliferation in lung tumors in a dose-dependent manner, though the 5-LOX inhibitor A-79175 was more effective. Interestingly, A-79175 revealed also a synergistic anti-tumor effect when combined with aspirin. However, both inhibitors were ~10 times more effective than aspirin as *in vivo* anti-proliferative agents [86, 87]. The MK-886-FLAP inhibitor has also been demonstrated to induce bronchial carcinoma cells apoptosis [88]. LTB₄, a more terminal product of the 5-LOX metabolic pathway than 5(S)HETE, has also revealed its procarcinogenic activity in several studies. Moreover, the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone increased plasma LTB₄ in animal lung tumorigenesis [89]. In addition, smokers' alveolar macrophages are primed to generate higher amounts of LTB₄ upon activation. Interestingly, concomitant COX-2 inhibition with SC58236 did not significantly impact LTB₄ production, while zileuton, the 5-LOX inhibitor, blocked LTB₄ generation in a dose-dependent manner [90].

Both, 12- and 15-LOX-2 are expressed in lung cancer cells, though not uniformly. Gonzalez *et al.* reported 15-LOX-2 expression only in some examined non-small cell lung carcinomas (in 14% large cell carcinomas, 48% adenocarcinomas, 63% bronchioalveolar carcinomas, none in neuroendocrine tumors). Moreover, 15-LOX-2 expression was greater in better differentiated non-small cell lung carcinomas, and inversely correlated with tumor grade, as well as tumor cell proliferation [89].

Kidney Diseases

Since liver and kidney are the major organs for CYP oxygenation activity [91], Astex Technology Ltd. (a drug discovery company) claims that CYP is responsible for metabolism of ~90% of marketed drugs [9]. Other researchers have established that activation of xenobiotics in human kidney occurs primarily *via* the lipoxygenase pathway [92]. In reality, all three oxygenases coexist in kidney [93]. CYP has been located in a proximal convoluted tubule and the thick ascending limb of Henle (TALH). COX presence was determined mainly in the collecting duct, the interstitial cells, and less in the descending/ascending limbs and TALH. Kidney lipoxygenases were detected in the glomerulus, in the cytosolic fraction of the renal cortex as well as the microsomal fraction of the renal medulla/papilla [92]. In mouse kidney, platelet 12-LOX has been found in mesangium and tubules, including the distal ones [94].

Eicosanoids are involved in many kidney functions and renal diseases. Below are examples concerning hp-12-LOX and 12-HETE.

Kidney Blood Flow, Glomerular Filtration Rate (GFR), Chronic Kidney Disease (CKD)

12S-HETE is important for the full expression of the renal hemodynamic actions in response to angiotensin II [95]. It acts as a vasoconstrictor in small renal arteries [94]. In particular, research on rats shows that arachidonic acid induces an endothelium-dependent vasodilation of the rat small mesenteric arteries, which is neither suppressed by nitric oxide synthase nor cytochrome P450 enzyme inhibitors, but is enhanced by COX inhibitors, and abolished by LOX inhibitors [96]. Esculetin (a LOX inhibitor) increases renal blood flow and medullary laser Doppler flux (MLDF), but not in the presence of ibuprofen (a COX inhibitor). COX inhibitors contribute to the maintenance of arterial pressure and renal medullary perfusion under 'nitric oxide clamp' conditions but not to renal haemodynamic responses to vasoconstrictors, while the LOX inhibitor (esculetin) may blunt noradrenaline-induced vasoconstriction [97].

Inflammatory Diseases

12/15-LOX metabolites appear to protect the kidney during various inflammatory diseases [95, 98]. Inflammation in the glomeruli is called glomerulonephritis, or simply nephritis. LOX expression was found in biopsies from patients with glomerulonephritis. Inhibition of LOX activity in patients with immune-mediated glomerulonephritis was associated with reductions in proteinuria and restoration of glomerular size permselectivity. To reduce the renal risk associated with anti-inflammatory drugs, COX-LOX dual inhibition was suggested instead of COX-selective inhibition, as a possibly less disruptive means of interfering with prostanoid-dependent inflammatory mechanisms [99, 100]. The LOX inhibitor, MK-591, restores glomerular size selectivity and reduces proteinuria in human glomerulonephritis [101].

Renal Cell Carcinoma (RCC)

The expression of 12-LOX in human renal cell carcinoma (RCC), normal kidney tissues, and 3 kinds of RCC cell lines (Caki-1, A498, RC-1), and its effects on cell proliferation in 3 RCC cell lines were examined by Yoshimura *et al.* [102]. The 12-LOX inhibitor, baicalein, caused a marked inhibition of all 3 kinds of RCC cells in a concentration- and time-dependent manner. Cells treated with the 12-LOX inhibitor showed chromatin condensation, cellular shrinkage, small membrane-bound bodies (apoptotic bodies), and cytoplasmic condensation. These results suggest 12-LOX may play a role in the progression of RCC in human tissue, and its inhibitors may become anticancer agents in human RCC. The 12S-HETE's key role in the metastasis, tumor cell motility, proteolysis, invasion and angiogenesis has been firmly established [103-106]. It has been found that 5-, 8- and 12-LOX are procarcinogenic, while 15-LOX-1 and possibly 15-LOX-2 show anticarcinogenic activity. Therefore, a novel approach for cancer chemoprevention should include LOX modulators, which can induce the anticarcinogenic and/or reduce procarcinogenic LOXs [107]. Novel therapeutics directed

toward vascular endothelial growth factor (VEGF) show promising anti-tumor effects in the treatment of renal cell carcinoma in the initial trials [108]. VEGF and endothelial growth factor receptor (EGFR) are overexpressed in >70-90% of RCC cells. Experimental evidence shows a strong interdependence between 12-LOX and EGFR/VEGF expression, indicating that inhibiting growth factors signaling pathway and 12-LOX effects angiogenesis, and prevents urological cancer cell growth [41, 102, 109-112].

Hypoxia/Reoxygenation-Induced Renal Cell Injury

A study was undertaken to examine the role of eicosanoids in hypoxia/reoxygenation (H/R)-induced renal cell injury in rabbit renal cortical slices using AA metabolic inhibitors. Results suggest that inhibitors of cyclooxygenase and lipoxygenase pathways exert a direct protective effect against the H/R-induced cell injury in renal tubules [113].

Uremia

Platelets of patients with uremia show an increased activity of phospholipase A2 and produce increased amounts, with respect to the controls, of lipoxygenase metabolite hydroxyeicosatetraenoic acid, an inhibitor of the platelet function, while cyclooxygenase activity is significantly lower in hemodialysis patients [114, 115].

Proteinuria

Most proteins are too large to pass through the kidney's filters into the urine unless the kidneys are damaged. The main protein that is most likely to appear in urine is albumin. Proteins from the blood can escape into the urine when the filters of the kidney, called glomeruli, are damaged. Albumin's function in the body includes the retention of fluid in the blood. It acts like a sponge, soaking up fluid from body tissues. It is also a major carrier in the drug delivery system. Processes that can damage the glomeruli and cause proteinuria include inflammation, diabetes, hypertension, and other forms of kidney diseases. Proteinuria is associated with cardiovascular disease and it is the best predictor of progressive kidney failure in people with type 2 diabetes. Increased levels of 12-HETE were discovered in patients with micro- or macroalbuminuria/hyporenimic hypoaldosteronism [116].

Diabetes

A several fold increase in 12S-HETE production was determined in human aortic endothelial cells cultured under high-glucose conditions, which demonstrates that the 12-LOX pathway may play a role in the increased susceptibility of diabetics to atherosclerosis [117]. 12-HETE was increased in diabetics with normal renal function as well as in those with micro- or macroalbuminuria patients (69 ± 18 vs. 250 ± 62 vs. 226 ± 60 and 404 ± 131 ng/g creatinine; $P < 0.01$). Calcium did not stimulate PGI₂, but increased 12-HETE in diabetics with microalbuminuria, in contrast to levels in normal subjects. Hence, very early involvement of the kidney in diabetes is associated with fixed or suppressed production of PGI₂, causing an increase in the vasculotoxic lipoxygenase product 12-HETE [116]. Also streptozotocin-induced diabetes has been associated with increased renal lipoxygenase activity [118].

Hyperlipidaemia, Cardiovascular Disease (CVD), Hypertension [119]

An increase in the chronic kidney disease (CKD) has been noticed worldwide as a growing public health problem due to the greater prevalence of obesity and diabetes mellitus. Recent studies confirm a close relation between CKD and CVD and show that once the GFR falls below 60 mL/min, the risk for cardiovascular events increases dramatically [120]. Severity in the abnormalities in lipid levels correlates with the degree of proteinuria and is a common complication in patients with chronic kidney disease and nephritic syndrome. Most patients do not develop kidney failure but die as a result of cardiovascular disease that begins at the early stages of kidney disease. CVD is the major cause of mortality and morbidity of such patients, therefore they should be considered at very high risk for cardiovascular disease and treated accordingly [121]. The connection between elevated content of lipids and the risk for cardiovascular diseases is well established. It is known that HDL and oxidized LDL are antagonists in atherothrombosis [122]. Increased LDL oxidation is associated with coronary artery disease, while HDL prevents atherosclerosis by reverting the stimulatory effect of oxidized LDL on monocyte infiltration, smooth muscle cell migration and proliferation. There are two HDL-associated enzymes, paraoxonase and PAF-acetyl hydrolase, important in protecting against atherosclerosis, but both are inhibited by oxidized LDL. Circulating oxidized LDL does not originate from extensive metal ion-induced oxidation in the blood, but from mild oxidation in the arterial wall by cell-associated lipoxygenase and/or myeloperoxidase. Although no one disputes the importance of cholesterol, normal or low levels have been found in half of the heart attack victims. The new federal recommendations point to an above-average inflammation in such patients (that puts those otherwise healthy middle-age Americans at unusual risk for heart attacks and strokes) and asks for the development of the new markers for its detection. Protein C, suggested as a marker for that lurking inflammation, is related to kinases and 12S-HETE, which is a protein kinase C activator [123, 124].

Renal injury is one of the most frequent complications associated with cardiac surgery on cardiopulmonary bypass (CPB) with a reported incidence between 1% and 30%. Furthermore, up to 4% of patients with normal pre-operative renal function develop acute renal failure (ARF) requiring dialysis and the associated mortality has been reported to be 40–100%. CPB is associated with polymorphonuclear neutrophil activation resulting in the release of reactive oxygen-derived species (ROS), which have been shown to impair renal function. Thus, ROS might be involved in the etiology of CPB-related renal function impairment [125].

A striking correlation has been found between platelet 12S-HETE production and systolic blood pressure [126]. Increased levels of platelet 12-LOX and the urinary excretion of 12S-HETE were observed in patients with essential hypertension [127]. The authors were studying basal and thrombin-induced platelet 12S-HETE production and urinary 12S-HETE excretion. There were no significant differences observed for thrombin-stimulated generation of this eicosanoid, while both platelet 12-LOX protein levels and the urinary excretion of 12S-HETE were significantly higher

in patients with hypertension than in control subjects. Experimental evidence also indicates that the lipoxygenase pathway, may have a critical role in the long-term maintenance of high blood pressure after renal artery stenosis [128].

Other Diseases

We have used respiratory and renal diseases as an illustration. Hundreds of publications can be found about LOXs and related eicosanoids in *cancers*. 5-LOX, hp-12-LOX and 12/15-LOX are related to *atherogenesis* [129]. Many NSAIDs are prescribed every day to elevate pain and stiffness of joints caused by *arthritis*. LOX genes are associated with *osteoporosis* and *periodontal disease* [4]. Iron-induced modulation of expression and activity of 12-LOX and catalase may be relevant to the increase in iron-related oxidative stress observed in smokers [130]. The frontal and temporal regions of brains affected with *Alzheimer's disease* have elevated 12/15-LOX, implying that these enzymes might contribute to the pathogenesis of this neurodegenerative disorder [3]. Hence inhibition of oxygenases can have a very broad impact on health.

RECENT TRENDS IN INHIBITION AND REGULATION OF OXYGENASES

It is known that body can utilize all three pathways (CYP, COX, LOX) for drug metabolism and that it can switch between them. We also know that these enzymes are masterful catalysts of biotransformation of xeno- and endobiotics and the following reactions have been listed as catalyzed by LOXs: oxidation, hydroxylation, epoxidation, sulfoxidation, desulfuration, dearylation and dealkylation [2]. Dual inhibitors targeting COX/5-LOX are just emerging [131]. Researchers are looking also at natural compounds as possible leads to develop drugs targeting oxygenases with the hope of avoiding side effects common for NSAIDs in use.

Drug Metabolism, Specific vs. Non-Specific Inhibitors

Nonsteroidal anti-inflammatory drugs (NSAIDs) have well-documented gastric ulceration and nephrotoxicity. Adverse renal effects occur because of a shunt of arachidonate to the LOX pathway [100]. NSAIDs also increase the risk of acute urinary retention (AUR) in men, according to the results of a population-based, case-control study. AUR is characterized by the sudden inability to urinate, which is usually extremely painful and requires catheterization. Eicosanoids play an important role in the genitourinary function as they provoke contractions of the detrusor muscle. As NSAIDs are known to have a direct effect on their synthesis, they have been tested in clinical trials for the treatment of detrusor instability. Compared with nonusers of NSAIDs, current users of NSAIDs had a risk of AUR 2.02-fold higher (95% confidence interval, 1.23 - 3.31). Patients who recently started taking NSAIDs at a dose equal to or higher than the recommended daily dose had the highest risk for AUR (adjusted odds ratio, 3.3; 95% confidence interval, 1.2 - 9.2) [132].

While nonselective inhibitors can affect all pathways (many natural compounds would belong to this category), highly selective and enzyme-specific inhibitors can target one chosen enzyme and have a great impact on the delicate balance between them. Many NSAIDs can and do act upon more than one enzyme, although with often different IC₅₀ toward them. In the case of highly specific compounds, the unfortunate events related to COX-2 specific inhibitors (i.e. impact on cardiovascular diseases in patients taking Vioxx as an effective anti-inflammatory drug) provided clear and strong evidence that we either do not understand or neglect the intricacies of the interplay between oxygenases. Although the red flag about risk of cardiovascular events associated with selective COX-2 inhibitors was already high in 2001 [133], it was not commonly known until Merck's voluntary withdrawal of this drug [134-136]. But by this time, according to Reuters (9/30/04), 91 millions prescriptions for Vioxx had been written in the U.S. alone.

The short description above, while only the tip of the iceberg, shows clearly the complexity of the intertwined relationship between different enzymes and mutual dependence of various processes. In our aging society more and more patients are prescribed NSAIDs and selective COX-2 inhibitors for rheumatoid arthritis or osteoarthritis. Recent findings from clinical studies document that patients with previous myocardial infarction have a 2-3 times higher mortality risk when taking NSAIDs [137]. The demographic predictions tell us that by 2020, an estimated 60 millions Americans will have arthritis and 11.6 millions will be disabled. Anti-inflammatory drugs have been shown to cause abnormalities in renal function, which presents an important concern in patients with cardiorenal risk factors, including hypertension, congestive heart failure, edema, renal impairment, and advanced age [138].

Is COX/5-LOX Dual Inhibition Enough?

Fatty acids and drugs metabolizing oxygenases coexist and function together as a team. Although different substrates may have preferences as to the pathway to follow, the body can switch between the routes and enhance production of other metabolites when the path 'of preference' is blocked. Eicosanoids derived from the LOX pathway often exhibit an action contrary to the impact of COX or CYP metabolites, for instance, vasoconstriction (LOX) vs. vasodilation (COX, CYP) [95]. Products of the COX and CYP pathways contribute to the renal vasoconstrictor response to endothelin-1 (ET-1), whereas COX- and LOX-derived eicosanoids contribute to the response to angiotensin II (ANG II), ETYA (5,8,11,14-eicosatetraenoic acid) inhibits all three pathways and diminishes ET-1 and ANG II induced vasoconstriction by ~60-70% [139]. While high doses (100-500 µM/L) of diclofenac and flurbiprofen (COX and CYP inhibitors) increase platelet adhesion, the LOX inhibitor, nordihydroguaiaretic acid (NDGA), completely blocks this effect [140]. Studies on the proliferation of mouse fibrosarcoma cells *in vitro* have brought very interesting observations. LOX inhibitors (NDGA, esculetin and baicalein) markedly suppressed the number of cells and effected cell cycle distribution in a dose dependent manner. A CYP inhibitor (proadifen) applied in low concentration increased the number of cells, while in high concentration

inhibited cell proliferation and changed the cell cycle. COX inhibitors (ibuprofen, flurbiprofen and diclofenac) had a very moderate effect on cell number suppression and caused no changes in the cell cycle. Therefore, the authors concluded that the proliferating ability of sarcoma cells depends on the intact functionality of LOX and CYP [141]. Considering that baicalein can inhibit COX [142] or upregulate its expression [143], and that flurbiprofen is known to bind to CYP [6], the effects observed with LOX inhibitors might be skewed by COX presence, and results reported for COX inhibitors might be tinted by CYP. Ebselen, mentioned before as the r15-LOX inhibitor at the nM range, can also inhibit human 5-LOX, platelet 12-LOX and COX when in the mM quantities [144]. The above examples, a few out of many available, clearly show that regulation of oxygenases is challenging and a highly complicated task.

While there is a rich collection of literature on CYPs and COXs, LOXs are much less understood and were overshadowed by the other two in research funding. Structure/function studies of cyclooxygenase have culminated in the discovery of numerous inhibitors with therapeutic potential. Blocking the COX pathway makes more arachidonic acid substrate available for the LOXs governed catalysis. Investigations into the structure of lipoxygenase and its complexes with small molecules, e.g. substrate, product, and inhibitors, for the generation of therapeutically useful compounds have not reached the same level of development. It was quite obvious even during the last meeting (9th International Conference "Eicosanoids & other bioactive lipids in cancer, inflammation and related diseases", San Francisco, September 9-14, 2005) that research on lipoxigenases is seriously lagging. The development of a drug, licofelone, a dual inhibitor targeting COX/5-LOX, was described together with the results from clinical trials (abstract #68, S. Laufer, Eberhard-Karls-University Tuebingen, Germany). It is presumed that licofelone is a substrate analog, yet the manner of its binding is not known, nor was its impact on other LOXs or CYPs reported. In a recent article about CYP inhibition [145], the authors point out that competitive/noncompetitive/irreversible inhibition of drug-metabolizing enzymes by one drug can result in elevated plasma/tissue concentrations of other drugs, in common nowadays in clinical practice multi-drug therapy. Hence, the inhibition of other drug-metabolizing enzymes should be routinely tested before the drug candidate is considered for the clinical stages of development.

Phytochemicals

Natural compounds are gaining a lot of attention as potential drugs or 'leads' for drug development. Examination of their properties shows that most of them have a very broad impact and can effect more than one enzyme. Curcumin (major component of a common spice turmeric, also present in ginger) can serve as an example to illustrate such complex behavior. Search in Phytochemical and Ethnobotanical Databases [142] brings three pages long list of biological activities, description of some can be found in a review article [146]. It can inhibit kinases, proteases, tissue necrosis factor, topoisomerases, VEGF, while inducing lipase and quinone-reductase. The oxygenases

inhibitory properties of curcumin have been confirmed for 5-, 8-, 12-LOX, COX-2 and CYP with $IC_{50} \sim 2\text{-}20 \mu\text{M}$, and anticancer activity have been observed in breast, colon, duodenum, liver, mammary, skin, stomach, bladder and prostate cancers. Its anti-inflammatory dose of 1200 mg/day could be easily accommodated in Indian cuisine and is commonly used in Ayurveda, but is seldom met in Western diet. It is worth noticing that it is comparable in strengths and action to steroidal and non-steroidal drugs, but does not show their side effects and reduces risk for cardiovascular disease. Unlike NSAIDs, curcumin acting as the COX inhibitor blocks production of TXA₂, a promoter of platelet aggregation, while sparing or slightly increasing production of prostacyclin [147], which is a natural inhibitor of platelet aggregation and a major AA metabolite in heart. Hence, it might be beneficial for patients prone to vascular thrombosis and requiring anti-inflammatory therapy. Curcumin is a dominant ingredient among curcuminoids that make the yellow pigment present in 3-5% in the tuberous roots of the plant *Curcuma longa*, commonly known as turmeric. Natural remedies almost never consist of a single ingredient and usually are a mixture of many in proper proportions, with a synergistic effect of their simultaneous action being absolutely necessary for beneficial medicinal results. Thus, one should proceed with caution, since the action of a selected single compound may not be the same. Recent review articles [148-150] summarize natural products with inhibitory properties toward COX and LOX.

SUMMARY

Lipoxygenases constitute putative targets for pharmacologic intervention, which might have a better chance of development if the structures of enzymes be known. Solving the structures of human lipoxygenases could greatly contribute to the understanding of their action, the intricacies of their structural differences/similarities, and their regio- and stereo-specificity. It might be a turning point not only in the rational drug design targeting these enzymes, but also a great step forward in understanding the interplay between fatty acids metabolizing oxidoreductases, their catalytic behavior and interaction with drugs. The examples provided here illustrate the enormity and seriousness of consequences that we face when trying to regulate fatty acids and drugs metabolizing oxygenases, without making an effort to understand the roles of the players involved in the process, and neglecting to take into account their participation and stimulation/interactions with other proteins and growth factors. However, that understanding cannot come without knowledge, hence research leading to information about coexistence, mutual influence of oxygenases, their inhibition and interactions with other biological systems can greatly contribute into our understanding of the complexity by which eicosanoids impact different diseases and drug metabolism.

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ABBREVIATIONS

12S-HETE	=	12S-hydroxyeicosa-5Z,8Z,10E,14E-tetraenoic acid
13-HpODE	=	13-hydroperoxy-9Z,11E-octadecadienoic acid
AA	=	arachidonic acid
BAL	=	Bronchoalveolar Lavage
CF	=	cystic fibrosis
CKD	=	chronic kidney disease
COPD	=	chronic obstructive pulmonary disease
COX	=	cyclooxygenase
CPB	=	cardiopulmonary bypass
CVD	=	cardiovascular disease
CYP	=	cytochrome P450 (or epoxyoxygenase)
EGFR	=	endothelial growth factor receptor
FA	=	fatty acid
FLAP	=	5-lipoxygenase activating protein
GFR	=	glomerular filtration rate
LOX	=	lipoxygenase
5-LOX	=	5-lipoxygenase
hp-12-LOX	=	human platelet 12-lipoxygenase
h-15-LOX	=	human 15-lipoxygenase
LT	=	leukotriene
Cys-LT	=	cysteinyl leukotrienes
LX	=	lipoxin
MLDF	=	medullary laser Doppler flux
NSAID	=	non-steroidal anti-inflammatory drug
PDB	=	Protein Data Bank
PG	=	prostaglandin
PLAT domain	=	the abbreviation is after polycystin-1, lipoxygenase and alpha-toxin
PUFA	=	polyunsaturated fatty acid
RCC	=	renal cell carcinoma
ROS	=	reactive oxygen-derived species
SAXS	=	small angle X-ray scattering
TALH	=	thick ascending limb of Henle
TX	=	thromboxane
VEGF	=	vascular endothelial growth factor

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