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Biology and pharmacology of platelet-type 12-lipoxygenase in platelets, cancer cells, and their crosstalk

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Abstract:	Platelet type lipoxygenase (pl12-LOX), encoded by ALOX12, catalyzes the production of the lipid mediator 12S-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (12S-HpETE), which is quickly reduced by cellular peroxidases to form 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12S-HETE). Platelets express high levels of pl12-LOX and generate considerable amounts of 12S-HETE from arachidonic acid (AA; C20:4, n 6). The development of sensitive chiral liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods has allowed the accurate quantification of 12S-HETE in biological samples. Moreover, advances in the knowledge of the mechanism of action of 12S-HETE have been achieved. The orphan G-protein-coupled receptor 31 (GPR31) has been identified as the high-affinity 12S-HETE receptor. Moreover, upon platelet activation, 12S-HETE is produced, and significant amounts are found esterified to membrane phospholipids (PLs), such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC), promoting thrombin generation. Platelets play many roles in cancer metastasis. Among them, the platelets' ability to interact with cancer cells and transfer platelet molecules by the release of extracellular vesicles (EVs) is noteworthy. Recently, it was found that platelets induce epithelial-mesenchymal transition(EMT) in cancer cells, a phenomenon known to confer high-grade malignancy, through the transfer of pl12-LOX contained in platelet-derived EVs. These cancer cells now generate 12-HETE, considered a key modulator of cancer metastasis. Interestingly, 12-HETE was mainly found esterified in plasmalogen phospholipids of cancer cells. This review summarizes the current knowledge on the regulation and functions of ALOX12 in platelets and cancer cells and their crosstalk. Novel approaches to preventing cancer and metastasis by the pharmacological inhibition of pl12-LOX and the internalization of mEVs are discussed.
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Biology and pharmacology of platelet-type 12-lipoxygenase in platelets, cancer cells, and their crosstalk

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Keywords: platelet type 12-LOX, platelets, extracellular vesicles, cancer, metastasis, 12-LOX inhibitors

Abstract

Platelet type lipoxygenase (pl12-LOX), encoded by ALOX12, catalyzes the production of the lipid mediator 12S-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (12S-HpETE), which is quickly reduced by cellular peroxidases to form 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12S-HETE). Platelets express high levels of pl12-LOX and generate considerable amounts of 12S-HETE from arachidonic acid (AA; C20:4, n 6). The development of sensitive chiral liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods has allowed the accurate quantification of 12S-HETE in biological samples. Moreover, advances in the knowledge of the mechanism of action of 12S-HETE have been achieved. The orphan G-protein-coupled receptor 31 (GPR31) has been identified as the high-affinity 12S-HETE receptor. Moreover, upon platelet activation, 12S-HETE is produced, and significant amounts are found esterified to membrane phospholipids (PLs), such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC), promoting thrombin generation. Platelets play many roles in cancer metastasis. Among them, the platelets' ability to interact with cancer cells and transfer platelet molecules by the release of extracellular vesicles (EVs) is noteworthy. Recently, it was found that platelets induce epithelial-mesenchymal transition(EMT) in cancer cells, a phenomenon known to confer high-grade malignancy, through the transfer of pl12-LOX contained in platelet-derived EVs. These cancer cells now generate 12-HETE, considered a key modulator of cancer metastasis. Interestingly, 12-HETE was mainly found esterified in plasmalogen phospholipids of cancer cells. This review summarizes the current knowledge on the regulation and functions of ALOX12 in platelets and cancer cells and their crosstalk. Novel approaches to preventing cancer and metastasis by the pharmacological inhibition of pl12-LOX and the internalization of mEVs are discussed.

Introduction

A vast variety of lipid mediators involved in many physiological and pathophysiological processes are generated from arachidonic acid (AA; C20:4, n 6), a polyunsaturated fatty acid (PUFA), via the activity of lipoxygenases (LOX), cyclooxygenases (COX), or cytochrome P450 enzymes (Figure 1) [1-4]. In the cases of COXs (COX-1 and COX-2), AA is first converted to prostaglandin G_2 (PGG_2) by the cyclooxygenase reaction, and then the peroxidase activity of the enzyme catalyzes a two-electron reduction of PGG_2 to PGH_2. PGH_2 is converted to different prostanoids such as PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2 (prostacyclin) and thromboxane (TX)A_2 by specific synthases. Prostanoids interact with their receptors [DP1 and CTRH2 (also known as DP2), EP1–4, FP, IP, and TP, respectively] and exert various biological actions [3]. AA can be bound in the cyclooxygenase active site of COXs in at least three different catalytically competent arrangements that lead to PGG_2 but also 11-hydroperoxyeicosatetraenoi acid (HpETE), and 15R/S-HpETE as minor products (Figure 1) that can be reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs), at least in part, via the peroxidase activity of the enzymes[5,6]. COXs are the target of an important class of drugs, the nonsteroidal antiinflammatory drugs (NSAIDs) widely used in the symptomatic management of rheumatic diseases characterized by chronic musculoskeletal pain and diverse forms of acute pain [3,7]. Among the NSAIDs, Aspirin (acetylsalicylic acid, ASA) irreversibly inhibits COX-1 and COX-2 through the acetylation at serine 529 and 516, respectively [8,9]. The persistent irreversible inhibition of platelet COX-1 by low-dose aspirin explains its efficacy in preventing atherothrombosis and possibly colorectal cancer (CRC) in humans [7,10]. It has been reported that acetylated COX-2 not producing PGH_2 can lead to the generation of 15R-HETE [9, 11], which can be converted to the “aspirin-triggered lipoxin” 15-epi-lipoxin (LX)A_4 [12]. The relevance of this pathway in vivo in humans requires further evaluation.

Cytochrome (CYP) P450 enzymes can hydroxylate AA at many different positions [13], and three types of products are formed:

1. ω-hydroxy metabolites

2. Bisallylic hydroxylation products, in which the hydroxyl group is added to a methylene group situated between two cis double bonds
3. HETEs, in which the hydroxyl group is adjacent to a conjugated diene system.

Among them, 20-HETE is noteworthy for its role in kidney functions. CYP4A11, CYP4F2, CYP4F3B, and CYP4F11 catalyze the formation of 20-HETE and are expressed in the liver and kidney [14]. GPR75 has been recently identified as the first 20-HETE receptor [15]. Selective synthesis inhibitors and orally active receptor antagonists are now available for experimental animal models [16-20]. Their use has allowed elucidation of other roles of 20-HETE in cardiovascular disease, angiogenesis, and cancer [16, 21, 22]. Given these many actions, the safety of 20-HETE inhibitors or antagonists remains to clarify when used to treat chronic diseases, such as hypertension.

Lipoxygenases (LOX) are a family of non-heme iron proteins or dioxygenases, which catalyze the abstraction of hydrogen atoms from *bis*-allylic positions (1*Z*,4*Z*-pentadiene groups) of PUFA followed by stereospecific addition of dioxygen to generate hydroperoxides [23]. The human genome contains six functional LOX genes (*ALOX15*, *ALOX15B*, *ALOX12*, *ALOX12B*, *ALOX5*, and *ALOXE3*), expressed in various tissues [24, 25]. LOXs were traditionally named according to their positional specificity for AA [26]. However, human 15-LOX-1 (ALOX15) exhibits dual reaction specificity with AA as a substrate since 12S- and 15S-HpETE have been identified as oxygenation products [27] (Figure 1). Thus, gene names alongside enzyme names are recommended [23, 26]. Cellular peroxidases can reduce HpETE to monohydroxy fatty acids (HETEs) [2].

5-LOX and its products are of pharmacological importance since they are the target of clinically used antiasthmatic drugs [1, 28]. The enzyme converts AA to 5S-HpETE and then to leukotriene (LT)A_4 (Figure 1). LTA_4 is enzymatically converted to LTB_4, a neutrophil chemoattractant, via the activation of the BLT1 receptor. LTC_4, the precursor of LTD_4 involved in asthma, is also generated from LTA_4. LTD_4 activates cysLT1 and cysLT2 receptors [1]. Antagonists targeting the receptor of

CysLTs (CysLT1) are currently used as antiasthmatic drugs. LTA_4 has been reported to be the precursor for other lipoxygenases, such as 12-LOX or 15-LOX, to generate LXA_4, which interacts with the ALX/formyl peptide receptor 2(FPR2) and LXB_4 [29, 30]. However, it is noteworthy that FPR2/ALX, a G protein-coupled receptor belonging to the formyl peptide receptor (FPR) family, conveys the biological functions of a variety of ligands, including the proresolution mediators, annexin A1 (AnxA1) and LXA_4, as well as the proinflammatory protein serum amyloid A [31]. This review summarizes the current knowledge on the regulation, functions, and pharmacological modulation of ALOX12/platelet-type 12-LOX in platelets and cancer cells and their crosstalk.

Platelet-type 12-lipoxygenase roles in platelet function and thrombosis

The ALOX12 gene encodes for the platelet-type 12-LOX (pl12-LOX; 12S-LOX), which is expressed at high levels in blood platelets, skin epidermis, and some hematopoietic and solid tumors [32, 33]. Pl12-LOX seems to be the only LOX isoform present in platelets. Pl12-LOX oxygenates position C12 of AA to form 12S-HpETE. In vivo, 12-HpETEs is rapidly reduced to 12S-HETE by peroxidases, such as glutathione peroxidase [34,35].

12S-HpETE can be generated in mice also by ALOX15, and the enzyme was previously named leukocyte-type 12-LOX for its high expression in leukocytes; however, this nomenclature should not be used anymore, and it should be classified as 12-lipoxygenating ALOX15 isoforms [26].

As a consequence of platelet activation, the enhancement of intracellular calcium triggers the translocation of 12-LOX and the hydrolysis of PUFAs from the glycerolphospholipid via cytosolic phospholipase ($cPLA_2$) [36-41]. These events seem crucial for 12S-HETE generation in platelets. However, crosstalk exists among COX-1-dependent TXA_2, dense granule ADP release, and 12S-HETE generation in human platelets. Platelets also express high levels of COX-1 [3]. Tacconelli et al. [42] performed a targeted chiral lipidomics analysis of eicosanoids generated from endogenous AA in human whole blood allowed to clot for 60 min at 37°C [42]. Under this condition, thrombin is generated and induces the release of AA from membrane phospholipids of blood cells (mainly platelets) which is transformed into eicosanoids [43]. Using a chiral liquid chromatography-mass

spectrometry/mass spectrometry (LC-MS/MS) method, prostanoids (mainly TXB_2 and PGE_2) and different HETEs, generated enzymatically or nonenzymatically from AA, can be detected. Comparable levels of TXB_2 and 12S-HETE were detected and were the most abundant eicosanoids. PGE_2 and other HETEs were 4.14% of the average concentration of all molecules assessed (i.e., 700.08 ng/ml). Also, in platelet-rich plasma (PRP), TXB_2 and 12S-HETE were generated at high concentrations (ng/ml) in response to collagen or TRAP-6 [a peptide fragment that acts as a protease-activated receptor 1 (PAR-1) agonist][44]. PAR-1 is the highest-affinity thrombin receptor on human platelets, while murine platelets do not express it [45]. The other thrombin receptor PAR-4 responds to high concentrations of thrombin [45]. Humans and mice express PAR-4, but the two receptors are functionally different [45, 46]; thus it is challenging to translate PAR-4 studies using mice to human platelet physiology.

Pharmacological studies in vitro showed that during platelet aggregation induced by collagen 2 μg/ml in human PRP, TXA_2 generation drives the biosynthesis of 12S-HETE in part via the ADP release. Thus, aspirin, a selective inhibitor of COX-1, indirectly reduced 12S-HETE production via the inhibition of TXA2 generation. The inhibition of collagen-induced platelet aggregation by ASA (100 μM) was rescued by the addition of U46619 (the stable TXA_2 mimetic), but not 12S-HETE, (at concentrations generated endogenously), thus showing that at a low collagen concentration, 12-HETE does not play a functional role in platelet aggregation [42]. It has been shown that the pl12-LOX pathway plays an important role in normal platelet function through PAR-4 [47], which promotes the late phase of the platelet aggregation process by extending the increase of intracellular Ca^{2+} levels [44]. Thus, pl12-LOX inhibition could be beneficial in some clinical conditions where PAR-4 plays a role [47]. This may include patients with acute coronary syndromes who are insensitive to current antiplatelet drugs, possibly due to an increased population of procoagulant platelets.

In platelets, 12S-HETE can act through the NOX (NADPH oxidases) stimulation and the enhancement of the production of ROS (reactive oxygen species), which are known to potentiate platelet activation [34].

However, approximately one-third of the 12-HETE generated by platelets is riesterified into the membrane phospholipids (PL-12-HETEs) [48]. Incorporation of HETEs into lipids has also been observed in many other cell types, including epithelial and endothelial cells [49,50].

Activation of platelets leads to the rapid activation of pl12-LOX and the incorporation of 12S-HETE into phosphatidylethanolamine (PE) and phosphatidylcholine (PC) to give the following plasmalogen and acyl lipids: 16:0p_12S-HETE-PE, 18:1p_12S-HETE-PE, 18:0p_12S-HETE-PE, 18:0a_12S-HETE-PE, 16:0a_12S-HETE-PC, and 18:0a_12S-HETE-PC [51]. This phenomenon enhances tissue factor-dependent thrombin generation and might be involved in enhancing thrombus formation [51, 52].

12S-HETE can also act by activating the orphan receptor GPR31 [53]. The GPR31 is coupled to Gi, and its activation by 12S-HETE potentiates platelet aggregation, calcium flux, and dense granule release induced by the stimulation of PAR4 and thrombin [53]. Interestingly, a GPR31 pepducin antagonist (GPR310), a synthetic 21mer peptide conjugated to palmitate to form an N-palmitoylated lipopeptide targeting the i3 loop of GPR31, has been developed. GPR310 significantly reduced arterial thrombosis without affecting hemostasis in mice [54].

Genetic manipulation in mice to determine the oxygenases and their lipid contributions to vascular and platelet functions has considerably progressed over the last two decades. These tools have greatly enhanced our understanding of the varying roles of oxylipins, mainly in platelet biology and different pathophysiological processes.

Knockout (KO) mice for the platelet-type 12-LOX ($ALOX12^{-/-}$) were specifically generated and showed a viable phenotype, but they exhibited prolonged tail vein bleeding compared to wild-type (WT) mice [47]. Johnson et al. [55] showed that $ALOX12^{-/-}$ murine platelets could not synthesize 12-HETE from exogenous or endogenous AA; differently, TXA_2 synthesis remained unchanged.

However, platelets from mice lacking pl12-LOX were hyperresponsive to ADP, thus suggesting that platelet 12-LOX has an inhibitory role in ADP-induced aggregation[55]. Moreover, ALOX12$^{-/-}$ mice were more sensitive to thrombosis elicited by intravenous ADP injection [55]. More recently, it was shown that platelets from LOX12$^{-/-}$ mice present lower aggregation when compared to platelets from WT mice in response to collagen, PAR4-activating peptide (AP), and also ADP [47, 56, 57], Moreover, 12-LOX $^{-/-}$ mice showed a decrease in thrombus formation compared to WT mice, in response to laser-induced vascular injury [57].

Overall, these results suggest complex biological effects of 12S-HETE in platelets. It seems that 12S-HETE plays a role at low concentrations of agonists with a particular preference for PAR4 activation.

Dietary supplementation with dihomo-γ-linolenic acid (DGLA), an ω-6 PUFA, has been shown to inhibit platelet aggregation ex vivo [58-60]. Yeung et al. [57] showed that DGLA, inhibited platelet thrombus formation in vivo in a model of vessel wall injury. Using mice with the deletion of ALOX12, it was demonstrated the involvement of pl12-LOX in mediating the antithrombotic effects of DGLA. Moreover, they demonstrated that the 12S-hydroxy-8,10,14-eicosatrienoic acid (12S-HETrE), generated by pl12-LOX from DGLA as substrate, is responsible for its antithrombotic effects, but it did not affect primary hemostasis or caused increased bleeding. 12-HETrE acted via activation of $G\alpha_s$ signaling pathway leading to the formation of cAMP and PKA activation in the platelet [57].

However, it is to be considered that the results using mouse models of thrombosis in the background of ALOX12-/- should be taken into consideration with caution due to important differences with humans regarding the expression of LOX isoforms and the generation of 12S-HETE from other LOX enzymes [26].

Roles of platelet-type 12-lipoxygenase in cancer and metastasis

Several lines of experimental evidence have shown the role of pl12-LOX and 12S-HETE in cancer pathophysiology [61-63]).

Both ALOX-12 and Pl12-LOX expression have been detected in various cancer cell lines of different tissue origin. Pl12-LOX is overexpressed in various cancers, including melanoma, skin carcinoma, and prostate carcinoma [64-66]. Moreover, it has been shown the increased expression of ALOX-12 in breast carcinoma [67]. pl12-LOX and its metabolite 12S-HETE are important regulators of ovarian cancer growth, as shown in vitro [68]. In prostate cancer patients, the expression levels of ALOX12 correlated with tumor stage and grade [69]. High levels of ALOX-12 have been found to be of prognostic value for staging in breast cancer [70,71].

12S-HETE has a protective role for cancer cells, preventing them from undergoing apoptosis and leading to mechanisms of invasion, motility, and angiogenesis in several cancer types as demonstrated in vitro [62, 72-74].

Several studies conducted on prostate and epidermal cancer cells have shown that 12S-HETE is implicated in increasing tumor cell adhesion to microvascular endothelial cells by upregulating the surface expression of αvβ3 integrins [75]. Further studies have also shown that melanoma cells release 12S-HETE in sufficient quantities to induce a reversible, non-destructive, time- and concentration-dependent retraction of the endothelial cells themselves by stimulating cytoskeletal protein rearrangement in a PKC-dependent manner [72, 75, 76].

Dilly et al. [77] demonstrated that pl12-LOX and its metabolite 12S-HETE positively affected the levels of the metalloproteinase MMP9, which has a pivotal role in prostate cancer invasion and metastasis [78]. Overexpression of ALOX12 in PC-3 prostate cancer cells increased MMP9 production through activation of the PI3K/Akt/NF-κB pathway; this phenomenon led to increased chemoattraction of endothelial cell, which was reversed by inhibitors of 12-LOX (baicalein), PI3K (LY294002) or NF-κB (MG-132) [77].

Furthermore, prostate cancer cells overexpressing ALOX12 led to increased secretion of the vascular endothelial growth factor (VEGF) [79], which was inhibited by the LOX inhibitor baicalein. The role of 12-LOX in angiogenesis in vivo was shown by the finding that 12S-HETE activates the (ERK1/2) mitogen-activated protein (MAP) kinase signaling cascade that underlies the increase in VEGF [80].

12S-HETE is also involved in pancreatic cancer development by inducing tyrosine kinase phosphorylation and cell proliferation [81].

During metastasis, tumor cells interact with platelets, endothelial cells, and matrix proteins, undergoing various cell-host interactions and several lines of evidence suggest that the ability of tumor cells to synthesize 12S-HETE strongly correlates with their metastatic potential [82].

12S-HETE can contribute to cancer cell survival by directly affecting the tumor and endothelial cells' promotion of metastases. In particular, 12S-HETE can induce the release of cathepsin B by tumor cells, contributing to the degradation of the subendothelial membrane, thereby promoting the migration of the cells themselves in and out of the bloodstream [82], a distinctive feature in the development of metastases. 12S-HETE contributes to the dissemination of metastases by increasing the motility of tumor cells through PKC-dependent cytoskeletal rearrangements [83] and by activating endothelial cells. Endothelial cells cultured with tumor cells release 12S-HETE, which influences cytoskeletal rearrangement and cell retraction [82].

In MCF-7 cell (a breast cancer cell line) spheroids, 12S-HETE induces transmigration of lymphatic endothelial cells through local interruptions in the endothelial monolayer [84]. The role of 12S-HETE in breast cancer has been further demonstrated in xenograft tumors in mice using pharmacological inhibitors (pan-LOX inhibitor nordihydroguaiaretic acid or baicalein) or short interfering RNA (sh-RNA) treatment, that prevent the production of 12-HETE [84].

The correlation between 12S-HETE production and metastatic potential was also evaluated in different tumor cell types [85].

12-LOX has been shown to stimulate prostate cancer tumor growth by influencing angiogenesis [86]. The addition of exogenous 12S-HETE alters the cytoskeleton of tumor cells through phosphorylation of cytoskeletal proteins [87], thus increasing adhesion and spread of tumor cells on matrices in vitro [83,88], inducing proteinase secretion [89], integrin expression [75,90] and increases invasion. Pretreatment of tumors with 12S-HETE stimulates cell motility [91] and increases the surface expression of αvβ integrins, in particular αvβ3 integrin [92]. The adhesion of B16 murine melanoma cells to microvascular endothelial cells was enhanced through the up-regulation of αvβ3 integrin following treatment with 12S-HETE [92]. Pidgeon et al. [93] found that 12-LOX regulates cell survival and apoptosis by influencing the expression and localization of αvβ3 and αvβ5 integrins in PC3 and A431 human cancer cells. It has been reported that the metastasis of cancer cells is enhanced by low-dose radiation associated with elevated biosynthesis of 12S-HETE [94]. Another positive correlation was observed between the biosynthesis of 12S-HETE and the metastatic potential of murine B16a cells [95]. 12S-HETE is a crucial intracellular signaling molecule involved in the activation of various signaling pathways [96-98] It activates PKCα via PLCγ1 and stimulates PKCζ via inositide kinase. It also activates MAPK kinase and mediates the biological functions of many growth factors such as basic Fibroblast Growth Factor (bFGF), Platelet-derived growth factor (PDGF), Epidermal Growth Factor (EGF), and Autocrine Motility Factor (AMF) [83, 99, 100].

Recently, it has been identified an orphan receptor GPR31, which shows a high affinity for 12S-HETE (Kd, 5 nM) and a lower affinity for other HETEs [53]. The activation of this receptor by 12S-HETE activates PKC-ERK1/2 axis. In GPR31-transfected CHO and COS7 cells, 12S-HETE leads to ERK1/2 and NFκB activation, whereas knockdown of GPR31 blocks the stimulatory effect of 12S-HETE on the invasive capability of tumor cells through Matrigel, associated with an inhibition of MEK activation [53].

Biosynthesis and functions of 12S-HETE in platelet/cancer cell crosstalk

The role of platelets in cancer and metastasis development is sustained by the efficacy of antiplatelet agents, such as low-dose aspirin, in humans [10]. Plenty of mechanisms can be used by platelets to promote tumorigenesis and metastasis: (i) upon activation, platelets release many molecules, including growth and angiogenic factors, lipids, and extracellular vesicles (EVs) [101, 102], and activate numerous cell types, including vascular and immune cells, fibroblasts, and cancer cells [103]; (ii) platelets can shroud disseminated tumor cells from recognition and elimination by natural killer cells [103]; (iii) platelets can promote cancer cell malignancy and migratory properties via the induction of epithelial-mesenchymal transition (EMT) thus facilitating extravasation and colonization of distant organs[103].

Platelets release EVs accounting for 70–90% of circulating EVs in the peripheral blood of healthy people [101, 104]. Medium-size (100–1000 nm) EVs (mEVs) are also called microparticles (MPs) [105].

Membrane-derived EVs are involved in cell-cell communication through many mechanisms: (i) directly stimulate cells, (ii) transfer membrane receptors, proteins, mRNA, and organelles (e.g., mitochondria) between cells, (iii) deliver infectious agents into cells (e.g., human immunodeficiency virus, prions) [106], (iv) platelet-derived MPs can influence target cells via transcellular lipid metabolism, i.e., transferring AA to endothelial cells to induce the expression of COX-2 and the synthesis of prostanoids [107].

12-LOX expressed in PRP has been reported to be a promising diagnostic and prognostic biomarker of prostate cancer [108, 109]. Contursi et al. [48] have shown that circulating mEVs (mainly from platelets) collected from patients with colorectal adenomas/adenocarcinomas contain pl12-LOX protein. An enhanced number of platelet-derived EVs is detected in the bloodstream of patients with CRC and other cancer types [110, 111]. Thus, the crosstalk between mEVs expressing pl12-LOX with circulating cancer could enhance their prometastatic potential in vivo.

In cocultures of human platelets and human colon adenocarcinoma HT-29 cells, free 12-HETE is detected in the medium and derived mainly from platelets [48]. However, one-third of 12-HETE generated is esterified into membranes PLs of platelets, mainly in PC species with acyl-linked 16:0 or 18:0 at Sn1 and PE 18:0a. In the interaction of platelets and cancer cells, platelet-derived mEVs are released [48]. They present only small amounts of 12-HETE esterified in PLs but express pl12-LOX, which is catalytically active. HT29 cells cultured alone neither expressed 12-LOX nor generated 12-HETE (either as a free form or esterified in membrane PLs. In cocultures of HT29 cells and platelets, cancer cells acquired pl12-LOX protein via the uptake of platelet-derived mEVs (expressing pl12-LOX) and the ability to generate 12-HETE [48]. Interestingly, 12-HETE was mainly esterified into membrane PLs. The pattern of 12-HETE esterification into the six PE, PC species of HT29 cells was different from that found in platelets [48]. Higher levels of plasmalogen forms were detected versus platelets, specifically PE 18:0p_12-HETE and PE 16:0p_12-HETE [48]. These results provide the rationale to characterize the production of 12-HETE-PLs in colorectal cancer lesions and verify whether the pattern of 12-HETE-PLs identifies patients with metastatic potential. These findings show that platelets induce 12-HETE generation in colon cancer cells and its esterification into membrane phospholipids via MP-mediated delivery of pl12-LOX (Figure 2). Interestingly, dm-amiloride, which inhibits the Na^+/H^+ exchanger involved in macropinocytosis [112] reduced mEV uptake by HT29 associated with the reduction of the levels of 12-LOX in HT29 cells cocultured with platelets [48]. Thus, the promotion of platelet-dependent malignancy in cancer cells could be counteracted by drugs affecting platelet-derived MP uptake. It is noteworthy that Mir-939, transferred by platelet mEVs to ovarian epithelial cancer cells, induces their proliferation and migration [113].

The role of pl12-LOX in EMT induction has been demonstrated in human gastric cancer cells with ALOX12 overexpression [114]. In esophageal cancer cell lines, the downregulation of ALOX12 expression or the pharmacological inhibition of 12-LOX activity is associated with reduced EMT markers and cell migration [115]. Contursi et al. [48] showed that in platelet-HT-29 cell cocultures,

the 12-LOX inhibitor CDC and other chemically distinct 12-LOX inhibitors [such as esculetin and baicalein (42, 116, 117)] prevented the induction of EMT genes, thus strengthened the role of 12-LOX activity in platelet-dependent induction of EMT in cancer cells. CDC is not a selective inhibitor of 12-LOX, also impacting 5-LOX [118, 119]. However, in HT29 cell-platelet cocultures, 5-HETE was undetectable

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12-LOX Inhibitors

The first 12-LOX inhibitors acted as redox inhibitors or fatty acid analogs [34] (Table).

Early 12-LOX inhibitors [baicalein, nordihydroguaiaretic acid (NDGA), 5,8,11,14-eicosatetraynoic acid, OPC-29030, L-655,238 and BW755C] are not 12-LOX selective, but they can also target cPLA2, COX-1, COX-2, and other LOXs (15-LOX-1, 15-LOX-2, 5-LOX) [120-124]. NDGA, BW755C, and baicalein [125] are redox inhibitors [126]. The activity of all LOX isoforms requires the activation of non-heme iron. Redox inhibitors prevent the oxidation of non-heme iron at the catalytic site, thus affecting its conversion from inactive iron (Fe^{2+}) to active iron (Fe^{3+}) [127]. The flavonoid baicalein, a major component in the root of Scutellaria baicalensis, is not a selective inhibitor of 12-LOX but affects also 15-LOX, due to its catecholic scaffold. The catecholic alcohol ligates the iron and causes an inner sphere reduction on the active site iron, with baicalein undergoing oxidation to its quinone form [116]. A structurally related flavonoid, apigenin, which differs from baicalein because it does not contain a cathecol moiety, is a not reductive inhibitor of LOXs. However, it can inhibit LOX due to a hydrogen bond between its terminal alcohol group and residue T591. In fact, this residue may anchor apigenin in an orientation that blocks substrate accessibility to the iron. Like baicalein, NGDA is a nonselective LOX inhibitor of LOXs with micromolar and sub-micromolar IC_{50}. It possesses two catechol rings, which confer a potent antioxidant activity; thus, by halting iron in the Fe^{2+} state, it can break the redox cycle of LOXs, resulting in their inactivation [123].

Another LOXs inhibitor is N-(4-chlorophenyl)-N-hydroxy-N′-(3-chlorophenyl)urea (CPHU), which can reduce various lipoxygenases and stimulates the degradation of lipid hydroperoxide catalyzed by these enzymes (pseudoperoxidase activity) [120]. Since phenols and hydroxamates react with the iron center of LOXs [120], they represent poor choices as potentially specific inhibitors.

Another subset of LOX inhibitors is fatty acid analogs. Sun et al. [128] synthesized a series of acetylenic fatty acids. One of them, 5,8,11,14-eicosatetrainoic acid (ETYA), resulted in an inhibitor of COX and 12-LOX. In contrast, 4,7,10,13-ETYA was found to be a potent and selective inhibitor of pl12-LOX. 5,8,11,14-ETYA resulted ten times more potent than 4,7,10,13-ETYA in inhibiting AA-induced aggregation in platelet-rich plasma and washed platelets [128].

Another class of 12-LOX inhibitors is 12-LOX translocation inhibitors (OPC-29030 and L-655,238) (Table) [129]. They were synthesized to inhibit the translocation of 12-LOX to the glycerophospholipid membrane involved in the synthesis of 12-HETE. These compounds can block 12-HETE formation without directly inhibiting enzymatic activity. In response to U46619 stimulation in vitro, OPC-29030-treated platelets have a decrease in Ca^{2+} mobilization, granule secretion, aIIbb3 activation, and aggregation compared to control-treated platelets, resulting in a decrease in thrombus formation in canine models of thrombosis [129].

The use of all these LOX inhibitors has permitted the understanding of the effects of pl12-LOX in potentiating platelet activation in vitro. In fact, they reduced platelet activation and aggregation in response to a wide variety of platelet agonists, including collagen, thrombin, ADP, and U46619 [34], but their lack of selectivity has limited their in vivo utility.

Development of novel 12-LOX inhibitors

Recently, by using a quantitative high-throughput screen (qHTS) on a library of about 150000 compounds, novel small molecule inhibitors of human pl12-LOX have been synthesized [130]. Interestingly, two scaffolds have been identified, i.e., 8-hydroxyquinoline-based scaffold and 4-((2-hydroxy-3-methoxybenzyl)amino)-benze-nesulfonamide-based scaffold, which showed nanomolar

potency against the purified enzyme 12-LOX and >50- fold selectivity over other LOX isozymes and COXs. Moreover, they had micromolar potency for the human platelets pl12-LOX [130]. Medicinal chemistry optimization strategies applied to 8-hydroxyquinoline-basedscaffold have led to the synthesis of two lead compounds, NCTT-956 (N-((8-hydroxy-5-nitroquinolin-7-yl)(thiophen-2-yl)methyl)propionamide) and ML-127 (N-((5-bromo-8-hydroxy-quinolin-7-yl)(thiophen- 2-yl)methyl) acetamide), which are potent noncompetitive inhibitors of 12-LOX and highly selective (>50-fold) over other LOX isozymes. NCTT-956 was also able to inhibit intracellular Ca2+, $\alpha_{IIb}\beta_3$ activation, and platelet aggregation mediated by thrombin or collagen. However, its development has been interrupted after structural modifications performed to reduce its cytotoxic effects have impaired its selectivity [130, 131]. In platelets stimulated with Activating peptides for PAR1 (PAR1-AP), it has been demonstrated that compound ML-127 inhibits 12-HETE generation[131], but its effects on platelet activation and thrombosis remain to be evaluated. The development of other benzenesulfonamide-based compounds for limiting 12-LOX activity and platelet function while maintaining enzyme selectivity remains an area of active research.

ML-355 ((N-benzo[d] thiazol-2-yl)-4((2-hydroxy-3 methoxybenzyl)amino)benzenesulfonamide) has been obtained by the structure-activity relationship studies with benzenesulfonamide-based scaffold [132]. This compound, which reversibly inhibits 12-LOX, is highly selective (>50-fold) over other oxygenases. It reduces calcium mobilization and platelet aggregation induced by a PAR4 agonist [56] and can also inhibit platelet activation mediated by the immune receptor, FcγRIIa [56]. It can inhibit human platelet aggregation in response to low concentrations of agonists, but its antiplatelet effects can be overcome by higher concentrations of agonists [56, 132]. Ex vivo flow chamber assays confirmed that human platelet adhesion and thrombus formation at arterial shear over collagen were mitigated in whole blood treated with ML-355 comparable to aspirin [133]. Pharmacokinetic studies with ML-355 in mice showed that it is orally bioavailable and has no observable toxic effects [133]. ML-355 significantly inhibited thrombosis formation in WT mice in several in vivo thrombosis models, but it has only a marginal effect on hemostasis; these effects

were different from those observed in 12-LOX deficient mice, which showed prolonged tail bleeding times. Adili et al. [133] developed two new in vivo hemostatic assays to evaluate both hemostatic plug formation and bleeding through the assessment of plasma extravasation caused by vascular wall rupture in real-time [133]. In these hemostasis models, the injury is intense, and it completely rips the vascular wall, resulting in hemorrhage from the vessel into the extravascular space. By using these models, hemostasis has been quantitatively assessed in mouse large and small vessels after ML-355 treatment, showing that the compound has inhibitory effects on platelet activation and clot formation in vivo; however, the complete rupture of the vessel wall causes a significant activation of the coagulation pathway and thrombin generation which can overcome the inhibitory effects of ML-355. This residual platelet activation in the presence of ML-355 at high concentrations of endogenous agonists under pro-coagulative conditions could have an important role in restoring normal coagulation and hemostasis, thus suggesting that ML-355 can potentially act as a safer antiplatelet agent, which has minimal effects on hemostasis. Noteworthily, even if these in vivo hemostatic models may reproduce the hemostasis process, they have limitations and may not fully reflect hemostasis in the human vessel. Altogether, available data on ML-355 support the idea that it can potentially represent a first-in-human approach to control platelet activation following vascular insult or injury, with the important advantage of minimizing the increased risk of bleeding associated with antiplatelet therapy. However, it will be urgent to explore potential off-target effects and toxicity prior to human studies.

Among the different strategies used to identify selective platelet 12-LOX inhibitors, the structural modification of known 5-LO inhibitors to shift selectivity on platelet 12-LOX has been shown to be successful [118]. Thus, a series of 3,4-dihydroxy-a-cyanocinnamoyl esters derived from the 5-LO inhibitor caffeic acid has been proposed as potent and selective platelet 12-LOX inhibitors. Among them, the synthetic compound cinnamyl-3,4-dihydroxy- α-cyanocinnamate (CDC) was reported to inhibit platelet 12-LOX (IC50 63 nM) and to be selective approximately 30 and 53 times over 5- and 12/15-LOX-, respectively (Table). Thus, CDC has been widely used as a pharmacological tool

to study 12-LOX mediated effects because it has been considered as a rather selective 12-LOX inhibitor [96, 134-138]. However, further studies reported a divergent selectivity profile of CDC than initially described and defined 5-LOX as a preferential target [119]. In fact, CDC inhibited 5-LOX in both polymorphonuclear leukocytes and monocytes and showed similar efficiencies as the 5-LOX inhibitor zileuton [119]. Platelet 12-LOX activity was inhibited by CDC when thrombin and collagen (and to a lesser extent ionophore) were used as stimuli, but not in presence of exogenous AA [119]. CDC has radical scavenging properties, which suggest the reducing feature as the mechanism responsible for LOX inhibition. This could represent an important limitation because the systemic use of redox type 5-LOX inhibitors, as CDC, can be hampered by interference with other redox systems or by the production of ROS with consequent side effects (e.g., hemolysis or methemoglobin formation) [139]. Thus, an accurate analysis of the potential toxicity of CDC should be performed to justify further developments for systemic use.

Esculetin (curcumin; 6,7-dihydroxycoumarin), is a phenolic compound with antioxidant properties [140], inhibiting 12-HETE production in both human and rat platelets [117]; however, it also affects the 5-LOX activity of human polymorphonuclear leukocytes [141].

Recently, Tacconelli et al. [42] have studied the effects of ML-355, esculetin, and CDC on the biosynthesis of prostanoids (such as TXB_2 and PGE_2) and HETEs in serum obtained from human whole blood allowed to clot for one h at 37 °C, thrombin-stimulated washed human platelets and during platelet aggregation in human PRP in response to collagen or TRAP6. The use of a targeted chiral lipidomics analysis by LC-MS/MS of serum allowed the simultaneous assessment of prostanoids and HETEs, thus, providing important information on the mechanism of action of ML-355, esculetin, and CDC on nonenzymatic and enzymatic pathways of AA metabolism (i.e., COX-1, 12-, 15- and 5-LOX) [42]. In this study, the pharmacological characterization of the compounds was performed in the presence of plasma proteins and endogenous sources of AA released in response to thrombin (generated endogenously during blood clotting) [7, 43]. ML-355 did not affect serum eicosanoid biosynthesis up to 260 μM, and platelet aggregation and eicosanoid generation in

PRP in response to low and high concentrations of collagen (2 and 10 μg/ml) or TRAP-6 (a PAR-1 agonist) [42]. In contrast, the antiplatelet agents, aspirin, and ticagrelor inhibited platelet aggregation [42]. Esculetin affected 12S-HETE production in serum and washed platelets in a concentration-dependent fashion[42]; however, esculetin also inhibited TXB2 biosynthesis, thus suggesting that it can act by affecting the release of AA. When used at concentrations ≤ 100 μM, esculetin preferentially inhibited platelet 12-LOX in washed platelets [42]. Platelet aggregation induced in PRP by collagen or TRAP-6 was not significantly affected by 300 μM of esculetin, due to the incomplete inhibition of eicosanoid generation [42]. The influence of antioxidant plasma components can explain the reduced inhibition of eicosanoid biosynthesis by esculetin in serum or PRP.

CDC was assessed in the same models [42]. In serum, the compound was selective for 12S-HETE, but the maximal effect was incomplete. In washed platelets stimulated with thrombin, the compound caused a comparable concentration-dependent inhibition of 12S-HETE and TXB2 [42]. In PRP stimulated with collagen or TRAP-6, CDC (300 μM) caused an incomplete inhibitory effect on 12S-HETE and other eicosanoids and no effect on platelet aggregation [42]. These data show the importance of plasma components in the inhibitory effects of pl-12LOX and the selectivity of CDC. The results reported by Tacconelli et al. [42] enlighten the importance of using experimental models resembling the in vivo situation (particularly the presence of proteins and other plasma components) for the pharmacological characterization in vitro or ex vivo of novel antiplatelet compounds. In particular, the impact of 12-LOX inhibitors on eicosanoids generated in serum can provide important information on their enzyme selectivity, off-target effects, and the potential interference of plasma components on their pharmacological effects.

Conclusions

The role of platelet type 12-LOX in human health and disease is still not completely understood. The major limitation is the unavailability of selective inhibitors to use in animal models and treat

patients. KO mice for ALOX12 have the limitation that 12S-HpETE/HETE can be generated by the expression of ALOX15, which encodes a 12-LOX enzyme [24,25]. Moreover, it is now clear that genetically engineered mouse (GEM) phenotypes can be background-dependent [142]. The different findings obtained in ALOX12$^{-/-}$ mice in thrombosis models are noteworthy. Johnson et al. [55] found that mice with the deletion of ALOX12 were more sensitive to thrombosis in response to intravenous ADP injection. Differently, Yeung et al. [57] found reduced platelet aggregation and thrombus formation in response to laser-induced vascular injury in ALOX12$^{-/-}$ versus WT mice. The ALOX12/12S-HETE pathway seems to contribute to platelet function through PAR4 activation or by low concentrations of agonists.

The development of ML-355, an orally and selective pl12-LOX inhibitor [133], can help to clarify the role of 12S-HETE in atherothrombosis and other pathological conditions. However, Tacconelli et al. [42] could not find an inhibitory effect on platelet-dependent 12S-HETE biosynthesis in whole blood allowed to clot at 37°C for 1 h or in aggregating PRP in response to low and high collagen concentrations or TRAP-6. Thus, the compound should be more carefully characterized before its administration to humans.

It remains to clarify also the mechanism involved in platelet activation by 12S-HETE. Identifying the orphan receptor GPR31 as a 12S-HETE receptor [53] and developing GPR31 pepducin antagonist (GPR310) [54] will be helpful for enhancing knowledge of ALOX/12S-HETE pathway in human pathology.

The discovery that platelet activation leads to the incorporation of 12-HETE into PLs and augments thrombin generation has opened the way to study novel functions of ALOX12 [48, 51, 52]. Interestingly, a new mechanism by which platelets can promote EMT in cancer cells via the transfer of pl12-LOX contained in platelet-derived mEVs has been described [48]. These cancer cells now generate 12S-HETE, a modulator of cancer metastasis. Interestingly, 12-HETE is mainly esterified in plasmalogen phospholipids of cancer cells [48]. Modifying cancer cell phospholipids by 12S-HETE may functionally impact cancer cell biology and represent a novel

target for anticancer agent development. Selective inhibitors of 12-LOX or macropinocytosis inhibitors[112], which prevent the internalization of mEVs, have the potential to represent novel anticancer tools.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. The cascade of arachidonic acid (AA). Free AA can be metabolized to eicosanoids through three major pathways: the cyclooxygenase (COX) pathway, the lipoxygenase (LOX) pathway, and the cytochrome P450 (CYP) pathway. In the CYP pathway, AA is converted to epoxyeicosatrienoic acids (EETs) and 20-HETE by CYP epoxygenases and CYP ω-hydroxylases, respectively. In the COX pathway, AA is first converted to prostaglandin G_2 (PGG_2) by the cyclooxygenase reaction, and then the peroxidase activity of the enzyme catalyzes a two-electron reduction of PGG_2 to PGH_2. PGH_2 is converted to different prostanoids. AA can be bound in the cyclooxygenase active site of COXs in at least three different catalytically competent arrangements that lead to PGG_2 but also 11-hydroperoxyeicosatetraenoi acid (HpETE), and 15R/S-HpETE as minor products, reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs), at least in part, via the peroxidase activity of the enzymes. LOXs catalyze AA to HpETEs, which may be converted to HETEs and leukotrienes (LT).

*pl12S-LOX: platelet type 12-LOX; *generated from aspirin-acetylated COX-2; h, human; m, mouse; LX: lipoxin*

Figure 2. Platelet/cancer cell crosstalk and the generation of 12S-HETE and 12-HETE-phospholipids. The interaction of platelets and cancer cells in vitro causes platelet activation and the rapid generation of 12S-HpETE/HETE via the activity of platelet-type 12-LOX (pl12-LOX) highly expressed in platelets. A large amount of 12-HETE is esterified in six different species of phospholipids (PLs) producing phosphatidylethanolamine (PE) 16:0p_12-HETE, PE 18:1p_12-HETE, PE 18:0p_12-HETE, PE and phosphatidylcholine(PC) 18:0a_12-HETEs, and PC 16:0a_12-HETE. Medium-sized extracellular vesicles (mEVs) are released from platelets and contain active pl12-LOX. Colorectal cancer cells do not express pl12-LOX and do not produce 12-HETE or 12-HETE-Pls. However, these cells uptake platelet-derived mEVs and the pl12-LOX is transferred to

cancer cells which can produce 12-HETE that is rapidly esterified in membrane PLs to generate the six 12-HETE-PLs detected in platelets. The plasmalogen HETE-PEs (PE18:0p_12-HETE and PE 16:0p _12-HETE) were significantly higher in cancer cells cultured with platelets than in platelets alone. The interaction of platelets and cancer cells promotes epithelial-mesenchymal transition (EMT) in cancer cells. dm-amiloride, a Na+/H+ exchanger, which inhibits macropinocytosis, mitigates the internalization of platelet mEVs by cancer cells (resulting in reduced levels of 12-LOX), and 12-LOX inhibitors (which inhibit pl12-LOX activity) can prevent the induction of EMT marker genes in colon cancer cells cocultured with platelets [48].

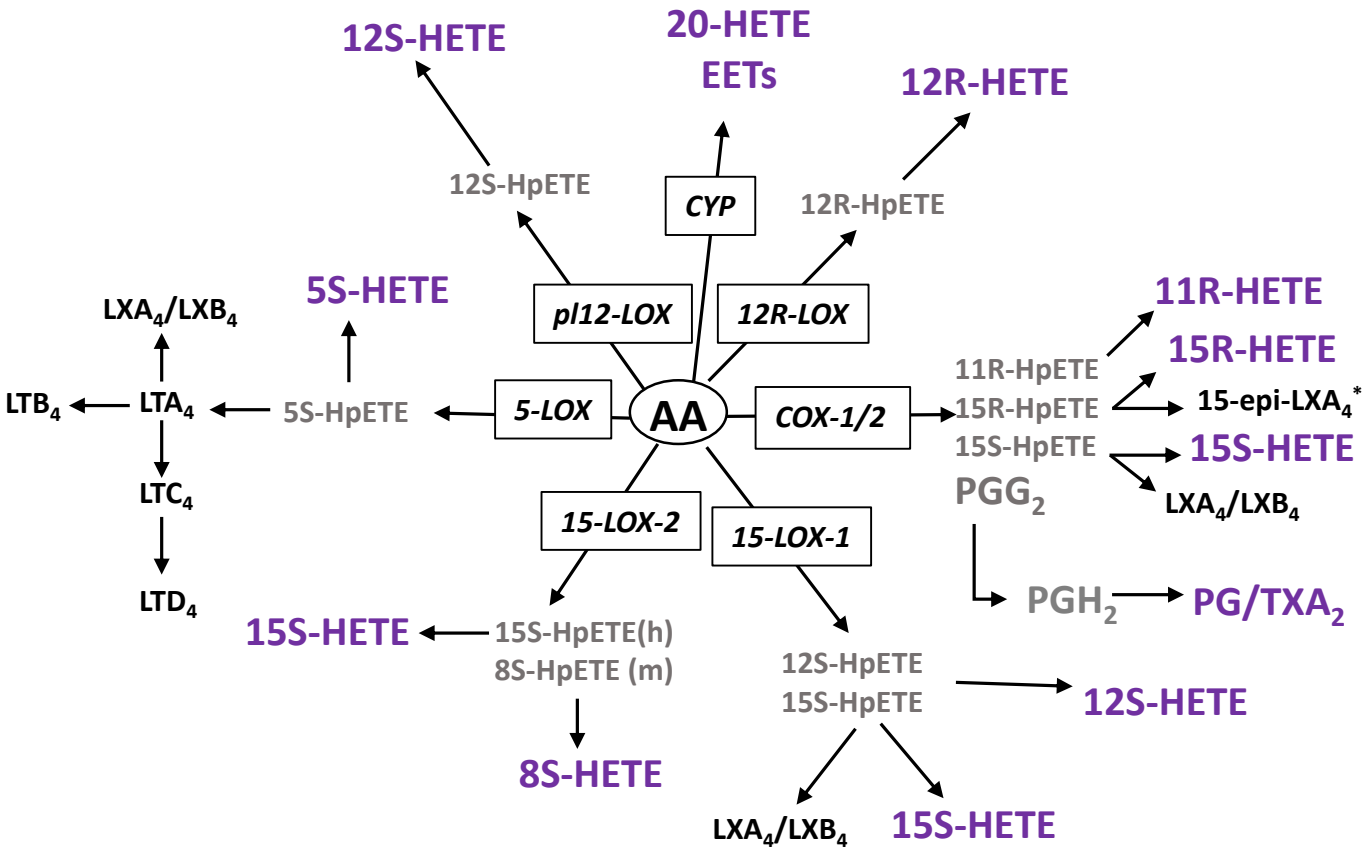

12S-HETE
20-HETE
EETs
12R-HETE
12S-HpETE
CYP
12R-HpETE
5S-HETE
LXA4/LXB4
pl12-LOX
12R-LOX
11R-HETE
15R-HETE
LTB4
LTA4
5S-HpETE
5-LOX
AA
COX-1/2
11R-HpETE
15R-HpETE
15-epi-LXA4*
15S-HpETE
15S-HETE
LTC4
PGG2
15-LOX-2
15-LOX-1
LXA4/LXB4
LTD4
PGH2
PG/TXA2
15S-HETE
15S-HpETE(h)
8S-HpETE (m)
12S-HpETE
15S-HpETE
12S-HETE
8S-HETE
LXA4/LXB4
15S-HETE

Figure 1.

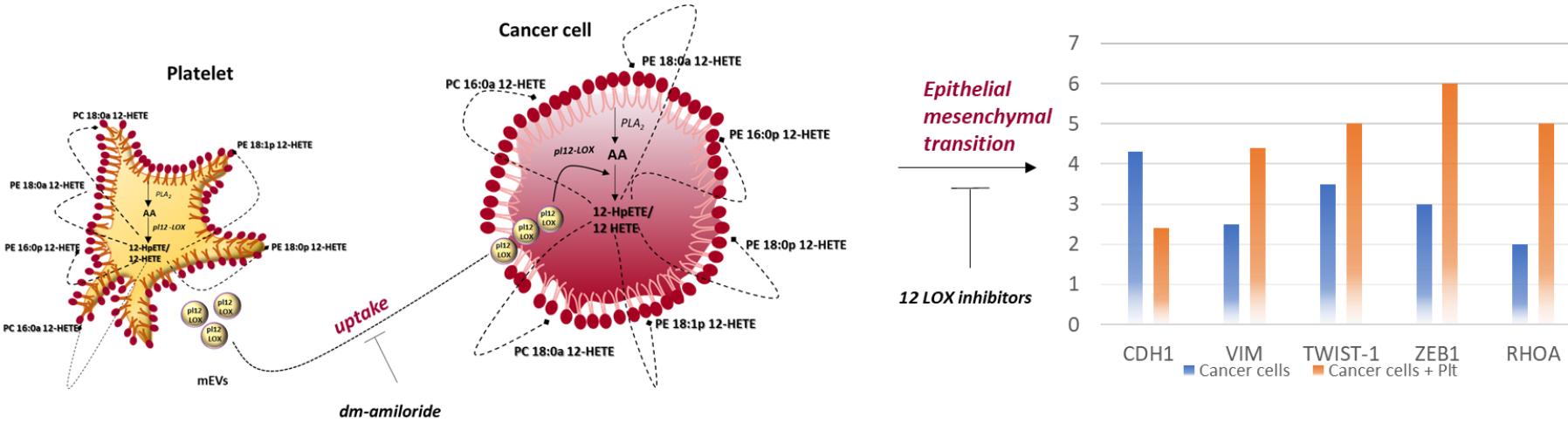
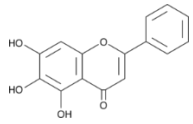
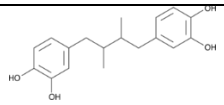
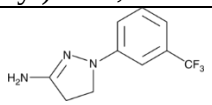
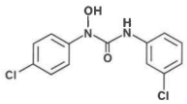
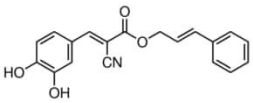
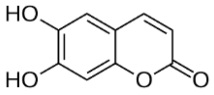
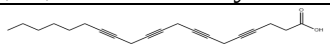
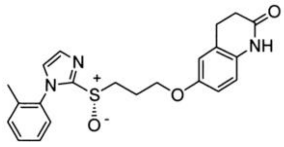
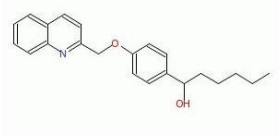
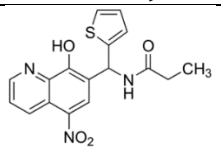
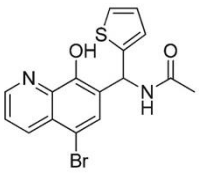
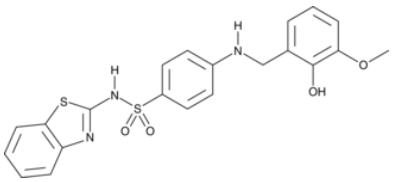
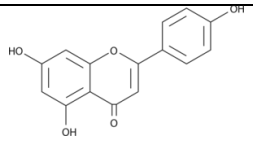


Figure 2

Table: Classification of LOX inhibitors according to their mechanism of action

	Chemical structure and formal name	Targets	References
Redox inhibitors			
Baicalein	 2,6,7-trihydroxyflavone	Human platelet 12-LOX, IC_{50}=0.64μM; Human Reticulocyte 15-LOX-1, IC_{50}=1.6μM Rat leukocite 5-LOX, IC_{50}=7.13μM	116, 122
Nordihydroguaiaretic acid (NDGA)	 4,4'-(2,3-dimethyl-1,4-butanediyl)bis-1,2-benzenediol	Human platelet 12-LOX, IC_{50}=3-5μM 15-LOX, IC_{50}=1.7 μM Human platelet 5-LOX, IC_{50}=2.3μM	123
BW755C	 4,5-dihydro-1-[3-(trifluoromethyl)phenyl]-1H-pyrazol-3-amine	Inhibitor of rat leukocite 12-LOX, COX-1 and rabbit leukocite 5-LOX (IC_{50} is not reported)	124
CPHU	 N-(4-chlorophenyl)-N-hydroxy-N′-(3-chlorophenyl)urea	Porcine 12-LOX, IC_{50}=15μM Soybean 15-LOX-1, IC_{50}=2 μM Human recombinant 5-LOX, IC_{50}=0.10μM	, 120
CDC	 Cinnamyl-3,4-dihydroxy-α-cyanocinnamate	Platelet 12-LOX, IC_{50}=0.063μM Rat leukocyte 15-LOX, IC_{50}=3.33μM Rat leukocyte 5-LOX, IC_{50}=1.89μM Recombinant human platelet 12-LOX, IC_{50}:0.5μM; Recombinant human 15-LOX-1, IC_{50}: 0.3μM; Recombinant human 5-LOX, IC_{50}: 0.015μM	118 119

Esculetin	 6,7-dihydroxycoumarin	Platelet 12-LOX, IC_{50}=0.65μM; platelet COX-1, IC_{50}=450μM; Human leukocytes 5-LOX, IC_{50}=0.4μM	117, 141
Fatty acid analogues			
5,8,11,14-eicosatetraynoic acid (ETYA)	 5,8,11,14-eicosatetraynoic acid	Human platelet 12-LOX, IC_{50}=0.03μM, COX, IC_{50}=3.2μM	128
4,7,10,13-ETYA	 4,7,10,13-eicosatetraynoic acid	Human platelet 12-LOX, IC_{50}=0.009 μM, COX, IC_{50}=8 μM	128
Translocation inhibitors			
OPC-29030	 (S)-(+)-3,4-Dihydro-6-[3-(1-o-tolyl-2-imidazolyl)sulfinylpropoxy]-2(1H)-quinolinone	Human platelet 12-LOX, IC_{50}=0.06μM; no effect on platelet COX-1 activity; suppression of 5S-HETE production by 50% at 0.1-10μM in rat basophilic leukemia cells	129
L-655,238	 α-pentyl-3-(2-quinolinylmethoxy)-benzenemethanol	Human platelet 12-LOX, IC_{50}=0.171μM 5-LOX, IC_{50}=0.135μM (rat basophilic leukemia cells)	129
Non-reductive, non-competitive inhibitors			
NCTT-956	 N-((8-hydroxy-5-nitroquinolin-7-yl)(thiophen-2-yl)methyl)propionamide	Human platelet 12-LOX, IC_{50}=0.80μM Human Reticulocyte 15-LOX-1, IC_{50}=>25μM	130
ML-127	 N-((5-bromo-8-hydroxyquinolin-7-yl)(thiophen-2-yl)methyl)acetamide	Human platelet 12-LOX, IC_{50}=1μM Human Reticulocyte 15-LOX-1, IC_{50}=>100μM Human epithelial 15-LOX-2, IC_{50}=>100μM Human recombinant 5-LOX, IC_{50}=>100μM	130

ML-355	 N-2-benzothiazolyl-4-[[(2-hydroxy-3-methoxyphenyl)methyl]amino]-benzenesulfonamide	Human platelet 12-LOX, IC_{50}=0.34μM Human Reticulocyte 15-LOX-1, IC_{50}=9.7μM Human epithelial 15-LOX-2, IC_{50}=>100μM Human recombinant 5-LOX, IC_{50}=>100μM COXs=no inhibition at the tested concentration (15μM)	132
Other mechanisms			
Apigenin	 5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one	Human platelet 12-LOX, IC_{50}=81 μM Human Reticulocyte 15-LOX-1, IC_{50}=3.4μM	116

UNIVERSITA' DEGLI STUDI DI CHIETI "G. D'ANNUNZIO"
Scuola di Medicina e Scienze della Salute
DIPARTIMENTO DI NEUROSCIENZE, IMAGING E SCIENZE CLINICHE

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To: Prof Oliwer Werz
Friedrich Schiller University Jena, Germany
Editor: Lipid Mediators Special Issue of "Biochemical Pharmacology"

RE: Submission of the manuscript "Biology and pharmacology of platelet-type 12-lipoxygenase in platelets, cancer cells, and their crosstalk"

Dear Editor:

I am writing as the corresponding author and on behalf of all coauthors of the manuscript entitled "Biology and pharmacology of platelet-type 12-lipoxygenase in platelets, cancer cells, and their crosstalk" by Annalisa Contursi, Stefania Tacconelli, Ulrika Hofling, Annalisa Bruno, Melania Dovizio, Patrizia Ballerini and Paola Patrignani.

We wish to submit the manuscript for consideration to publication in the Lipid Mediators Special Issue of "Biochemical Pharmacology".

All authors confirm that the paper is not under consideration elsewhere and none of the paper's contents have been previously published. All authors have read and approved the manuscript.

With best wishes,

Yours sincerely,

Paola Patrignani
Professor of Pharmacology

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