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Development of 5-lipoxygenase inhibitors—lessons from cellular enzyme regulation

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Abstract

5-Lipoxygenase (5-LO) catalyzes the first steps in the conversion of arachidonic acid (AA) into leukotrienes (LTs) that are mediators of inflammatory and allergic reactions. Recently, the 5-LO pathway has also been associated with atherosclerosis and osteoporosis. Thus, in addition to the classical applications including asthma and allergic disorders, LT synthesis inhibitors might be of interest for the treatment of cardiovascular diseases and osteoporosis. Recently, it has been shown that cellular 5-LO activity is regulated in a complex manner that can involve different signalling pathways. 5-LO can be activated by an increase in intracellular Ca^{2+} concentration, diacylglycerols, phosphorylation by MAPKAP kinase-2 and ERK. Previous work could demonstrate that cellular 5-LO activity is repressed in a protein kinase A-dependent manner and by glutathione peroxidases. This comment focuses on the impact of these stimulatory and inhibitory pathways on the efficacy of 5-LO inhibitors and suggests additional criteria for the development of this class of compounds.

Contents

1. LO and disease

The arachidonic acid (AA) transforming enzyme 5-lipoxygenase (5-LO) catalyzes the conversion of AA into LTA4. This unstable intermediate can be further converted into LTB4 by LTA4 hydrolase or into LTC4 by LTC4 synthase and the LTC4 synthase isoenzymes MGST2 or MGST3. LTs have been identified as mediators of a variety of inflammatory and allergic reactions including rheumatoid arthritis, inflammatory bowel disease, psoriasis, allergic rhinitis but their major pathophysiological implication was linked to bronchial asthma [1,2]. Recently, the 5-LO pathway has also been associated with atherosclerosis [3–5], osteoporosis [6] and certain types of cancer like neuroblastoma [7] and prostate cancer [8]. Thus, in addition to the classical indications including asthma and allergic disorders, LT synthesis inhibitors might be of interest for the treatment of these diseases.

During the recent decades, there was a considerable progress in the understanding of the cellular regulation of the 5-LO pathway. Thus, knowledge on 5-LO regulation allows now to define additional criteria for the identification of highly active 5-LO inhibitors [9] and provides us with new strategies and concepts for the development of

Abbreviations: AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-lipoxygenase-activating protein; fMLP, *N*-formyl-methionyl-leucyl-phenylalanin; GM-CSF, granulocyte macrophage colony-stimulating factor; GPx, glutathione peroxidase; LPS, lipopolysaccharide; 5-LO, 5-lipoxygenase; LT, leukotriene; MAPK, mitogen-activated protein kinase; MK, MAPKAP kinase (mitogen-activated protein kinase-activated protein kinase); MGST, microsomal glutathione *S*-transferase; PC, phosphatidylcholine; PMNL, polymorphonuclear leukocytes; PKA, protein kinase A; TNFα, tumor necrosis factor alpha

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such compounds. This commentary focuses on the enzymology and cellular regulation of 5-LO and discusses potential consequences for the pharmacology of the 5-LO pathway.

2. Activation of 5-LO by Ca^{2+} and ATP

Initially, LT synthesis was observed after cell stimulation of neutrophils by Ca^{2+} ionophores [10], which suggested that the increase in $[Ca^{2+}]_i$ is an important determinant for cellular enzyme activity. The role of Ca^{2+} in 5-LO activation is complex. Although no obvious Ca^{2+}-binding motif in the primary sequence of 5-LO is apparent, reversible binding of Ca^{2+} to 5-LO was recently found by different techniques, including Ca^{2+} overlay, gel filtration in the presence of Ca^{2+}, equilibrium dialysis, and Ca^{2+}-induced mobility shift in gel electrophoresis [11]. Mutation of N43, D44 and E46 which are located within the β-barrel (C2-like) domain caused reduced Ca^{2+} binding and a requirement for higher Ca^{2+} concentrations to stimulate enzyme activity [12]. The C2-like domain seems to be involved in the Ca^{2+}-dependent interaction of the enzyme with membrane structures. Thus, Ca^{2+} stimulates 5-LO translocation to the nuclear envelope as well as association of 5-LO with membranes [13,14], preferentially the nuclear membrane, which is rich in phosphatidylcholine (PC) [15]. This scenario leads to a model suggesting that Ca^{2+} promotes membrane association which then facilitates the transfer of AA to 5-LO via 5-lipoxygenase-activating protein (FLAP) [16] (Fig. 1). However, in vitro, a requirement of Ca^{2+} for enzyme activity is not absolute and strongly depends on the cellular stimulus, the cell type, and the assay conditions. In homogenates of human polymorphonuclear leukocytes (PMNL) and rat basophilic leukemia-1 cells, considerable 5-LO product synthesis is detected in the absence of Ca^{2+}, whereas Ca^{2+} is required for 5-LO activity in homogenates of monocytic Mono Mac 6 cells under the same assay conditions [17,18]. In Mono Mac 6 cells, glutathione peroxidase (GPx)-1 was identified as endogenous inhibitor of cellular 5-LO [18] that renders 5-LO activity Ca^{2+}-dependent [17] and it was found that the C2-like domain mediates the Ca^{2+}-dependent resistance of 5-LO activity against inhibition by GPx-1 [19].

Interestingly, Ca^{2+} is not required for 5-LO activity at high PC vesicle concentrations or at high concentrations of AA in vitro [20]. Mg^{2+} can substitute for Ca^{2+} regarding binding and activation of 5-LO, although it is less efficient [21]. Moreover, 5-LO product synthesis in PMNL can be induced by cell stress, such as sodium arsenite or osmotic shock, which is Ca^{2+}-independent and involves enzyme phosphorylation [22].

In contrast to other LOs, the 5-LO catalytic activity is stimulated by ATP and to a lesser extent by other nucleotides including ADP, AMP, cAMP, CTP, and UTP [23]. The extent of 5-LO stimulation at 0.1–2 mM ATP is in the range of two- to six-fold, is partially Ca^{2+}-dependent, the K_a value for ATP binding was 31 μM [24], and the stoichiometry of ATP-binding was estimated 1 mol ATP/1 mol 5-LO [25]. By means of photolabelling, two tryptophane residues (Trp75 and Trp201) have been identified that specifically interact with ATP-analogues [25]. At the

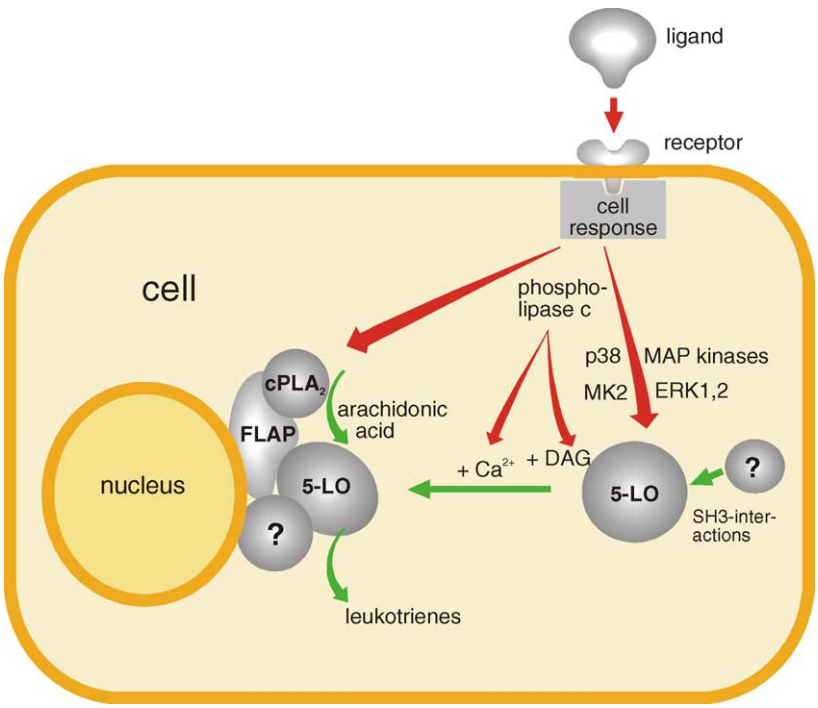

Fig. 1. Pathways involved in 5-lipoxygenase activation.

moment, the physiological function of ATP-binding to 5-LO is unknown.

3. Activation of 5-LO by phosphorylation

LTs display a great variety of biological effects. Thus, it is not surprising that both, 5-LO expression and cellular LT biosynthesis are tightly regulated [26]. Cellular formation of these mediators is controlled by (1) liberation of AA which is mainly catalyzed by cytosolic phospholipase (cPL)A_2 and (2) regulation of 5-LO activity. In contrast to Ca^{2+}-ionophore A23187, naturally occurring inflammatory mediators like *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), C5a, platelet-activating factor, or LTB_4 are poor activators of 5-LO metabolism in neutrophils [27]. The response of neutrophils to these stimuli can be increased if cells are preincubated with priming agents like granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)α or bacterial lipopolysaccharides (LPS) which are unable to induce 5-LO activity by themselves, for review see [28]. It was suggested that these priming effects are related to increased AA release and 5-LO activation.

Phosphorylation of $cPLA_2$, induced by priming of PMNL or macrophages, renders the enzyme more susceptible to Ca^{2+} stimulation which results in substantial AA release also at moderate increases in $[Ca^{2+}]_i$. Priming with phorbol myristate acetate also upregulates 5-LO activity by mechanisms such as phosphorylation of 5-LO [29]. In LPS-primed cells, but not in unprimed cells, $cPLA_2$ as well as 5-LO translocate to the nuclear envelope after stimulation with fMLP, coinciding with enhanced release of AA and LTs [30]. Thus, redistribution of 5-LO might be one mechanism of how priming increases the capacity of LT generation.

Recently, p38 mitogen-activated protein kinase (MAPK)-regulated mitogen-activated protein kinase-activated protein kinases (MAPKAPKs, MKs) and extracellular signal-regulated kinase (ERK) 1/2 were identified as 5-LO kinases that phosphorylate 5-LO in vitro at Ser271 and Ser663, respectively, and mediate cellular activation of 5-LO [31,32] (Fig. 2). The efficiency of 5-LO phosphorylation by both kinases is strongly stimulated by polyunsaturated fatty acids [32,33]. ERKs and p38 MAPKs are activated by a number of proinflammatory cytokines, chemotactic factors, phorbol esters and Ca^{2+} mobilizing agents, but also by cell stress, such as osmotic shock, genotoxic stress (sodium arsenite (SA)), UV light and heat shock. Agents that induce LT synthesis, i.e. ionophore and fMLP as well as TNFα, GM-CSF, LPS and phorbol esters that prime leukocytes for enhanced LT synthesis, activate ERKs, p38 MAPK and downstream MKs in leukocytes. Therefore, these stimuli could activate 5-LO for product formation via enzyme phosphorylation by ERKs and p38 MAPK-regulated MKs. Furthermore, both phosphorylation reactions seem to induce nuclear translocation of 5-LO [34,35]. In BL41-E95-A cells, stimulation with ionophore plus AA causes only moderate 5-LO product formation and fails to activate p38 MAPK or to phosphorylate 5-LO. However, cell stress strongly enhances cellular 5-LO activity and activates p38 MAPK as well as downstream MKs, and inhibition of p38 MAPK by SB203580 abolishes stress-induced LT synthesis in BL41-E95-A cells without inhibition of 5-LO catalytic activity in cell free systems [36].

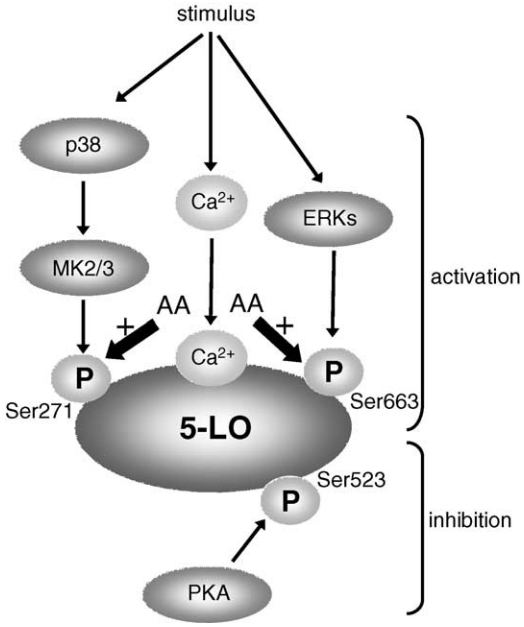

Fig. 2. Regulation of 5-lipoxygenase activity by phosphorylation and Ca^{2+}.

In human PMNL, stimulation of p38 MAPK and MKs by cell stress itself (no further stimulus) is sufficient to activate 5-LO. In contrast to simulation with Ca^{2+}-mobilizing agents like ionophore A23187, the stress-induced activation of 5-LO and of p38 MAPK occurs also after Ca^{2+} depletion and the p38 MAPK inhibitor SB203580 blocks stress-induced 5-LO product formation more efficiently than ionophore- or thapsigargin-induced formation of 5-LO products [22]. SB203580 and U0126 (an ERK pathway inhibitor) also inhibit AA-induced LT biosynthesis efficiently in PMNL under conditions that do not lead to substantial mobilization of Ca^{2+} [32]. Apparently, depending on the cell type, ERKs and MKs stimulate 5-LO by phosphorylation under conditions that do not lead to substantial increases in $[Ca^{2+}]_i$, but also can act together with Ca^{2+} to activate 5-LO (see Figs. 1 and 2). In semipurified enzyme preparations, phosphorylation does not affect enzyme activity which suggests that phosphorylation by ERKs and MKs rather modulates or regulates the interaction of 5-LO with other cellular components than to directly affect its catalytic properties.

Based on a theoretical model of the tertiary structure of 5-LO, Ser271 and Ser663 are located on the enzyme

surface and should be well accessible to both kinases. In addition to MKs, also other serine-directed kinases that recognize a R–X–X–S motif, such as CaMKII and protein kinase A (PKA) also phosphorylate Ser271 in vitro, although only at low level. Recently, it was found that PKA activation inhibits 5-LO translocation and LT biosynthesis in human neutrophils [37,38]. PKA phosphorylates 5-LO at Ser523 which reduces cellular enzyme activity and also decreases activity of the recombinant enzyme in vitro. Ser523 is localized at the periphery of the catalytic domain and phosphorylation of this residue may cause allosteric changes that reduce 5-LO enzyme activity [38] (Fig. 2), and might alter binding of 5-LO inhibitors to the enzyme.

4. Regulation of cellular 5-LO activity

It has been shown earlier that 5-LO activity is stimulated by several cellular fractions including membrane preparations. The membrane fraction that upregulates 5-LO activity, can be replaced by synthetic lipid vesicles consisting of PC. The C2-like domain of 5-LO selectively binds PC, which is conferred by tryptophane residues (Trp13, Trp75, and Trp102) located in the putative Ca^{2+}-binding loops [15]. Recently, it was found that the Ca^{2+}-binding C2-like domain is involved in the ionophore-induced association of 5-LO with the nuclear envelope [39]. Particularly, the PC selectivity of the C2-like domain seems to account for the specific targeting of 5-LO to the nuclear membrane [15]. Interestingly, 1-oleoyl-2-acetylglycerol not only primes leukocytes for enhanced LT release upon subsequent agonist stimulation, but also functions as a direct agonist for PMNL, stimulating 5-LO product formation in the presence of exogenously added AA by novel, undefined mechanisms [40] (Fig. 1).

Another important parameter for cellular 5-LO activity is the redox tonus. Activation of resting 5-LO requires the oxidation of the active site iron from the ferrous to the ferric state (Fig. 3). Among various lipid hydroperoxides, 5- and 12-hydro(pero)xyeicosatetraenoic acid, and 13-hydroperoxyoctadecadienoic acid activate crude 5-LO in homogenates, whereas hydrogen peroxide and several other organic hydroperoxides fail in this respect. Lipid hydroperoxides can shorten the lag phase of 5-LO, which is observed after addition of substrate to crude 5-LO in homogenates or for purified enzyme, and conditions that promote lipid peroxidation stimulate 5-LO activity in leukocytes. Selenium-dependent GPx reduce the cellular peroxide content and are potent suppressors of 5-LO activity [41,42]. Phospholipid hydroperoxide GPx (PhGPx) was demonstrated to suppress 5-LO activity in A431 cells [43], B-cells [42] and granulocytes [41], whereas GPx-1 is an endogenous 5-LO inhibitor in monocytic cells [18]. Differentiation of myeloid cells enhances the resistance of 5-LO activity against peroxidase inhibition which might be due to increased production of peroxides after cell stimulation or to enhanced cell signalling in differentiated Mono Mac 6 or HL-60 cells. In Mono Mac 6 cell homogenates, Ca^{2+} is required for 5-LO activity and GPx-1 was identified as a factor that renders 5-LO activity Ca^{2+}-dependent [19]. Furthermore, we could demonstrate that the C2-like domain mediates the Ca^{2+}-dependent resistance of 5-LO activity against inhibition by GPx-1 [19].

In conclusion, Ca^{2+}, the cellular peroxide tonus, phosphorylation, binding of PC and/or DAGs seem to regulate the activity of 5-LO in a complex manner (Fig. 1). Additionally, a number of 5-LO interacting proteins like FLAP, coactosin like protein, TRAP-1 or Dicer might affect cellular 5-LO activity and could modulate the efficacy of 5-LO inhibitors [44].

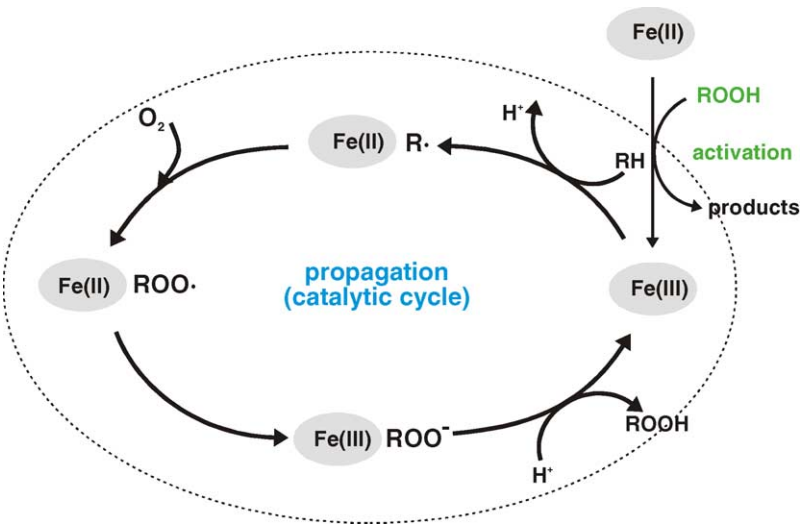

Fig. 3. 5-Lipoxygenase catalytic cycle (redox regulation).

5. Classification of 5-LO inhibitors

5-LO inhibitors can be classified into three main groups: redox-active compounds, iron-ligand inhibitors with weak redox-active properties and non redox-type inhibitors. Early screening programs identified many redox-active compounds like nordihydroguaiaretic acid, coumarins or flavonoids (e.g. cirsiliol) as 5-LO inhibitors. These compounds act as nonselective antioxidants and many of them show severe side effects like methemoglobin formation or are rapidly metabolized, precluding further development. Reducing agents interfere with the activation and the catalytic cycle of 5-LO (Fig. 3). The active site of the 5-LO enzyme contains a non-heme iron. In the resting state, the iron is in the ferrous form (Fe^{2+}). Activation of 5-LO by hydroperoxides oxidizes the iron into the ferric form (Fe^{3+}) that allows the enzyme to enter the catalytic cycle. This cycling is interrupted by redox-type 5-LO inhibitors which are able to reduce the active-site ferric iron.

The rational development of compounds that are able to chelate the active site iron led to hydroxamic acid and *N*-hydroxy-urea derivatives. However, in vivo, the hydroxamate group is rapidly metabolized into an inactive carboxylate group, which precludes further development of this class of inhibitors. The most prominent *N*-hydroxyurea derivative is A-64077 (zileuton) which is the first and only 5-LO inhibitor up to now that came on the market for the treatment of asthma. Only low therapeutic benefits were observed for zileuton in other diseases such as allergic rhinitis, rheumatoid arthritis and inflammatory bowel disease [45], and no therapeutic benefit of zileuton was observed in ulcerative colitis. As other iron-chelators and redox-active compounds, zileuton supports the pseudoperoxidase reaction by 5-LO and is metabolized to form nitroxides [9].

The disadvantage of redox active inhibitors and iron ligands, i.e. the participation in redox reactions and lack of selectivity, initiated the development of nonredox-type inhibitors. It should be noted that the classification of compounds as nonredox-type inhibitors relates to the lack of redox activity of the drugs themselves but does not exclude that the inhibitory potency of the drugs is affected by the redox state of their target, the 5-LO. Optimization of lead structures led to ZD 2138 and ZM 230487 which are selective, orally active 5-LO inhibitors [46]. The compounds inhibit LT biosynthesis in human leukocytes and whole blood with IC_{50}-values of about 20–50 nM, respectively [47]. However, despite its strong potency in several ex vivo and in vitro models, ZD 2138 fails to strongly inhibit LT synthesis at sites of chronic inflammation [48]. A number of structurally related compounds, e.g. L-697,198 and L-739,010 were developed by other pharmaceutical companies.

The disappointing clinical results with inhibitors like ZD 2138 that have excellent in vitro potency raised the question whether inhibition of LT biosynthesis has any therapeutical potential at all. In this respect, it should be noted that the clinical use of LT receptor antagonists (that block LT action) provide a clear proof for a benefit of anti-LT therapy in inflammatory and allergic diseases, and that the pivotal roles of LTs in these disorders could be confirmed by studies using knock-out animals (for review, see [28]). Interestingly, when 5-LO inhibition by ZD 2138 or its *N*-ethyl analogue ZM 230487 was tested with purified 5-LO or in cell 100,000 g supernatants, the compound was more than 100-fold less active than in intact cells [47]. The apparent loss of 5-LO inhibition by ZM 230487 or the related Merck compound L-739,010 in cell homogenates is reversed by addition of glutathione or dithiothreitol, and efficient inhibition of purified 5-LO requires the presence of GPx [49]. Accordingly, conditions that lead to increased peroxide levels impair the potency of ZM 230487 in intact cells, but not of other types of LT synthesis inhibitors (MK886 or BW-A4C). Kinetic analysis of ZM 230487 revealed a competitive inhibition of 5-LO under non-reducing conditions, whereas the compound acts as a non-competitive inhibitor when the hydroperoxide level is low.

Moreover, the potency of several nonredox-type 5-LO inhibitors like ZM 230487 or the related Merck compound L-739,010 (but not of redox-type 5-LO inhibitors) in intact cells depends on the stimulus and the phosphorylation status of the 5-LO enzyme [50]. Thus, 5-LO product formation involving 5-LO kinase pathways require ~10- to 100-fold higher concentrations of ZM 230487 or L-739,010 for comparable 5-LO inhibition versus Ca^{2+}-mediated 5-LO activation using ionophore as stimulus. Furthermore, a significant difference in the potency of ZM 230487 and L-739,010 was determined between wild type 5-LO and the non-phosphorylatable mutant S271A/S663A-5-LO in HeLa cells. On the other hand, the inhibitory potency of CJ-13,610, another nonredox-type inhibitor, does not depend on the type of stimulus (phosphorylation or ionophore) but is very sensitive to increased peroxide levels [51]. Thus, phosphorylation events seem to impair the potency of some but not all nonredox-type inhibitors of 5-LO.

In the past, 5-LO inhibitors were usually screened by ionophore stimulation of resting leukocytes. However, from the recently obtained data, it becomes clear that the inhibitory potency of compounds can strongly depend on the cellular stimulus as well as on the signalling pathways and stimulating factors that in an orchestrated interplay determine cellular 5-LO activity (Fig. 4). This suggests that potential drug candidates should be screened in different cellular assays where Ca^{2+}- and phosphorylation-dependent stimuli are applied. Screening of compounds with ionophore stimulated granulocytes can be regarded as a reliable tool to verify the inhibitory potency of drug candidates when cellular 5-LO activity is elicited by an increase in $[Ca^{2+}]_i$. However, we found a dramatic loss of activity of several nonredox-type inhibitors when

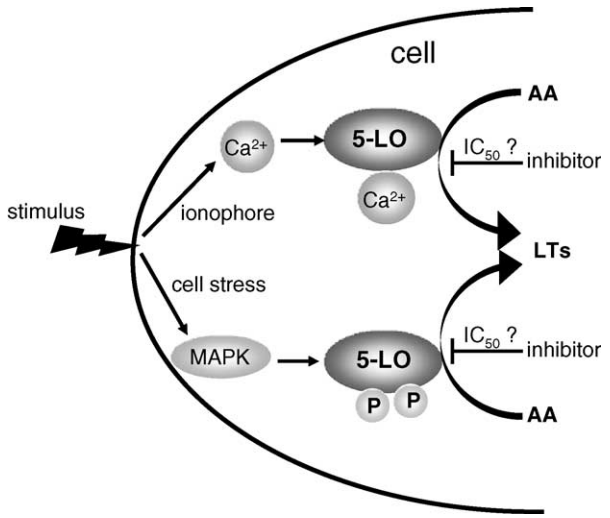

Fig. 4. Determinants for the efficacy of 5-lipoxygenase inhibitors.

cellular 5-LO activity was induced with stimuli that act via 5-LO phosphorylation. The most prominent shift in inhibitory potency was observed when hyperosmotic stress (300 mM NaCl) together with arachidonic acid (40 μM) was used for granulocyte stimulation [50]. Under these conditions, cellular 5-LO activity of granulocytes is strongly dependent on 5-LO phosphorylation by the p38 MAP kinase pathway [22]. On the other hand, both the ERK and the p38 pathways are involved when granulocytes are activated with AA alone (60 μM) [32]. These observations suggest that drug candidates should be screened with granulocytes stimulated with ionophore/AA, NaCl/AA and AA alone in order to establish an in vitro drug profile.

Furthermore, the dependence on peroxide levels seems to be another point that should be addressed during the in vitro characterization of potential drug candidates. Since glutathione peroxidases represent potent endogenous inhibitors of cellular 5-LO activity that control the cellular lipid hydroperoxide tone and since the 5-LO enzyme seems to have a regulatory fatty acid hydroperoxide binding site, it is worthwhile to check whether elevated hydroperoxide levels interfere the inhibitory potency of drug candidates. Glutathione peroxidases require millimolar concentrations of thiols (GSH and DTT) for their activity that act as cosubstrates. Thus, homogenization of granulocytes (at cell densities of 10^6 to 10^7 cells per ml) dilutes the endogenous thiols more than 1000-fold, so that these broken cell preparations lack glutathione peroxidase activity unless millimolar concentrations of DTT or GSH are added. Therefore, screening of compounds in granulocyte homogenates for 5-LO inhibition in the absence of exogenously added thiols provides an easy tool to study the potency of the drugs under conditions that lead to the generation of high concentrations of hydroperoxides. Addition of GSH or DTT (1 mM) to the granulocyte homogenates efficiently restores glutathione peroxidase activity and works well as control assay that mimics a low hydroperoxide tone [49]. Interestingly, both ZM 230487 and L-739,010 show identical IC_{50}-values for 5-LO inhibition in intact granulocytes and in cell homogenates in the presence of DTT or GSH [49], whereas the IC_{50}-values are up to 100-fold higher when both compounds are tested in homogenates in the absence of DTT and GSH. These observations suggest that screening should not only be done with purified 5-LO but also with granulocyte homogenates in the presence and absence of thiols in order check whether inhibition of 5-LO by a drug candidate is peroxidase-dependent.

Finally, the recent results raise the question whether the determination of ex vivo 5-LO inhibition in ionophore-stimulated whole blood is a sufficient or suitable parameter to assess the in vivo inhibition of LT biosynthesis by 5-LO inhibitors.

The recent progress in the understanding of the regulation of cellular 5-LO activity allowed us to define new criteria for 5-LO inhibitors and should help to select potential drug candidates for further clinical development in the future.

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