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Multi-dimensional target profiling of *N*,4-diaryl-1,3-thiazole-2-amines as potent inhibitors of eicosanoid metabolism

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ABSTRACT

Eicosanoids like leukotrienes and prostaglandins play a considerable role in inflammation. Produced within the arachidonic acid (AA) cascade, these lipid mediators are involved in the pathogenesis of pain as well as acute and chronic inflammatory diseases like rheumatoid arthritis and asthma. With regard to the lipid cross-talk within the AA pathway, a promising approach for an effective anti-inflammatory therapy is the development of inhibitors targeting more than one enzyme of this cascade. Within this study, thirty *N*-4-diaryl-1,3-thiazole-2-amine based compounds with different substitution patterns were synthesized and tested in various cell-based assays to investigate their activity and selectivity profile concerning five key enzymes involved in eicosanoid metabolism (5-, 12-, 15-lipoxygenase (LO), cyclooxygenase-1 and -2 (COX-1/-2)). With compound **7**, 2-(4-phenyl)thiazol-2-ylamino)phenol (**ST-1355**), a multi-target ligand targeting all tested enzymes is presented, whereas compound **9**, 2-(4-(4-chlorophenyl)thiazol-2-ylamino)phenol (**ST-1705**), represents a potent and selective 5-LO and COX-2 inhibitor with an IC_{50} value of 0.9 ± 0.2 μM (5-LO) and a residual activity of 9.1 ± 1.1% at 10 μM (COX-2 product formation). The promising characteristics and the additional non-cytotoxic profile of both compounds reveal new lead structures for the treatment of eicosanoid-mediated diseases.

1. Introduction

Lipid mediators like eicosanoids are pivotal messengers in immunity and inflammation. Deregulation in their biosynthesis and metabolism leads to different harmful diseases. Bronchial asthma, rheumatoid arthritis, atherosclerosis, cardiovascular diseases and certain types of cancer are a few of them [1–4]. These diseases are wide-spread and one therapeutic option is to regulate the metabolism of the disease triggering lipid mediators, by inhibiting an enzyme of their biosynthesis pathway.

Hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs) and prostaglandins (PGs) are subtypes of the eicosanoids, which are products of the so-called arachidonic acid (AA) cascade (Scheme 1). The first step in their biosynthesis is the release of AA from the cell membrane [5]. The subsequent conversion into the lipid mediators is realized by two different enzymatic pathways, the lipoxygenase and the cyclooxygenase pathway.

Human lipoxygenases (LO) are non-heme iron-containing enzymes. They catalyze the oxygenation of AA and are named after the position of the oxygenated carbon. The 5-lipoxygenase catalyzes two steps in leukotriene biosynthesis: 5-HPETE, the product of the first oxygenation, is then reduced to the corresponding alcohol 5-HETE or alternatively dehydrated to the unstable epoxide LTA_4 [6–8]. LTA_4 is further metabolized to LTB_4 or the cysteinyl-containing LTs C_4, D_4 and E_4. LTB_4 is an activator for phagocytes as well as a chemotactic and chemokinetic mediator [9], while the

Abbreviations: 12-HHT, 12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid; (5-)LO, (5-)lipoxygenase; AA, arachidonic acid; COX, cyclooxygenase; H(P)ETE, (*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; LDH, lactate dehydrogenase; LT(B_4), leukotriene (B_4); NDGA, nordihydroguaiaretic acid; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin; PMNL, polymorphonuclear leukocytes; SAR, structure–activity relationships; TXA_2, thromboxane A_2.

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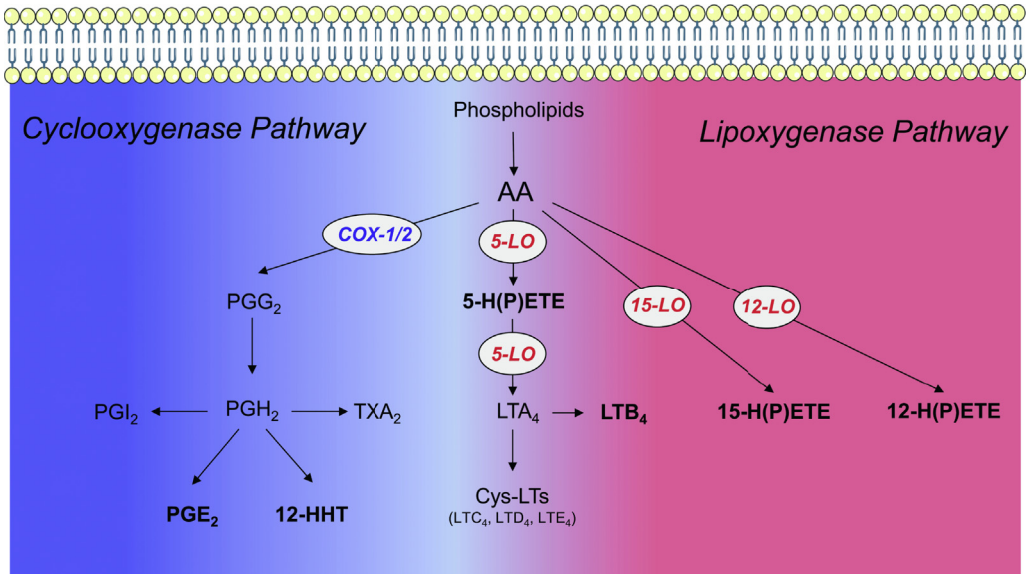
Cys-LTs cause bronchoconstriction and vascular permeability [5] explaining their role in the pathogenesis of e.g. asthma. Platelet 12-LO and 15-LO1 from reticulocytes and eosinophiles are key enzymes of the hydro(pero)xy-fatty acid biosynthesis of 12(*S*)- or 15(*S*)-hydro(pero)xyeicosatetraenoic acid (12-, 15-H(P)ETE). Both enzymes as well as 5-LO are found up-regulated in different cancer tissues like prostate or colon cancer [10,11]. Chronic inflamed tissue exhibits a significant risk to develop cancer. Inflammatory as well as immune cells infiltrate cancer tissue affecting malignant cells. Inflammatory processes are linked to almost every state of cancer development like initiation and metastasis [12,13]. However, for 12-LO and 15-LO1 the detailed pathophysiological role is still unclear: besides the pro-inflammatory and tumor-promoting effects of the HETEs these two enzymes are controversially discussed for pro-resolving effects in inflammation and tumor suppression [14–17].

AA is also a precursor for the prostaglandins. Released AA is converted to PGH_2 by the two isoforms of cyclooxygenase (COX), COX-1 and COX-2. PGH_2 is further metabolized by PGE_2 synthase (PGES) to PGE_2, which is a pivotal pro-inflammatory mediator [18–20], by PGI_2 synthase (PGIS) to PGI_2, a potent vasodilator and inhibitor of platelet aggregation [21], and by thromboxane A_2 (TXA_2) synthase to TXA_2, a potent stimulator of platelet aggregation and vascular constriction [22]. In activated human platelets PGH_2 is also converted by TXA_2 synthase to 12(*S*)-hydroxyheptadeca-5*Z*, 8*E*, 10*E*–trienoic acid (12-HHT) which acts as an intracellular messenger and can bind to the LTB_4 receptor-2 (BLT2) [23]. Both COX-1 and COX-2 are targeted by the non-steroidal anti-inflammatory drugs (NSAIDs). These drugs are used in the treatment of inflammatory processes and pain [24]. NSAIDs are partially non-selective which leads to harmful side effects like gastrointestinal irritation and a poor platelet activity resulting in increased bleeding times [25]. COX-1 is constitutively expressed in many tissues and responsible for basic housekeeping functions. In contrast, COX-2 expression is induced in inflammatory cells. The selective inhibition of COX-2 exhibits less side effects (mainly ulcerogenic) and results in potent anti-inflammatory effects [26,27]. However, some selective COX-2 inhibitors had to be withdrawn from the market, for example rofecoxib [28], as they failed to fully satisfy the criteria of safer anti-inflammatory drugs due to an increased risk of cardiovascular events [29–31]. Because more people are affected with inflammation and pain than any other disease state, treatment of these conditions constitutes significant medical needs [32]. With the aim to find safer but efficacious drugs, current research in the field now focuses on novel rational approaches for effective anti-inflammatory agents with better safety profiles.

The products of both AA pathways (5-LO and COX) play indisputable roles in several inflammatory diseases and cancer. Selective inhibition of just one of the closely related pathways may result in a shunt of substrate (AA) into the uninhibited pathway resulting in an increased synthesis of the respective eicosanoid, as could be shown for instance for celecoxib [33]. Thus inhibition of just one AA metabolizing enzyme would not reduce the overall level of pro-inflammatory mediators [34]. Instead, targeting multiple key enzymes with one single inhibitor is a promising approach for an effective anti-inflammatory therapy and, as shown for dual 5-LO/COX-2 inhibitors, for inhibition of cancer growth as well [35,36]. This so-called polypharmacology is nowadays representing a major paradigm shift in drug discovery [37,38] also and in particular when addressing the AA cascade [39–41]. Multi-target ligands help to understand the crosstalk between the different branches of the AA pathway [33,42,43]. They might as well be the key to a safer treatment strategy for eicosanoid-mediated diseases, not least due to the known advantages of a multi-target drug like better patient compliance, less safety concerns, and no drug–drug interactions compared to a drug cocktail [44].

N,4-Diaryl-1,3-thiazole-2-amines are privileged structures in medicinal chemistry and bear high potential for the above described therapeutic option. They revealed auspicious inhibitory potency on PGE_2 formation [45] as well as direct inhibition of rat 5-LO [46]. Interested in this promising potential we synthesized a series of *N*,4-diaryl-1,3-thiazole-2-amines with different substitution pattern via microwave-assisted Hantzsch thiazole synthesis [47] and optimized their selectivity profile with regard to key enzymes involved in eicosanoid biosynthesis, starting from 5-LO. Here, we present structure–activity relationships (SAR) for the



Scheme 1. Biosynthesis of eicosanoids, focusing on the cyclooxygenase and lipoxygenase pathway. AA, arachidonic acid; COX-1/2, cyclooxygenase 1/2; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; PGE_2, prostaglandin E_2; 12-HHT, 12(*S*)-hydroxyheptadecatrienoic acid; 5-/12-/15-LO, 5-/12-/15-lipoxygenase; 5-/12-/15-H(P)ETE, 5-/12-/15-hydro(pero)xyeicosatetraenoic acid; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; Cys-LTs, cysteinyl-leukotrienes; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; bold: analyzed metabolites of eicosanoid metabolism.

different targets of these novel potent multi-target lipid signaling inhibitors.

2. Results

2.1. Chemistry

N,4-Diaryl-1,3-thiazole-2-amines (compounds **1**, **3**–**9**, **14**, **15**, **19**–**25**, **27** and **28**, Scheme 2) were synthesized by a multistep procedure adopted to the previously described synthesis route by Rasmussen et al. [48]. Bromination of acetophenone derivatives (**A**) in a unipolar solvent resulted in reactive α-bromoketones (**B**). *N*-Arylthioureas (**E**) were prepared by the reaction of benzoyl chloride with aniline derivatives (**C**) in the presence of NH_4SCN in acetone and following debenzoylation of the resultant *N*-aryl-*N*′-benzoylthiorureas (**D**) under basic conditions. In the final step, microwave-assisted Hantzsch thiazole synthesis of α-bromoketones (**B**) and *N*-arylthioureas (**E**) in ethanol provided the desired *N*,4-diaryl-1,3-thiazole-2-amines (**F**).

2.2. Activity assays

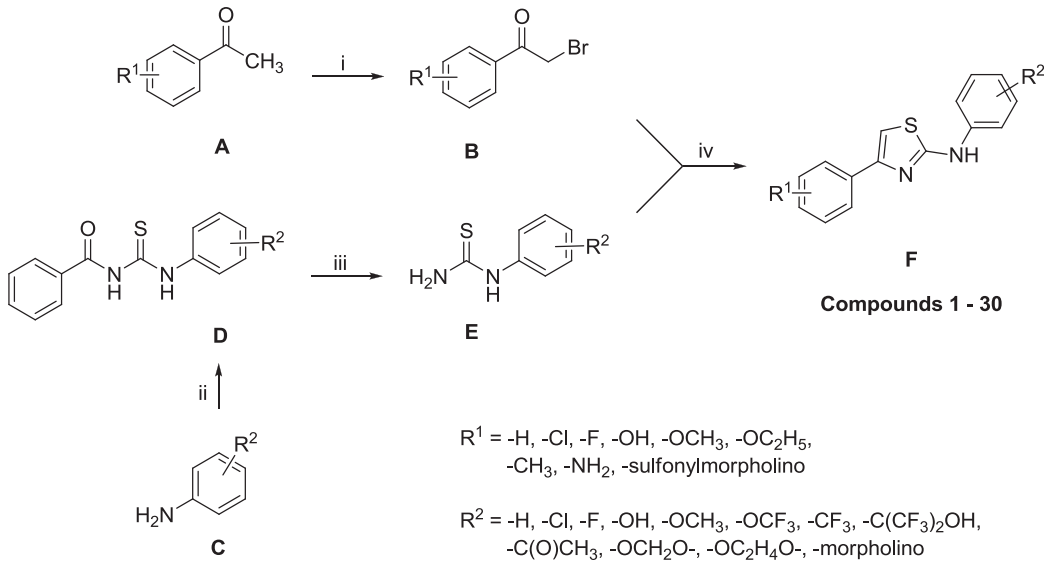
The aim to address either one or several different enzymes linked to inflammation with only one compound requires the detailed knowledge about possible inhibitory activities and the selectivity profiles of the derivatives. Therefore, we performed different cell-based assays, allowing simultaneously assessing the intracellular bioavailability of the test compounds. Freshly isolated human blood cells were used for determination of 5-LO and 15-LO1 product formation (polymorphonuclear leukocytes (PMNL)) and for 12-LO and COX-1 product formation (platelets). The cervix carcinoma cell line HeLa was used to investigate the influence of test compounds on COX-2 mediated extracellular PGE_2 concentration. To distinguish between direct inhibition of 5-LO and indirect effects, e.g. interference with upstream enzymes, a cell-free assay system using purified recombinant 5-LO was applied. As all compounds inhibiting cellular 5-LO product formation are direct 5-LO inhibitors as well (see Supplementary Data), we here focus the discussion about SARs and selectivity on cellular activity data.

All tested compounds bear an *N*,4-diaryl-1,3-thiazole-2-amine-core. To gain knowledge about SARs for the different targets, we kept the core stable and varied substituents at position R^1 and R^2 (Table 1).

With the first set of compounds (**1**–**7**) we investigated the role of the 4-chlorophenyl at position R^1. Therefore, we kept the 4-hydroxy group at R^2 and varied the substituents of the R^1 phenyl. Whether the electron-drawing 4-Cl (**1**) was replaced by 4-F (**3**), 4-CH_3 (**5**), 4-OC_2H_5 (**6**) or even omitted (4-H, **7**), no significant change in the inhibitory potency for 5-LO product formation could be observed. Furthermore, the addition of a second 3-Cl (**2**) did not alter the activity resulting in IC_{50} values for intact PMNL between IC_{50} = 0.3 ± 0.1 μM and 0.9 ± 0.5 μM. Substitution with a 4-amino, (**4**), led to a slight decrease in inhibitory activity with an IC_{50} value of 4.4 ± 1.5 μM.

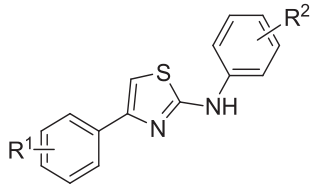
Most compounds of this subset were selective for 5-LO and showed no significant inhibitory potency on 15-LO1 product formation at 50 μM, except two derivatives: compound **5** showed weak inhibitory potency whereas compound **7** decreased the residual activity of 15-LO1 product formation to 13.0 ± 4.3%. Compounds **2** and **7** showed a slight inhibitory activity on 12-LO product formation resulting in residual activities of 32.6 ± 2.0% (**2**) and 34.9 ± 17.3% (**7**) at a concentration of 50 μM.

With no significant change in inhibitory activity of 5-LO product formation despite of different substituents at R^1, we studied the importance of the 4-hydroxyphenyl group at R^2 and kept the 4-chlorophenyl at R^1 constant (**8, 9**, **12**–**25**). Interestingly, the hydroxyl group in position 3 (**8**) led to a lower inhibition with an IC_{50} value of 5.6 ± 1.4 μM in intact cells whereas the 2-hydroxyphenyl (**9**) restored the IC_{50} (0.9 ± 0.2 μM). Similar observations could be made for compounds **7** and **10**: The hydroxyl group in position 3 (**10**) worsened the inhibitory activity to an IC_{50} value of 8.3 ± 2.4 μM compared to 4-hydroxy (**7**). The missing chlorine at the R^1 phenyl (**7**, **10**) only marginally decreased the inhibition compared to compounds **1** and **9**. The methylether derivatives of compounds **1**, **8** and **7**, the 4-methoxyphenyl derivatives **12**, **13**, and **11**, exhibited a more than tenfold loss of inhibitory activity in intact PMNL. In addition, removing the 4-Cl at R^1 (**11**) restored the IC_{50} in intact cells to 4.7 ± 2.9 μM compared to **12**. Rotating the methoxy moiety at R^2 to the 2-position (**13**) resulted in decreased inhibitory activity



Scheme 2. General synthesis procedure for synthesized compounds. Reagents and conditions: i) Bromine, chloroform, 0 °C – RT, 2 h, yield: 37–92%, ii) Benzoyl chloride, NH_4SCN, acetone, 60 °C, 45 min, yield: 63–98%, iii) NaOH (2 M), 100 °C, 1 h, yield: 64–87%, iv) Ethanol, 80 °C, microwave irradiation, 30 min, yield: 28–87%.

Table 1
Inhibitory activities of test compounds on multiple enzymes of the arachidonic acid cascade. IC_{50} values for inhibition of 5-LO product formation by test compounds in intact PMNL, residual activities of 12-LO and 15-LO1 as well as COX-1 and COX-2 product formation; $n = 3$, mean ± SEM.



			5-LO product formation IC_{50} [μM]	Product formation (residual activity at 50 μM, % of control)			
	R^1	R^2	PMNL	12-LO	15-LO1	COX-1	COX-2
1	4-Cl	4-OH	0.7 ± 0.1	>70%	>70%	28.6 ± 3.4%	4.1 ± 2.3%
2	3,4-diCl	4-OH	0.9 ± 0.2	32.6 ± 2.0%	>70%	69.4 ± 3.7%[c]	15.1 ± 6.1%[c]
3	4-F	4-OH	0.7 ± 0.1	56.7 ± 17.0%	>70%	26.3 ± 3.0%	2.2 ± 1.1%
4	4-NH_2	4-OH	4.4 ± 1.5	>70%	>70%	26.7 ± 2.7%	3.3 ± 1.3%
5	4-CH_3	4-OH	0.9 ± 0.5	>70%	44.6 ± 11.5%	18.5 ± 1.5%	3.4 ± 1.5%
6	4-OC_2H_5	4-OH	0.6 ± 0.2	>70%	>70%	16.6 ± 0.5%	2.1 ± 1.2%
7[a]	H	4-OH	0.3 ± 0.1	34.9 ± 17.3%	13.0 ± 4.3%	16.2 ± 4.9%	3.1 ± 1.5%
8	4-Cl	3-OH	5.6 ± 1.4	>70%	57.0 ± 8.5%	19.5 ± 9.6%	2.7 ± 1.4%
9[b]	4-Cl	2-OH	0.9 ± 0.2	>70%	>70%	67.4 ± 13.5%[c]	9.1 ± 1.1%[c]
10	H	3-OH	8.3 ± 2.4	>70%	>70%	4.1 ± 0.4%	2.4 ± 1.2%
11	H	4-OCH_3	4.7 ± 2.9	>70%	>70%	2.3 ± 1.0%	3.2 ± 1.8%
12	4-Cl	4-OCH_3	>30	>70%	>70%	25.6 ± 2.3%	3.7 ± 1.3%
13	4-Cl	2-OCH_3	22.5 ± 6.3	>70%	>70%	55.8 ± 7.2%	5.3 ± 2.2%
14	4-Cl	H	5.6 ± 1.6	>70%	>70%	7.7 ± 0.4%	5.0 ± 2.2%
15	4-Cl	3,4-diOCH_3	5.8 ± 1.0	>70%	>70%	–	8.6 ± 2.7%
16	4-Cl	2,4-diOCH_3	7.3 ± 6.8	>70%	>70%	>70%	18.4 ± 6.3%
17	4-Cl	3-C(O)CH_3	>30	>70%	>70%	38.7 ± 1.2%	59.9 ± 12.1%
18	4-Cl	3,5-diCl	23.5 ± 5.7	>70%	>70%	2.3 ± 0.6%	30.9 ± 6.9%
19	4-Cl	4-CF_3	5.9 ± 2.3	>70%	>70%	2.7 ± 0.1%	>70%
20	4-Cl	4-OCF_3	2.9 ± 0.6	>70%	>70%	1.1 ± 0.3%	17.8 ± 1.0%
21	4-Cl	3,5-diCF_3	3.3 ± 1.4	>70%	>70%	13.7 ± 0.7%	45.1 ± 8.9%
22	4-Cl	4-C(CF_3)$_2$OH	5.0 ± 0.4	>70%	>70%	2.6 ± 0.3%	42.9 ± 3.1%
23	4-Cl	3-OC_2H_4O-4	3.1 ± 0.8	>70%	>70%	6.0 ± 1.8%	5.5 ± 2.2%
24	4-Cl	3-OCH_2O-4	3.2 ± 0.8	>70%	>70%	3.2 ± 0.1%	3.8 ± 0.8%
25	4-Cl	4-morpholino	>30	>70%	>70%	>70%	>70%
26	4-OCH_3	H	1.6 ± 1.3	>70%	50.3 ± 19.0%	2.0 ± 1.3%	2.3 ± 1.0%
27	4-OH	4-Cl	3.1 ± 1.6	>70%	43.1 ± 9.7%	3.5 ± 0.3%	2.1 ± 0.4%
28	4-OH	4-F	3.5 ± 1.4	4.2 ± 1.7%	53.7 ± 21.6%	2.1 ± 0.6%	2.1 ± 0.2%
29	4-F	4-C(O)CH_3	4.9 ± 2.6	>70%	>70%	56.1 ± 10.8%	9.1 ± 1.2%
30	4-sulfonyl-morpholino	4-Cl	>30	>70%	41.3 ± 3.7%	>70%	39.1 ± 2.4%
Reference compounds			65.6 ± 3.8%[d]	1.5 ± 0.7%[e]	23.1 ± 7.5%[e]	2.8 ± 0.5%[f]	10.5 ± 1.6%[g]

[a] Compound **ST-1355**.
[b] Compound **ST-1705**.
[c] Measured at a concentration of 10 μM.
[d] BWA4C 0.08 μM, residual activity.
[e] NDGA 100 μM.
[f] ASA 100 μM.
[g] Rofecoxib 1 μM
– no determination.

with IC_{50} of 22.5 ± 6.3 μM. Starting with the 4-methoxy derivative **12** we inserted an additional methoxy-group in 3- or 2-position (**15**, **16**). The loss of inhibitory activity of compound **12** could be eliminated resulting in IC_{50} values of 5.8 ± 1.0 μM, and 7.3 ± 6.8 μM, respectively. Introduction of a methyl ketone-group in position 3 at the free phenyl group of compound **14** impaired the inhibitory potency (**17**).

To study the influence of bulky halogen derivatives, we synthesized 4-trifluoromethyl (**19**), 4-trifluoromethylether (**20**), two trifluoromethyl moieties in position 3 and 5 (**21**) and the 4-hexafluorohydroxypropyl (**22**) at R^2. All compounds led to a similar decreased inhibitory activity of 5-LO product formation. The IC_{50} values range between 2.9 ± 0.6 to 5.9 ± 2.3 μM in PMNL. The same potency is reached with compounds **23** and **24**, bearing a 1,4-dioxane and a 1,3-dioxolane ring. The addition of a 4-morpholine at the R^2 phenyl (**25**) erased the inhibitory activity in PMNL. As this derivative is inactive in all cell-based assays (12-/15-LO, COX-1/-2), but showed a prominent IC_{50} value of 0.05 ± 0.01 μM for purified 5-LO (Supplementary Data), suggesting that cell membrane penetration might be impaired.

Regarding the lipoxygenase selectivity of the derivatives bearing a 4-chlorophenyl at position R^1, all but one compound (**8**) were selective for the 5-LO isoenzyme: Only the 3-hydroxy derivative (**8**) caused a weak inhibition (about 50%) of 15-LO1 product formation at a concentration of 50 μM.

In the last set we tested some compounds with different substituent combinations at R^1 and R^2. Replacement of 4-Cl (**14**) with 4-methoxyphenyl at R^1 and unsubstituted phenyl at R^2 (**26**) slightly improved the inhibitory potency for 5-LO product formation in intact cells (IC_{50} 1.6 ± 1.3 μM) and resulted in a measurable inhibition of 15-LO1. The interchange of both substituents at both phenyl rings resulting in 4-hydroxyphenyl at R^1 and 4-chlorophenyl (**27**) or 4-fluorophenyl (**28**) at R^2 led to twenty-fold worsening of inhibition of 5-LO product formation compared to **1** (IC_{50} 3.1 ± 1.6 μM and 3.5 ± 1.4 μM). The methyl ketone group in position 4 at R^2 (**29**) led to an improved IC_{50} value of 4.9 ± 2.6 μM in intact cells compared to the 3-methyl ketone group (**17**). The replacement of chlorine (**17**) with fluorine (**29**) does not seem to have an effect regarding compounds **1** and **3** that exhibit almost equal inhibitory activities. Compounds **26**–**28** slightly inhibited 15-

LO1 product formation, but compound **28** almost completely blocked 12-LO product formation at a concentration of 50 μM, down to 4.2 ± 1.7% residual activity. This fluorine substituted derivative (**28**) is the only one with marked 12-LO inhibition. Interestingly, its chlorine analogue (**27**) did not show any inhibition of 12-LO at all. Compound **30**, although active in the cell-free 5-LO enzyme assay (Supplementary Data), showed no (PMNL, 12-LO, COX-1) or only weak (15-LO1, COX-2) inhibition of the respective product formation. It is possible that the sulfonyl-morpholine moiety hinders cell-membrane passage, as suggested above for the second morpholine derivative, **25**.

Looking at the cyclooxygenases, compounds **1**–**9** and **12** provoked slight inhibition of the COX-1 pathway at 50 μM, resulting in residual activities between 16% and about 28%. In contrast, all these substances showed prominent inhibition of the COX-2 pathway at 50 μM: The concentration of COX-2 mediated PGE_2 was decreased to about 5% (**1**–**9** and **12**) compared to control. Compound **13**, **16** and **29** still inhibit COX-2 more potent than COX-1 but showed a weaker inhibition overall. However, compounds **10**, **11**, **14** as well as **23**, **24**, **26**–**28** showed potent inhibitory activities on both COX-1 and -2 product formation with residual activities under 10% at 50 μM. The derivatives with bulky halogen groups at R^2 (**18**–**22**) yielded prominent inhibition of COX-1 product formation with remaining activities under 15%, but showed only weak activity on COX-2 product formation. Only the 4-trifluoromethylether (**20**) restored the inhibitory activity on COX-2 product formation to 17.8 ± 1.0% residual activity. The inhibitory potency for both COX pathways is negatively affected by the 3-methyl-ketone group (**17**) at R^2 which led to a less confined product formation.

2.3. Cell viability

To gain knowledge if these compounds influence cell viability and might have cytotoxic effects, we screened selected compounds (Table 2) in an LDH (Lactate dehydrogenase) assay with U937 cells. With this assay, the leakage of LDH from cells after treatment with test compounds, which points to a loss of cell membrane integrity, is measured. U937 cells were treated with test compounds up to a concentration of 30 μM for 48 h. Rev-5901 (100 μM) was used as a cytotoxic control [49]. For comparison, the only marketed 5-LO inhibitor zileuton (100 μM) was also analyzed, exhibiting a non-cytotoxic profile (Table 2).

Compound **1** and **5** showed high LDH leakage at 30 μM, 17.5 ± 1.9% and 14.0 ± 6.3%, respectively. In contrast, compound **7** only slightly impaired cell viability with an LDH leakage of 5.5 ± 2.2%. Similarly, compounds **9**, **12**, **24** and **27** showed no effect on cell viability with a maximal LDH leakage of 1.6 ± 0.9%, which is comparable to zileuton (1.5 ± 0.6%). Instead, the trifluoromethyl substituted derivatives **21** and **22** led to a critical influence on cell viability, almost similar to the cytotoxic control compound Rev-5901, with an LDH leakage of 18.2 ± 2.6% and 21.7 ± 1.2%, respectively. Compound **9** remains, with its promising inhibitory profile (dual 5-LO/COX-2 inhibition (Fig. 1)) and the missing cytotoxic effects (Table 2), one of the most interesting compounds within this series.

Table 2
Cytotoxicity determination of test compounds. Cytotoxicity was measured in an LDH assay; $n = 3$, mean ± SEM.

	LDH release[c] [%]
1	17.5 ± 1.9
5	14.0 ± 6.3
7[a]	5.5 ± 2.2
9[b]	0.9 ± 1.5
12	1.6 ± 0.9
21	18.2 ± 2.6
22	21.7 ± 1.2
24	n.d.
27	1.4 ± 1.3
Rev-5901	28.3 ± 3.2
Zileuton	1.5 ± 0.6

Rev-5901 and Zileuton at 100 μM; nd: not detectable.
[a] Compound **ST-1355**.
[b] Compound **ST-1705**.
[c] Measured at 30 μM.

3. Discussion

We analyzed the SAR of a series of newly synthesized *N*,4-diaryl-1,3-thiazole-2-amines with regard to their selectivity profile towards several key enzymes of the arachidonic acid cascade. It turned out that the 4-hydroxyphenyl at R^2 is essential for inhibition of 5-LO product formation. Changing this group always resulted in higher IC_{50} values. Together with the R^2 morpholine derivative **25**, at least in the cell-free assay, the hydroxyl derivatives are the most potent representatives of this series with regard to 5-LO inhibition. Different substituents at R^1 were tolerated when R^2 is substituted with 4- or 2-hydroxyphenyl regarding 5-LO product formation. The 4-chlorophenyl at position R^1 retained 5-LO selectivity over the other lipoxygenases. Remarkably, almost all compounds showed selectivity for just one lipoxygenase, 5-LO, while two out of the 30 compounds prominently inhibited 12-LO (**28**) and 15-LO1 (**7**) product formation, respectively.

21 of the 30 compounds of this set are able to completely inhibit the COX-2 pathway in terms of PGE_2 formation, yielding remaining enzyme activities below 10%. With compound **1**–**9** and **29** we were able to identify several selective dual inhibitors of 5-LO and COX-2 (Scheme 3). Among them, compound **9** constitutes a selective dual 5-LO and COX-2 pathway inhibitor with potent inhibition of 5-LO at a concentration of less than 1 μM and almost complete inhibition of COX-2 at 10 μM (Fig. 1) without having cytotoxic properties. The derivatives of this subset show weak inhibitory activity on COX-1 product formation. Dual inhibitors of 5-LO and COX-2 product formation combined with weak inhibition of the COX-1 pathway may have different therapeutic options and advantages: On the one hand, the inhibition of both enzymatic pathways will avoid the observed shift of AA to one and the other enzyme, resulting in a prominent inhibition of inflammatory mediators. On the other hand, both enzymes are observed to be up-regulated in different tumor tissues and concerted inhibition seems to have promising anti-tumor properties [50–52]. The prominent COX-2 inhibition will avoid drawbacks of the classical NSAIDs like gastrointestinal complications whereas the weak COX-1 inhibition may balance the disadvantages of selective COX-2 inhibitors regarding cardiovascular diseases and platelet aggregation [53,54].

The product formation of both COX isoenzymes is prominently inhibited by compound **10**, **11**, **14**, **23**, **24** and **26**–**28** (Scheme 4). All these compounds showed a weaker 5-LO inhibitory activity compared to the first set. Dichloro- or trifluoromethyl-substituents at the R^2 phenyl (**18**–**22**) always resulted in stronger inhibition of COX-1 than COX-2 (Table 1). These compounds may be useful for the treatment of pain, inflammation and cardiovascular diseases where an anti-platelet-aggregation effect is desired [55,56]. They might act comparable to low-dose acetylsalicylic acid, which is the most commonly used anti-platelet drug for secondary prevention in patients with coronary artery disease and cerebrovascular events. Selective COX-1 inhibitors at low dose react pre-systemically with COX-1 in thrombocytes and do not reach systemic, 'house-keeping' COX-1. Furthermore, combining the inhibition of both the 5-LO and the COX pathway may result in smaller therapeutic doses, which should be a benefit compared to classical

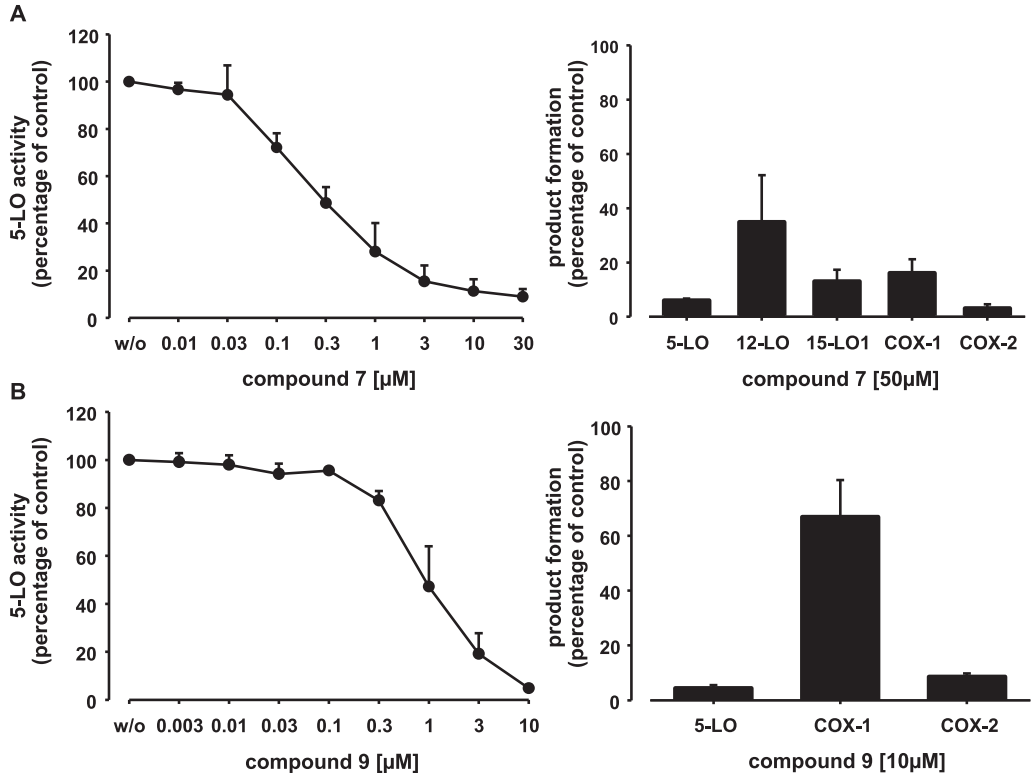
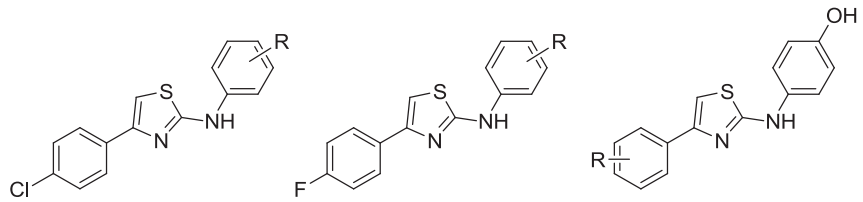
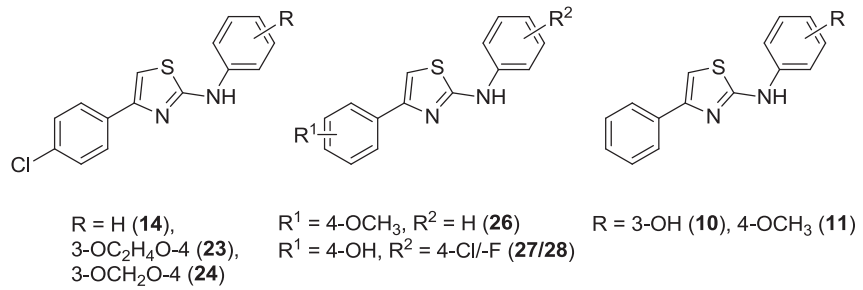


Fig. 1. Inhibitory activity and selectivity profile of compound **7** (**ST-1355**, **A**) and **9** (**ST-1705**, **B**). Dose–response curve for inhibition of 5-LO activity by test compounds, determined with 5×10^6 PMNL in 1 ml PBS/Glucose containing 1 mM $CaCl_2$. 5-LO activity was stimulated with 2.5 μM calcium ionophore A23187 and 20 μM AA, after 10 min at 37C° the reaction was stopped and 5-LO product formation (as percentage of vehicle control) was measured via HPLC. Inhibition of 15-LO1 activity was measured concomitantly. 12-LO and COX-1 activity was assessed using 10^8 human platelets resuspended in 1 ml PBS/Glucose containing 1 mM $CaCl_2$ stimulated with 10 μM AA for 10 min at 37 °C. Product formation was analyzed via HPLC. COX-2 activity was measured in 3×10^4 HeLa cells seeded in a 24-well plate and stimulated with 1 ng/ml IL1β and 5 ng/ml TNFα for 24 h. Cells were washed three times with 1 ml PBS containing 1 mg/ml BSA, incubated for 30 min at 37 °C with 500 μl medium containing 1 ng/ml IL1β, 5 ng/ml TNFα and 10 μM AA and COX-2 product formation was analyzed by PGE_2 ELISA. Compound **9** showed no significant inhibition of 12-LO and 15-LO1 activity.



Scheme 3. Dual 5-LO/COX-2 inhibitors.



Scheme 4. COX-1/-2 inhibitors.

NSAIDs. Dual or multi-target ligands might have lower side effects as they can be used at lower doses due to the fact that partial inhibition of the individual targets can be sufficient for the pharmacological effects but will still allow enough residual activity for maintenance of physiological functions of the individual pathways [57]. For sure, the two of the compounds (**21** and **22**) that possess strong cytotoxicity first have to be optimized prior further evaluation as anti-inflammatory agents.

Recently, one starting derivative of this series, compound **1**, was also reported to inhibit the activity of sphingosine kinases 1 and 2 [58,59]. However, none of the compounds under investigation in our study was able to directly inhibit any of the isoenzymes significantly in a purified enzyme assay (data not shown). This is in line with the observation that compound **1** can activate the intracellular degradation of sphingosine kinase, which in turn leads to reduced production of sphingosine-1-phosphate [60,61] and therewith to cellular inhibitory activity.

With compound **7** (**ST-1355**) we found a non-cytotoxic ligand targeting all tested enzymes of the arachidonic acid cascade, albeit inhibition of 12-LO is weaker than for the other enzymes (Fig. 1). With respect to the so called lipid-crosstalk and substrate shift between AA metabolizing enzymes [33,42,43], this compound represents an ideal candidate for further development of a multi-target inhibitor for the lipid signaling cascade.

4. Conclusion

With this work we gave a broad insight into the inhibitory activity and selectivity profile of a class of *N*-4-diaryl-1,3-thiazole-2-amines as inhibitors of different enzymes linked to eicosanoid biosynthesis. We systematically synthesized different substitution patterns to reveal SARs of this compound class. Most of the compounds were, amongst the lipoxygenases, selective for 5-LO, only the 4-hydroxyphenyl (R^1) and 4-fluorophenyl (R^2) derivative (**28**) does potently inhibit 12-LO product formation. The derivative lacking any substitution at R^1, but bearing a 4-hydroxy group at R^2 (**7**, **ST-1355**) targets all tested enzymes (Fig. 1). This promising profile of a multi-target ligand with no influence on cell viability represents a new lead structure for the treatment of inflammation and contributes to the understanding of the cross-talk within the arachidonic acid cascade. In total, we identified 20 novel dual 5-LO/COX inhibitors, most of them having a preference for COX-2 inhibition over COX-1. Additionally, with the 4-chloro- and 2-hydroxy-substituted compound **9** (**ST-1705**), we were able to identify a potent and selective dual 5-LO and COX-2 inhibitor with an IC_{50} value of 0.9 ± 0.2 μM for inhibition of cellular 5-LO and a residual activity of 9.1 ± 1.1% at 10 μM for COX-2 product formation (Fig. 1). The additional weak inhibitory activity of the COX-1 pathway (residual activity of 67.4 ± 13.5% at 10 μM) may balance the disadvantages of classical NSAIDs and selective COX-2 inhibitors. This high effectiveness for both pathways and the non-cytotoxic effects privileges this compound for further studies. Future experiments may address the availability of these compounds in human whole blood and in inflammatory tissues to affirm the therapeutic potential for inflammatory diseases and anti-cancer therapy aiming at a safer treatment strategy for eicosanoid-mediated diseases.

5. Materials & methods

5.1. Compounds and chemistry

Compounds **2**, **10**–**13**, **16**–**18**, **26**, **29** and **30** were purchased from SPECS (Delft, The Netherlands) or Chembridge (San Diego, USA) and exhibit ≥ 95% purity determined by the supplier using ^{1}H NMR and LC-MS. Compounds **1**, **3**–**9**, **14**, **15**, **19**–**25**, **27** and **28** were synthesized according to Rasmussen et al. [48] (by now, compound **1** is commercially available). Melting points for synthesized compounds were determined on a Buchi 510 melting point instrument (Buchi, Switzerland) and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker DPX 250 (250 MHz) spectrometer (Bruker, Germany). ^{1}H NMR data are reported in the following order: chemical shift (δ) in ppm downfield from tetramethylsilane as internal reference; multiplicity (br, broad; s, singlet; d, doublet; dd, double doublet; t, triplet; pt, pseudo triplet; m, multiplet); approximate coupling constant (J) in hertz (Hz); number and assignment of protons. ^{13}C NMR spectra were recorded on a Bruker DPX 250 (63 MHz) spectrometer (Bruker, Germany). ESI-MS was performed on a VG Platform II (Fisons Instruments, UK) in positive or negative polarity. Data are listed as mass number ($[M+H]^+$ or $[M-H]^-$). Elemental analysis results (C, H, N, S) were measured on a MicroCube (Elementar, Germany) and were within ± 0.4% of the theoretical values for all final compounds, which corresponds to ≥ 95% purity. Educts and all other reactants were commercially obtained from Sigma–Aldrich (Germany), ABCR (Germany), Alfa Aesar (Germany), Acros Organics (Germany) and Fluorochem (UK) and were used without further purification unless otherwise stated. Analytical TLC was performed with TLC plates (F254, Merck) and detection using a UV lamp and ninhydrine staining reagent.

5.1.1. General procedure for the preparation of compounds *1*, *3*–*9*, *14*, *15*, *19*–*25*, *27* and *28*

α-Bromoketones were synthesized via bromination (1 equiv. bromine) of acetophenone derivatives (1 equiv.) in chloroform at ambient temperature [62]. *N*-Arylthioureas were commercially available or prepared by the reaction of benzoyl chloride (1 equiv.) with aniline derivatives (1 equiv.) in the presence of NH_4SCN (1.1 equiv.) in acetone and debenzoylation of the resultant *N*-aryl-*N*′-benzoylthiorureas with aqueous sodium hydroxide (2 M) [48]. Microwave-assisted Hantzsch thiazole synthesis, a cyclic condensation of α-bromoketones (**B**) and *N*-arylthioureas (**E**) in ethanol, provided the desired *N*,4-diaryl-1,3-thiazole-2-amines (**F**). The classical reaction conditions (reflux, 3 h) of Hantzsch thiazole synthesis [47] could be improved regarding the reaction time (30 min).

Below we describe exemplarily the synthesis of compound **24** and its precursors.

5.1.1.1. 2-Bromo-1-(4-chlorophenyl)-ethanone (category B, Scheme 2). To a solution of 8.40 ml (64.70 mmol) 1-(4-chlorophenyl)-ethanone (**A**, Scheme 2) in 40 ml chloroform, 3.33 ml bromine (64.7 mmol) in 10 ml chloroform were added drop wise at 0 °C. The mixture was stirred at room temperature for 2 h and was washed with H_2O (3 × 50 ml) and saturated $Na_2S_2O_3$ solution (2 × 50 ml). The organic phase was dried over Na_2SO_4, filtered and the solvent was removed in vacuum. The crude product, 2-bromo-1-(4-chlorophenyl)ethanone (**B**, Scheme 2), was recrystallized from petrolether and gave 14.20 g of a white crystalline solid, yield: 92%. ^{1}H NMR (250 MHz, $CDCl_3$) δ = 7.94 (d, J = 8.7 Hz, 2H, 3*H*, 5*H*-Ph), 7.48 (d, J = 8.7 Hz, 2H, 2*H*, 6*H*-Ph), 4.41 (s, 2H, $-CH_2-$); m/z = 233.5 $[M+H]^+$ [62].

5.1.1.2. N-(Benzo[d][1,3]dioxol-5-ylcarbamothioyl)benzamide (category D, Scheme 2). Benzoyl chloride (1.75 ml, 15.20 mmol) was added over 5 min to a freshly prepared solution of 1.27 g (16.68 mmol) NH_4SCN in 20 ml acetone and the mixture was heated to reflux for 15 min. The *in situ* generated benzoyl isothiocyanate reacted with 2.08 g (15.20 mmol) added benzo[*d*][1,3]dioxol-5-amine (**C**, Scheme 2) in 20 ml acetone. The mixture was heated to reflux for 30 min, and then poured on ice with vigorous stirring. The

resulting solid, *N*-(benzo[*d*][1,3]dioxol-5-ylcarbamothioyl)benzamide (**D**, Scheme 2), was collected and washed with H_2O, followed by cold acetone, yield: 3.64 g (80%); ^{1}H NMR (250 MHz, DMSO-d_6) δ = 12.64 (s, 1H, -CS-N*H*-Ph-OH), 11.44 (s, 1H, −CO−N*H*−), 8.03 (d, *J* = 7.7 Hz, 2H, 2*H*,6*H*-Ph), 7.70−7.63 (m, 3H, 3*H*,4*H*,5*H*-Ph), 6.63 (d, 1H, *J* = 8.2 Hz, 7*H*-benzo[*d*][1,3]dioxol), 6.07 (s, 2H, -C*H*$_2$-), 6.03(s, 1H, 6*H*-benzo[*d*][1,3]dioxol), 5.89 (d, *J* = 8.4 Hz, 1H, 6*H*-benzo[*d*][1,3]dioxol); *m/z* = 301.1 $[M+H]^+$ [48].

5.1.1.3. 1-(Benzo[d][1,3]dioxol-5-yl)thiourea (category E, Scheme 2). A solution of 1.00 g (3.33 mmol) *N*-(benzo[*d*][1,3]dioxol-5-ylcarbamothioyl)benzamide (**D**, Scheme 2) in 5 ml aqueous sodium hydroxide (2 M) was heated to 100 °C for 1 h. The precipitating solid, 1-(benzo[*d*][1,3]dioxol-5-yl)thiourea (E, Scheme 2), was collected and washed with H_2O, yield: 529 mg (81%). ^{1}H NMR (250 MHz, DMSO-d_6) δ = 9.51 (s, 1H, -N*H*-), 7.32 (br s, 2H, -N*H*$_2$), 6.99 (s, 1H, 4*H*-benzo[*d*][1,3]dioxol), 6.86 (d, 1H, *J* = 8.2 Hz, 7*H*-benzo[*d*][1,3]dioxol), 6.68 (d, 1H, 6*H*-benzo[*d*][1,3]dioxol), 6.06 (s, 2H, -C*H*$_2$-); *m/z* = 197.2 $[M+H]^+$ [48].

*5.1.1.4. N-(Benzo[d][1,3]dioxol-5-yl)-4-(4-chlorophenyl)thiazol-2-amine hydrobromide (category F, Scheme 2) − Compound **24**.* A solution of 150 mg (0.64 mmol) 2-bromo-1-(4-chlorophenyl)-ethanone (**B**, Scheme 2) and 126 mg (0.64 mmol) 1-(benzo[*d*][1,3]dioxol-5-yl)thiourea (**E**, Scheme 2) in 2 ml ethanol was heated to 80 °C for 30 min under microwave irradiation. The precipitated white hydrobromide salt was collected, washed with H_2O and cold ethanol, yield: 182 mg (69%). ^{1}H NMR (250 MHz, DMSO-d_6) δ = 10.17 (s, 1H, −N*H*−), 7.90 (d, *J* = 8.5, 2H, 2*H*, 6*H*-Ph-Cl), 7.50−7.46 (m, 3H, 3*H*,5*H*-Ph-Cl + 5*H*-thiazole), 7.36 (s, 1H, 4*H*-benzo[*d*][1,3]dioxol), 7.05 (dd, 1H, ^4J = 2.3 Hz, ^3J = 8.5 Hz, 6*H*-benzo[*d*][1,3]dioxol), 6.89 (d, 1H, *J* = 8.4 Hz, 7*H*-benzo[*d*][1,3]dioxol), 5.99 (s, 2H, −C*H*$_2$−); ^{13}C NMR (63 MHz, DMSO-d_6) δ = 163.6, 148.6, 147.3, 141.6, 135.8, 133.3, 131.9, 128.6, 127.2, 109.6, 108.3, 103.3, 100.8, 99.5; *m/z* = 331.2 $[M+H]^+$; anal. calcd. for $C_{16}H_{11}ClN_2O_2S\cdot HBr$: C (46.68) H (2.94) N (6.80) S (7.79); found: C (46.63) H (3.02) N (6.87) S (7.75); mp = 251.0 °C [48].

5.2. Biological assays

5.2.1. Materials

AA, calcium ionophore A23187, BWA4C, DMSO, Albumin from bovine serum (fatty acid free), IL1β and trypan blue solution were purchased from Sigma−Aldrich (Munich, Germany). Rev-5901 and zileuton were purchased from Cayman Chemical (Ann Arbor, USA). HPLC solvents were purchased from Merck (Darmstadt, Germany). RPMI 1660 and DMEM medium was purchased from Gibco/Invitrogen (Paisley, UK). Penicillin and streptomycin were purchased from PAA laboratory GmbH (Pasching, Austria). Fetal calf serum (FCS) was purchased from Biochrom AG (Berlin, Germany). Fresh blood cell concentrates were provided by Städtische Kliniken Frankfurt-Höchst and Deutscher Blutspendedienst (Frankfurt, Germany).

5.2.2. Cell culture

The human leukemic monocyte cells U937 and human cervix carcinoma cells HeLa were purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). U973 cells were maintained in RPMI 1660 and HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium). Complete culture media contained 10% FCS, 100 μg/mL streptomycin and 100 U/mL penicillin. Cells were cultured at 37 °C in an atmosphere containing 5% CO_2.

5.2.3. Cell preparation

Human PMNL were freshly isolated from leukocyte concentrates obtained at Städtische Kliniken Frankfurt Höchst (Frankfurt, Germany). Leukocyte concentrates were prepared by centrifugation at 4000 × *g* for 20 min at RT. PMNLs were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described [63]. Cells were finally re-suspended in phosphate-buffered saline, pH 7.4 (PBS) containing 1 mg/mL glucose (purity of > 96−97%). Human Platelets were freshly isolated from platelet concentrates obtained at the Deutsche Blutspendedienst (Frankfurt, Germany). Platelets were re-suspended in PBS, pH 5.4 and centrifuged at 1849 × *g* for 15 min at room temperature (RT). The cells were re-suspended in PBS/NaCl (PBS, pH 5.4 and 0.9% NaCl, 1:1 dilution) and centrifuged at 1849 × *g* for 10 min at RT. Finally, platelets were re-suspended in PBS pH 5.4.

5.2.4. Determination of 5-LO product formation in intact cells

For whole-cell assay freshly isolated PMNL (5×10^6) were re-suspended in 1 mL PBS, pH 7.4, containing 1 mg/mL glucose and 1 mM $CaCl_2$ (PGC). After pre-incubation with the test compounds for 15 min at 37 °C, 5-LO product formation was stimulated by the addition of calcium ionophore A23187 (2.5 μM) and exogenous AA (20 μM). After 10 min at 37 °C, the reaction was stopped with the addition of methanol (1 mL) [64]. HCl (30 μL, 1 N), prostaglandin B_1 (200 ng) and PBS (500 μL) were added and 5-LO metabolites were extracted and analyzed by HPLC as described [65]. 5-LO product formation was determined as nanograms of 5-LO products per 10^6 cells, which includes leukotriene B_4 (LTB_4) and its all-*trans* isomers, and 5-H(P)ETE (5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid). Cysteinyl LTs C_4, D_4 and E_4 were not detected, and oxidation products of LTB_4 were not determined. Each compound was tested at least three times, and the mean ± S.E. were calculated. The direct 5-LO inhibitor BWA4C was used as control at a concentration of 0.08 μM resulting in an inhibition comparable to the literature.

5.2.5. Determination of 12-LO, COX-1 and 15-LO1 product formation in intact cells

Determination of 15-LO1 product formation was performed in PMNL preparations as described for 5-LO product formation in intact cells. For determination of 12-LO and COX-1 product formation 1×10^8 freshly isolated platelets were re-suspended in 1 ml of PGC buffer and were pre-incubated with the test compounds or vehicle (DMSO) at the indicated concentrations for 15 min at 37 °C. 12-LO and COX-1 product formation was stimulated by addition of 10 μM AA. After 10 min at 37 °C, the reaction was stopped with 1 ml of ice-cold methanol. 12-LO products include 12(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (12-H(p)ETE) and elute as one major peak. COX-1 product formation was measured as 12-hxdroxy-hepta-decatrienoic acid (12-HHT). 15-LO1 products, generated from 15-LO1 expressing eosinophils present in the PMNL preparations, were 15(*S*)-hydro(pero)xy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid, which elute as one major peak as well. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control (DMSO). For 12- and 15-LO1, the known lipoxygenase inhibitor NDGA (nordihydroguaiaretic acid, 100 μM) was used as control; for inhibition of COX-1 product formation acetylsalicylic acid (100 μM) was used.

5.2.6. Determination of COX-2 derived PGE_2 in supernatants of HeLa cells

HeLa cells were seeded in 24-well plates at a density of 3×10^4 cells/well. After 24 h incubation COX-2 expression was stimulated with culture media containing 1 ng/ml IL1β and 5 ng/ml

TNFα. After 24 h cells were washed three times for 5 min with 1 ml PBS containing 1 mg/ml albumin from bovine serum (fatty acid free) to remove accumulated PGE_2. Cells were incubated with culture media containing 1 ng/ml IL1β, 5 ng/ml TNFα and test compounds or vehicle (DMSO) at the indicated concentrations. After 30 min the reaction was stopped on ice and supernatants were taken and PGE_2 concentration was measured with PGE_2 ELISA kit (Enzo Life Science GmbH, Lörrach, Germany) according to the distributor's protocol using a microplate reader (Infinite M200, Tecan, Crailsheim, Germany). Data are expressed as percentage of control (DMSO). All experiments were done three times and mean ± S.E. were calculated. The known selective COX-2 inhibitor rofecoxib (1 μM) was used as control.

5.2.7. Expression and purification of 5-LO protein

Escherichia coli BL21 were transformed with plasmid pT3-5-LO. The recombinant 5-LO protein itself was expressed at 22 °C and purified from 1-L culture according to the ATP affinity chromatography procedure as described previously [66,67] the 5-LO protein was further purified via anion exchange chromatography as described previously [65]. In brief, the ATP-eluate (10 ml) was loaded on a ResourceQ 6 ml column (GE Healthcare, Uppsala, Sweden). Buffer A was phosphate buffer (PB) 0.05 mM, pH 7.4 containing 1 mM EDTA, buffer B was buffer A plus 0.5 M NaCl. Elution of 5-LO was performed in a gradient from 0 to 100% buffer B and the enzyme eluted at about 40% buffer B [68].

5.2.8. Determination of 5-LO product formation in a cell-free system

Partially purified 5-LO (3 μg) was set in 1 ml reaction mix (PBS, 1 mM EDTA, 1 mM ATP). After pre-incubation with the test compounds or vehicle (DMSO) at the indicated concentrations for 15 min at 4 °C the samples were pre-warmed for 30 s at 37 °C. The reaction was started after addition of 2 mM $CaCl_2$ and 20 μM AA. After 10 min at 37 °C 5-LO product formation was stopped with 1 ml ice-cold methanol and the formed metabolites were analyzed by HPLC as described for intact cells. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control (DMSO).

5.2.9. Lactate dehydrogenase (LDH) cytotoxicity assay

The LDH assay (cytotoxicity detection 1 kit; Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) was used to determine cell death after treatment of U937 cells with test compounds. LDH leakage was measured as an index of loss of cell membrane integrity. U937 cells were seeded in 96-well plates at a density of 1.5×10^4 cells/well and incubated with test compounds or vehicle (DMSO) for 48 h. Plates were centrifuged ($250 \times g$, 4 min) and an aliquot of the supernatant was transferred to a clean microplate. Cell toxicity was assessed according to the distributor's protocol using a microplate reader (Infinite M200, Tecan, Crailsheim, Germany). A control detergent supplied by Sigma–Aldrich (Saint Louis, Mo, USA) was used for maximum LDH release and set to 100%. All experiments were done three times and mean ± S.E. were calculated.

5.2.10. Statistics

For calculation of IC_{50} values, five to eight increasing concentrations of each compound were measured in 3–5 independent experiments. Measurements from each drug concentration were normalized to DMSO control condition. IC_{50} values ± S.E. were calculated with KNIME WebPortal (KNIME.com AG, Version 2.1) using the dose–response node of the HCS (High-Content Screening) Tools for IC_{50} determination. The node is based on the "drc" R package to build a four-parametric logistic model fitting the sigmoidal dose response curve [69].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.07.025.

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