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Synthesis and biological evaluation of a class of 5-benzylidene-2-phenyl-thiazolinones as potent 5-lipoxygenase inhibitors

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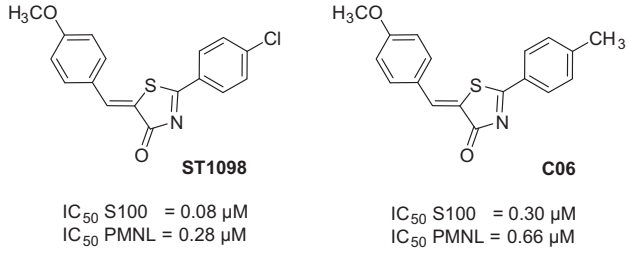
ABSTRACT

A class of 5-lipoxygenase (5-LO) inhibitors characterized by a central 5-benzylidene-2-phenyl-thiazolinone scaffold was synthesized as a new series of molecular modifications and extensions of a previously reported series. Compounds were tested in a cell-based and a cell-free assay and furthermore evaluated for their influence on cell viability. The presented substituted thiazolinone scaffold turned out to be essential for both the 5-LO inhibitory activity and the non-cytotoxic profile. With (*Z*)-5-(4-methoxybenzylidene)-2-(naphthalen-2-yl)-5*H*-thiazol-4-one (**2k**, **ST1237**), a potent, direct, non-cytotoxic 5-LO inhibitor with IC_{50} of 0.08 μM and 0.12 μM (cell-free assay and intact cells), we present a promising lead optimization and development for further investigations as novel anti-inflammatory drug.

1. Introduction

Different inflammatory diseases like allergic rhinitis, asthma, osteoporosis, cardiovascular diseases and cancer are linked to leukotrienes (LTs).[1–3] LTs are signalling lipids derived from polyunsaturated fatty acids acting as important regulators in immunity and inflammation.[4] One key enzyme of LT biosynthesis is 5-lipoxygenase (5-LO): after arachidonic acid (AA) is released from the membrane by cytosolic phospholipase A_2 ($cPLA_2$) and transferred via the 5-lipoxygenase-activating-protein (FLAP) to 5-LO, this non-heme iron containing enzyme catalyses the oxygenation of AA to 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE). Afterwards, 5-HPETE can be reduced to the corresponding alcohol 5-HETE or alternatively dehydrated to the unstable epoxide LTA_4.[5–7] LTA_4 is converted by LTA_4 hydrolase to LTB_4, which is a chemotactic, and chemokinetic agent as well as an activator for phagocytes.[8] Furthermore, LTA_4 can be conjugated with reduced glutathione by LTC_4 synthase, resulting in the cysteinyl-containing LTs C_4, D_4 and E_4, which cause bronchoconstriction and vascular permeability.[4]

Current studies showed that the ALOX5 gene is a critical regulator for leukemia stem cells in BCR-ABL-induced chronic myeloid leukemia (CML).[9] This together with the multiple pathophysiological actions of LTs explains the increasing interest to find an active agent for anti-leukotriene therapy. To date, zileuton,[10] which acts by chelating the active site iron of the enzyme, is the only 5-LO inhibitor that entered the market. Given that many 5-LO inhibitory drug candidates lack sufficient selectivity or show mechanism-based side effects[11–14] there is a strong need for novel 5-LO inhibitors with a distinct mode of action.



Scheme 1. Structures and 5-LO inhibitory activities of parent compounds **ST1098** and **C06.**[16]

Abbreviations: 5-LO, 5-lipoxygenase; AA, arachidonic acid; CML, chronic myeloid leukemia; $cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-LO-activating protein; 5-H(P)ETE, 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; LDH, lactate dehydrogenase; LT, leukotriene; PBS, phosphate-buffered saline pH 7.4; PMNL, polymorphonuclear leukocytes; S100, 100,000×*g* supernatant; SAR, structure–activity relationships; TLC, thin layer chromatography; TMS, tetramethylsilane.

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A promising structure series based on a scaffold that was originally identified in a virtual screening campaign[15] depict the thiazolinone-based compounds exemplified by **ST1098** (Scheme 1).[16] Compound **ST1098** is the most potent derivative of a series of thiazolinones we previously prepared and tested to investigate the influence of different substituents on the inhibitory activity of this thiazolinone scaffold.[16] One of the most promising compounds derived from this series, **C06** ((*Z*)-5-(4-methoxybenzylidene)-2-(*p*-tolyl)-5*H*-thiazol-4-one, Scheme 1), was also characterized by molecular biological and pharmacological approaches: it exhibits a selective, direct, allosteric, stimulus and substrate independent, non-cytotoxic 5-LO inhibitory mode of action different to other established 5-LO inhibitors.[17] To extend our knowledge of the structure–activity relationships (SAR) of this class of 5-LO inhibitors, we here present an additional set of synthesized derivatives with a wide range of variations, tested in a cell based and a cell free assay. Furthermore, selected compounds were tested for their influence on cell viability.

2. Results and discussion

2.1. Chemistry

Synthesis of the target compounds **1a–b** and **2a–l** and **3a–j** and **4a–f** is outlined in Scheme 2. The thiazolinones were prepared by a 'one-pot domino reaction' of thioglycolic acid, the corresponding benzonitrile and the corresponding benzaldehyde according to Zayed et al.[18] Multicomponent reactions like these are an excellent source for novel bioactive compounds.[19]

Since this domino reaction can be controlled at different steps it can also be performed in two-step synthesis route as done with the less reactive acetophenone derivatives resulting in the methyl-branched compounds **5a–b**. First thioglycolic acid and the corresponding benzonitrile were refluxed for one hour in ethanol with TEA to yield the thiazolinone core (Scheme 3).[18] The intermediate was then condensed with *p*-methoxy-acetophenone by heating it with ammonium acetate in toluene for 90 min at 130 °C under microwave irradiation (Scheme 3).[20]

2.2. Biological evaluation

2.2.1. 5-LO activity assay

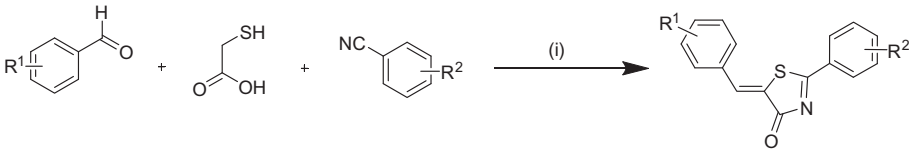
All compounds were evaluated in two different assay systems on 5-LO activity: (i) a cell-based test system using isolated human polymorphonuclear leukocytes (PMNL), and (ii) utilizing the cell-free supernatant of a 100,000×*g* ultracentrifuge run of homogenized PMNL, the S100 assay. The PMNL assay allows assessing intracellular bioavailability of the compounds. The cell-free assay (S100) was chosen to distinguish between direct inhibition of 5-LO (comparable inhibition in both assays) and indirect effects involving for example, FLAP or $cPLA_2$ interaction (lower inhibitory activity in the cell-free assay). The 5-LO inhibitor BWA4C,[21] another well-established iron-ligand type 5-LO inhibitor, was taken as reference compound showing IC_{50} of 0.05 ± 0.005 μM in PMNL S100 and IC_{50} of 0.14 ± 0.06 μM in intact PMNL (Table 1), which is similar to that in literature.[21]

To get further insight into the SAR of this class of 5-LO inhibitors, we developed compounds **1a–b**, **2a–l**, **3a–j**, and **4a–f** (Table 1), which were all synthesized via the 'one-pot reaction' mentioned above (Scheme 2). Initially, we synthesized compounds **1a–b** with exchanged residues at R^1 and R^2 compared to **C06** and **ST1098**. **1a–b** had a slightly higher inhibitory activity than their partners. The electron donating *p*-methoxy group at R^2 seems to be suitable at this position as well. Next, we changed the substitution pattern at R^2 while keeping the *p*-methoxybenzylidene moiety at R^1 constant (**2a–k**). The position of the chloro-residue at R^2 (**ST1098**, **2d**, **2e**) had only marginal influence on 5-LO inhibition as well as the type of halogen introduced (**ST1098**, **2b**, **2c**). Only the introduction of a fluorine at R^2 (**2c**) led to lesser 5-LO inhibition in the S100 assay, though a comparable potency in intact cells was observed. All of these halogen derivatives (**2b–e**) were able to completely abolish 5-LO product formation in both assays (Table 1). Derivatisation to *p*-propoxyphenyl or introduction of a benzophenone group at R^2 resulted in comparable potencies (**2a**, **2f**), although the *p*-propoxyphenyl derivative was not able to completely abolish 5-LO product formation (still ~20% residual activity left at 10 μM, Table 1) in contrast to the benzophenone-derivative. The disubstituted derivative **2g**, carrying a polar *p*-nitro and *m*-hydroxyl group, showed the lowest inhibitory activity in this set of compounds. The inhibitory activity decreased to IC_{50} values of 4.66 ± 0.74 μM in the S100 assay and to IC_{50} of 28.21 ± 4.86 μM in PMNL. The loss of activity in intact cells might be contributed to hampered membrane permeation of the potentially partially charged compound under test and also physiological conditions.

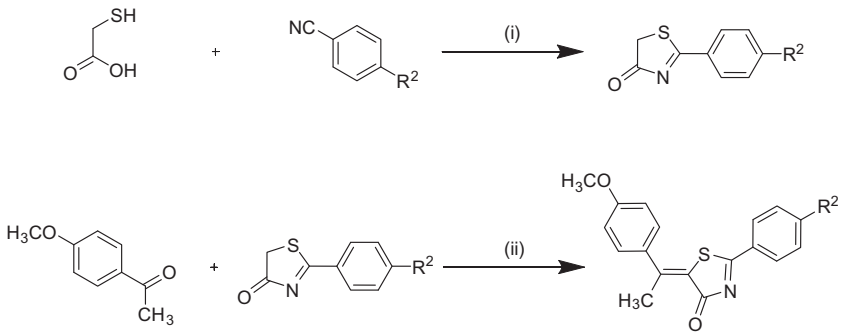
Substitution at the R^2 phenyl in meta position with a bulky adamantly coupled via a carboxamide linker (**2h**) did not alter activity at all (IC_{50} = 0.11 ± 0.02 μM, S100). The adamantyl-free analogue (**2i**) exhibited a five- to ten-fold loss of activity, whereas elongation with a benzyl group (**2j**) even resulted in a 100-fold loss of inhibitory activity in the S100 assay (IC_{50} = 11.05 ± 4.71 μM). Interestingly, inhibition of 5-LO product formation in intact PMNL was not hampered (IC_{50} = 0.26 ± 0.04 μM), suggesting interference with FLAP or $cPLA_2$ rather than 5-LO. Enlargement of the aromatic R^2 phenyl group to naphthalene (**2k**) did not alter activity at all. Further introduction of an electron donating *m*-hydroxyl group at the R^1 phenyl (**2l**) resulted in a six-fold loss of inhibitory potency in PMNL compared to **2k** yet without having any effects on the inhibitory activity in the S100 assay.

In the next set of derivatives the substitution at R^1 was varied while R^2 remained *p*-chloro (**3a–f**). Regardless of which substituent was introduced at position R^1, the inhibitory potency in the S100 assay of the first five derivatives (**3a–e**) did not change. In the PMNL assay, slight variations were observed, but compared to the inhibitory activity of **ST1098**,[16] no prominent effects of the substitutions were detected, except for compound **3e**. This derivative, which carries, together with **3f**, a methoxy naphthalene moiety as R^1, showed a three- to six-fold decreased activity in PMNL. All these halogen derivatives were either able to completely abolish 5-LO product formation in both assays (**3a–c**, Table 1) or resulted in only marginal residual 5-LO activity at 10 μM inhibitor concentration in the S100 assay (**3d–f**).

Next, the combination of a *p*-methoxy substitution at position R^2 and different bicyclic moieties as R^1 (**3h–j**) led to ineffective



Scheme 2. Synthesis of **1a–b**, **2a–l**, **3a–j**, and **4a–f**: (i) TEA, MeOH, reflux, 12 h.[18]



Scheme 3. Two-step reaction of the synthesis of methyl-branched compounds **5a**–**b**: (i) TEA, EtOH, reflux, 1 h,[18] (ii) NH_4OAc, toluene, microwave, 130 °C, 45–90 min.[20]

inhibitors with **3i** being the weakest derivative with an IC_{50} of 5.84 ± 0.62 μM in the S100 assay. Compound **3g** within this set, carrying a flexible *p*-propoxy substitution at R^1, also exhibited lower inhibitory potency with IC_{50} of 2.14 ± 0.84 μM (S100). In this case, the *p*-methoxy substitution at position R^2 seems to be detrimental as the inhibitory potency decreased up to 25-fold (S100) compared to **3a**, with the same R^1 moiety, but a *p*-chloro substitution at R^2. The same pattern was observed for the IC_{50} values in the S100 assays for **ST1098** and **ST1130**[16] and Table 1) and for **3f** and **3h**. These three compound pairs have exchanged the electron withdrawing *p*-chloro substitution at R^2 for an electron donating *p*-methoxy, which resulted in lower inhibitory potencies in the cell-free assay. Compound **3g** is somehow a negative outlier within these three compound pairs, as it also showed loss of activity in the intact cell assay.

One of the last steps on the compound optimization were performed on new combinations of bicyclic or biphenylic residues as R^1 and R^2 (**4a**–**f**) for a potentially increased aromatic enzyme/ligand interaction in combination with improved cell permeation. It resulted in a set of potent inhibitors with IC_{50} between 0.06–0.79 μM (S100). Only **4c**, **4e** and **4f** showed a loss of activity in the PMNL assay possibly due to the lipophilic and steric characteristics of the substitutions that might hamper membrane permeation in contrast to the initial drug design intentions, although the latter does not hold for all derivatives of this subset.

Additionally, two compounds with a methylated double-bond (**5a**–**b**) were synthesized (Scheme 3, Table 1). On the one hand, it has not been clear if the Michael acceptor function is needed for the enzyme inhibition and on the other hand, it is unclear if the restricted steric access at the enzyme can be enlarged by methyl-branching at the aromatic substituted methylene side-chain. Their syntheses followed the fore cited two-step reaction procedure (Scheme 3). In relation to their non-methylated analogues **C06** and **ST1131**,[16] the IC_{50} of **5a** and **5b** got worse (Table 1). Compound **5a**, the methylated derivative of **C06**, showed a seven-fold loss of activity in the S100 assay and a 1.5-fold loss of activity in intact PMNL. This observation was confirmed for the methylated analogue of **ST1131**[16], **5b**, which exhibited an up to 10-fold loss of inhibitory activity (Table 1). The Michael acceptor function is expected to have no effect in the physiological assay conditions, therefore, the additional space-filling methyl moiety seems to disturb the binding of the compound to the 5-LO. This fact highlights that the original core 5-benzylidene-2-phenyl-thiazolinone scaffold with an aromatic substituted methylene moiety is essential for the inhibitory activity of this compound class.

2.2.2. Cytotoxicity measurements

To determine possible negative influence on cell viability by the most potent compounds of this series, we selected a subset of seven derivatives with diverse scaffolds and wide variations of substituents (Table 2) and performed a WST-1 assay with U937 cells. This assay assesses mitochondrial metabolic activity after treatment with increasing concentrations of test compounds for 48 h. Inhibition of cell viability in this assay can be ascribed both to cytotoxic as well as anti-proliferative effects. The iron-ligand inhibitor BWA4C exhibited a non-cytotoxic profile with an IC_{50} >30 μM. Five of the tested compounds (**1a**, **2h**, **2k**, **3c**, **3f**) showed no effect on cell viability (IC_{50} >30 μM) as well. Compound **3a** showed a slight effect with an IC_{50} value of 21.06 ± 0.90 μM. The derivative with the methylated double-bond (**5a**) showed a high influence on cell viability in the WST-1 assay (IC_{50} = 12.10 ± 1.66 μM). To elucidate whether the decreased cell viability resulted from cytotoxicity like necrosis or rather from anti-proliferative effects, loss of cell membrane integrity of U937 cells was additionally measured. Cells were treated with test compounds up to a concentration of 30 μM for 48 h. An increased LDH leakage points to necrotic events. The known nonredox-type 5-LO inhibitor REV-5901[22] was used as cytotoxic control.[23] In this assay, BWA4C induced no LDH leakage at a concentration of 30 μM. Compound **3a** resulted in medium LDH leakage, whereas **5a** caused high LDH leakage. The latter is in contrast to the results for the non-methylated analogue **C06**, which showed no cytotoxic effects at all in both assays. The methyl group seems to be detrimental for cell viability.

3. Conclusions

Summarizing the results mentioned above, we gained further insight into the SAR of this class of thiazolinone-based 5-LO inhibitors. With (*Z*)-5-(4-methoxybenzylidene)-2-(naphthalen-2-yl)-5*H*-thiazol-4-one (**2k**, **ST1237**), we could identify a potent direct 5-LO inhibitor with IC_{50} of 0.08 ± 0.03 μM and 0.12 ± 0.02 μM in the cell-free S100 assay and intact PMNL, respectively. Therewith, it exhibits an about ten-fold higher inhibitory potency than zileuton (IC_{50} = 0.5–1 μM[10]). Furthermore **ST1237** showed no cytotoxic effects on treated cells. In line with the known non-cytotoxic effects of **C06**,[17] several additional compounds tested showed no influence on cell viability and the investigated SAR caused no negative influence on cytotoxicity except for the double-bond methylated derivative **5a**, which showed high cytotoxic effects. This additional methyl group also caused a decreased inhibitory activity on 5-LO product formation. The core 5-benzylidene-2-phenyl-thiazolinone scaffold is essential for the inhibitory activity of this compound class. With **ST1237**, we presented a promising lead optimization and development of a novel pharmacological tool for further investigations as novel anti-inflammatory drug.

4. Experimental section

4.1. Compounds and chemistry

^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AV 250 (^1H: 250 MHz; ^{13}C: 63 MHz), a Bruker AV 300 (^1H: 300 MHz; ^{13}C:

Table 1
Inhibition of 5-LO and 5-LO product formation by synthesized compounds[a]

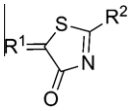
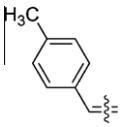
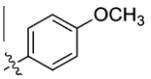
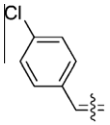
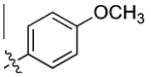
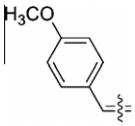
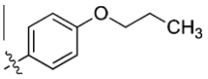
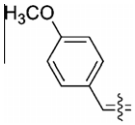
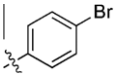
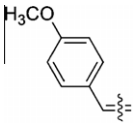
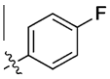
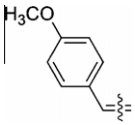
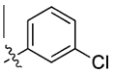
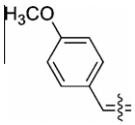
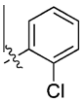
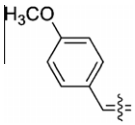
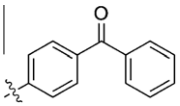
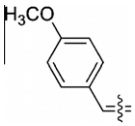
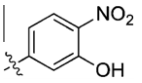
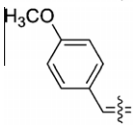
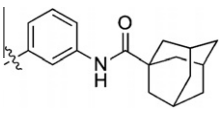
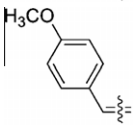
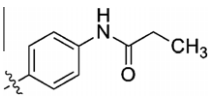
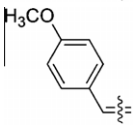
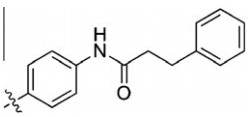
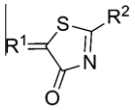
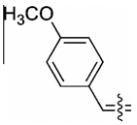
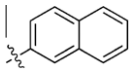
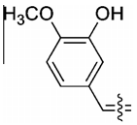
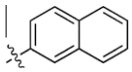
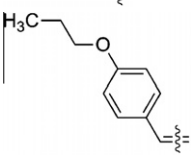
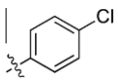
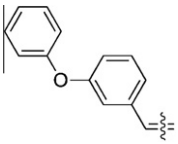
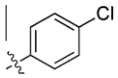
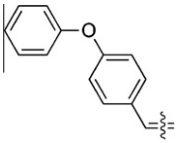
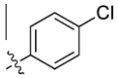
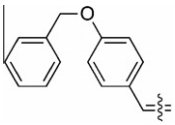
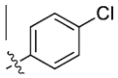
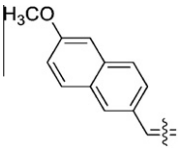
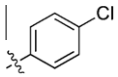
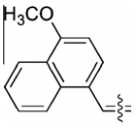
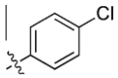
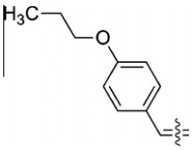
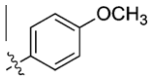
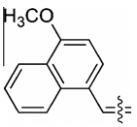
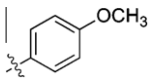
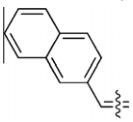
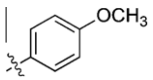
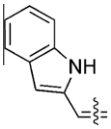
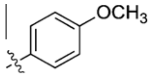
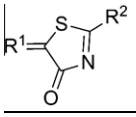
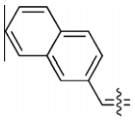
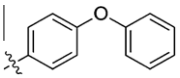
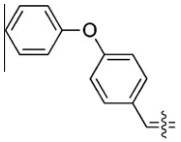
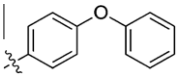
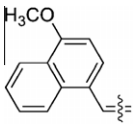
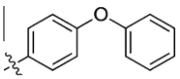
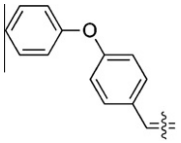
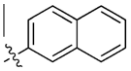
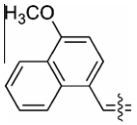
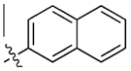
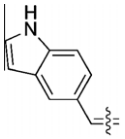
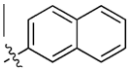
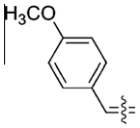
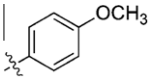
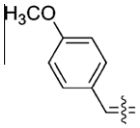
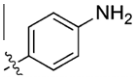
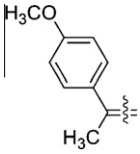
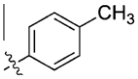
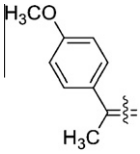
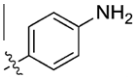
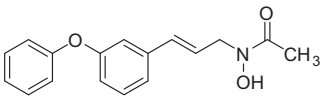
N°			5-LO product formation IC_{50} (μM)		Residual activity at 10 μM (%)	
	R^1	R^2	S100	PMNL	S100	PMNL
1a			0.21 ± 0.02	0.28 ± 0.04	20.9 ± 2.0	–
1b			0.09 ± 0.03	0.24 ± 0.08	30.1 ± 3.9	18.5 ± 3.8[b]
2a			0.21 ± 0.02	0.50 ± 0.13	27.6 ± 3.8	17.0 ± 1.7
2b			0.09 ± 0.01	0.18 ± 0.05	–	–
2c			0.55 ± 0.13	0.20 ± 0.02	21.3 ± 6.3	–
2d			0.10 ± 0.005	0.18 ± 0.04	–	–
2e			0.18 ± 0.06	0.51 ± 0.08	–	–
2f			0.15 ± 0.02	0.44 ± 0.04	–	–
2g			4.66 ± 0.74	28.21 ± 4.86	28.6 ± 7.4	61.8 ± 4.3
2h			0.14 ± 0.02	0.15 ± 0.01	16.2 ± 2.4	–
2i			0.76 ± 0.14	1.84 ± 0.63	20.1 ± 1.3	25.8 ± 5.6
2j			11.05 ± 4.71	0.26 ± 0.04	52.0 ± 7.8	14.8 ± 1.8

Table 1 (*continued*)

N°			5-LO product formation IC_{50} (μM)		Residual activity at 10 μM (%)	
	R^1	R^2	S100	PMNL	S100	PMNL
2k[c]			0.08 ± 0.03	0.12 ± 0.02	14.7 ± 2.8	—
2l			0.08 ± 0.01	0.78 ± 0.18	—	—
3a			0.08 ± 0.01	0.18 ± 0.03	—	—
3b			0.09 ± 0.01	0.32 ± 0.08	—	—
3c			0.08 ± 0.02	0.17 ± 0.03	—	—
3d			0.08 ± 0.003	0.41 ± 0.06	12.3 ± 1.7	26.6 ± 3.1
3e			0.07 ± 0.009	1.24 ± 0.61	15.7 ± 1.3	38.8 ± 2.9
3f			0.13 ± 0.02	0.64 ± 0.10	—	—
3g			2.14 ± 0.84	>30	43.3 ± 1.7	60.8 ± 3.8
3h			4.31 ± 1.24	0.90 ± 0.14	46.8 ± 1.4	28.9 ± 7.8
3i			5.84 ± 0.62	3.55 ± 1.62	38.3 ± 5.0	43.3 ± 3.7
3j			0.57 ± 0.14	0.67 ± 0.12	21.4 ± 1.1	20.5 ± 4.8

(*continued on next page*)

Table 1 (*continued*)

N°			5-LO product formation IC_{50} (μM)		Residual activity at 10 μM (%)	
	R^1	R^2	S100	PMNL	S100	PMNL
4a			0.13 ± 0.04	0.52 ± 0.09	—	26.9 ± 0.9
4b			0.09 ± 0.02	0.65 ± 0.34	14.5 ± 1.5	29.4 ± 2.3
4c			0.14 ± 0.01	2.15 ± 0.28	14.6 ± 2.4	42.3 ± 2.2
4d			0.06 ± 0.003	0.48 ± 0.15	—	23.7 ± 5.2
4e			0.17 ± 0.03	1.99 ± 0.50	15.3 ± 0.5	37.4 ± 2.2
4f			0.79 ± 0.42	5.78 ± 2.12	31.5 ± 3.7	45.8 ± 3.3
ST1130[d]			4.00	0.32	38.8 ± 2.4	21.4 ± 3.4[b]
ST1131[d]			0.63	1.93	37.2 ± 4.2[b]	72.9 ± 3.5[b]
5a			2.08 ± 0.44	1.02 ± 0.08	12.4 ± 2.1	—
5b			6.26 ± 1.39	3.34 ± 0.37	44.2 ± 7.2	14.4 ± 5.1
BWA4C			0.05 ± 0.005	0.14 ± 0.06	-	—

[a] Measured in cell-free S100 and intact PMNL, given as IC_{50} (μM) and residual 5-LO activity [percentage of control] as mean ± SE. '—': residual activity <10%.
[b] Residual activity at 1 μM.
[c] Laboratory code number is **ST1237**.
[d] Recently published.[16]

Table 2
Cytotoxicity determination of selected compounds as IC_{50} for inhibition of cell viability in a WST-1 assay and LDH release as percentage of the cytotoxic control Rev-5901[a]

N°	WST-1 assay IC_{50} (μM)	LDH release at 30 μM (%)
1a	>30	15.8 ± 8.5
2h	>30	8.4 ± 1.6
2k[b]	>30	4.1 ± 2.4
3a	21.06 ± 0.90	48.9 ± 6.5
3c	>30	12.0 ± 4.2
3f	>30	12.2 ± 2.7
5a	12.10 ± 1.66	106.0 ± 11.1
C06	>30[c]	2.7 ± 2.8
BWA4C	>30	0.8 ± 0.8

[a] Given as IC_{50} (μM) and LDH release [percentage of cytotoxic control] as mean ± SE.
[b] Laboratory code number is **ST1237**.
[c] Recently published.[17]

75 MHz), or a Bruker AV 400 (^{1}H: 400 MHz; ^{13}C: 100 MHz) spectrometer (Bruker, Germany). Chemical shifts (δ) are reported in ppm relative to tetramethylsilane (TMS) as internal reference; multiplicity: s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; approximate coupling constants (*J*) are shown in hertz (Hz); assignment: ada, adamantyl; ind, indol; naphth, naphthyl; ph, phenyl; thiaz, thiazol; ph, phenyl, where position 1 is the substituent. Mass spectra were obtained on a VG Platform II (Fisons Instruments, Great Britain) using electrospray ionization (ESI). Data are listed as mass number ([M±H$^+$]) and relative intensity (%). High resolution MS (HR-MS) were achieved on a LTQ Orbitrap XL (Thermo Fisher Scientific, USA). For final compounds the analyses were within ±5 ppm of the theoretical values. Elemental analyses (C, H, N, S) were measured on a Vario MicroCube (Heraeus, Germany) and were within ±0.4% of the theoretical values for all final compounds. Educts and all other reactants were commercially obtained from Sigma–Aldrich, ABCR, Alfa Aesar, and Acros Organics and were used without further purification. Analytical TLC (thin layer chromatography) was performed with TLC plates F_{254} (Merck, Germany) with detection using a UV-lamp. Flash chromatography separations were obtained on an IntelliFlash 310 Flash Chromatography Workstation with Column Station (Agilent, USA). The microwave syntheses were performed on a Biotage Initiator 2.0, 400 Watt (Biotage, Sweden).

4.1.1. General Procedure for preparation of compounds 1a–b, 2a-l, 3a–j, and 4a–f

A solution of the corresponding aromatic aldehyde (0.8–10.3 mmol), 2-sulfanylacetic acid (1.0–1.3 equiv) and the corresponding aromatic nitrile (1.0–1.3 equiv) and triethylamine (1.5–15.0 equiv) in methanol was refluxed over night. The reaction mixture was evaporated under reduced pressure and the crude product was recrystallized from ethanol and washed with acetone.[18] The synthetic procedures are described representatively for **2k** for the one-pot reaction and in details for the two-step synthesis for the methyl-branched derivatives **5a** and **5b**. Detailed synthesis and analytical data of all synthesized compounds are provided in the Supplementary data. For the Knoevenagel condensation, only the *Z*-isomer was obtained.[16]

4.1.1.1. (*Z*)-5-(4-Methoxybenzylidene)-2-(naphthalen-2-yl)-5*H*-thiazol-4-one (2k, ST1237). 0.53 g (3.92 mmol) *p*-methoxybenzaldehyde and 0.36 g (1.0 equiv) 2-sulfanylacetic acid and 0.60 g (1.0 equiv) 2-naphthonitrile were refluxed over night with 20 ml methanol and 2.0 ml (14.2 equiv) triethylamine. The reaction mixture was evaporated under reduced pressure and the pure product was recrystallized from ethanol and washed with acetone. Yield: 39%; orange solid; ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm): 8.65 (s, 1H, naphth-10*H*), 8.12 (dd, *J* = 8.6, 1H, naphth-*2H*), 7.86 (m, 4H, –C*H*═, naphth-*3,5,8H*), 7.53 (m, 4H, H_3CO-ph-*3,5H*, naphth-6,7*H*), 6.94 (d, *J* = 8,8, 2H, CH_3O-ph-2,6*H*), 3.81 (s, 3H, C*H*$_3$O); ^{13}C NMR (63 MHz, $CDCl_3$) δ (ppm): 186.35 (thiaz-4*C*), 183.50 (thiaz-2*C*), 162.15 (CH_3O-ph-1*C*), 138.47 (thiaz-5*C*), 136.67 (naphth-4*C*), 132.87 (CH_3O-ph-3,5*C*), 132.66 (naphth-9*C*), 130.56 (–*C*H═), 129.73 (naphth-1*C*), 129.35 (naphth-3*C*), 129.08 (naphth-5*C*), 128.03 (naphth-8*C*), 127.38 (CH_3O-ph-4*C*), 126.60 (naphth-2*C*), 124.14 (naphth-6,7*C*), 123.79 (naphth-10*C*), 115.00 (CH_3O-ph-2,6*C*), 55.61 (*C*H$_3$O); $C_{21}H_{15}NO_2S$, M_r = 345.4, ESI-MS (*m*/*z*): 346.3 [M+H$^+$]; Anal. calcd: C (73.02%), H (4.38%), N (4.06%), S (9.28%); found: C (73.14%), H (4.43%), N (3.96%), S (9.49%).

4.1.2. Preparation of (*Z*)-5-(1-(4-Methoxyphenyl)ethylidene)-2-(*p*-tolyl)-5*H*-thiazol-4-one (5a)

0.52 g (5.64 mmol) 2-sulfanylacetic acid and 0.66 g (1.0 equiv) *p*-tolunitrile were refluxed 1 h with 20 ml ethanol and 2.0 ml (2.5 equiv) triethylamine. The reaction mixture was evaporated under reduced pressure and the resulting solid was washed with acetone and isopropanol.[18] 75 mg (0.39 mmol) of this intermediate were heated 45 min with 177 mg (3.0 equiv) *p*-methoxyacetophenone and 91 mg (3.0 equiv) ammonium acetate in 3 mL absolute toluene in the microwave at 130 °C.[20] The reaction mixture was evaporated under reduced pressure and the crude product was purified by column chromatography eluting with hexane/ethyl acetate (5:1). Yield: 56%; ocher solid; ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 7.96 (d, *J* = 8.3, 2H, CH_3-ph-3,5*H*), 7.58 (d, *J* = 9.0, 2H, CH_3O-ph-3,5*H*), 7.42 (d, *J* = 8.0, 2H, CH_3-ph-2,6*H*), 7.09 (d, *J* = 8.8, 2H, CH_3O-ph-2,6*H*), 3.83 (s, 3H, C*H*$_3$O), 2.78 (s, 3H, C*H*$_3$); 2.41 (s, 3H, C*H*$_3$-ph); ^{13}C NMR (63 MHz, DMSO-d^6) δ (ppm): 184.88 (thiaz-4*C*), 180.10 (thiaz-2*C*), 160.38 (CH_3O-ph-1*C*), 155.43 (CH_3-ph-1*C*), 145.86 (CH_3-*C*), 135.67 (thiaz-5*C*), 130.04 (CH_3O-ph-3,5*C*), 128.99 (CH_3-ph-3,5*C*), 128.82 (CH_3-ph-4*C*), 127.89 (CH_3-ph-2,6*C*), 126.10 (CH_3O-ph-4*C*), 114.25 (CH_3O-ph-2,6*C*), 55.40 (*C*H$_3$O), 21.35 (*C*H$_3$-ph), 21.01 (*C*H$_3$-C); $C_{19}H_{17}NO_2S \times 0.25\ H_2O$, M_r = 323.4, ESI-MS (*m*/*z*): 324.5 [M+H$^+$]; Anal. calcd: C (69.59%), H (5.38%), N (4.27%), S (9.78%); found: C (69.70%), H (5.41%), N (4.13%), S (9.55%).

4.1.3. Preparation of (*Z*)-2-(4-Aminophenyl)-5-(1-(4-methoxyphenyl)ethylidene)-5*H*-thiazol-4-one (5b)

3.68 g (40.00 mmol) 2-sulfanylacetic acid and 4.73 g (1.0 equiv) *p*-aminobenzonitrile were refluxed one hour with 90 ml ethanol and 9.0 ml (1.6 equiv) triethylamine. The reaction mixture was evaporated under reduced pressure and the resulting solid was washed with acetone and isopropanol.[18] 0.40 g (2.08 mmol) of this intermediate were heated 90 min with 0.24 g (0.8 equiv) *p*-methoxyacetophenone and 0.19 g (1.2 equiv) ammonium acetate in 10.0 mL absolute toluene in the microwave at 130 °C.[20] The resulting precipitate was washed with methanol and purified with

a flash chromatography and dichloromethane/methanol as eluent. Yield: 21%; red-brown solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.95 (d, J = 8.7, 2H, NH_2-ph-3,5*H*), 7.47 (d, J = 8.9, 2H, CH_3O-ph-3,5*H*), 7.00 (d, J = 8.8, 2H, CH_3O-ph-2,6*H*), 6.70 (d, J = 8.7, 2H, NH_2-ph-2,6*H*), 3.92 (s, 3H, CH_3O), 2.88 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 184.67 (thiaz-4*C*), 181.60 (thiaz-2*C*), 160.38 (CH_3O-ph-1*C*), 153.15 (NH_2-ph-1*C*), 152.67 (CH_3-*C*), 136.71 (thiaz-5*C*), 130.87 (NH_2-ph-3,5*C*), 128.84 (CH_3O-ph-3,5*C*), 127.38 (CH_3O-ph-4*C*), 121.86 (NH_2-ph-4*C*), 114.15 (CH_3O-ph-2,6*C*), 114.06 (NH_2-ph-2,6*C*), 55.66 (CH_3O), 21.57 (CH_3-C); $C_{18}H_{16}N_2O_2S$, M_r = 324.4, ESI-MS (m/z): 325.0 [M+H$^+$]; HR-MS calcd: 325.10053; found: 325.10128 (−2.307 ppm).

4.2. 5-LO activity assay

4.2.1. Cell preparation

Human PMNL were freshly isolated from leukocyte concentrates obtained at Städtische Kliniken Frankfurt Höchst (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors with informed consent. Leukocyte concentrates were prepared by centrifugation at 4,000*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.[24] Cells were finally resuspended in phosphate-buffered saline, pH 7.4 (PBS) containing 1 mg/mL glucose (purity of >96–97%).

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

For whole-cell assay freshly isolated PMNL (5×10^6) were resuspended in 1 mL PBS, pH 7.4, containing 1 mg/mL glucose and 1 mM $CaCl_2$. After preincubation 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). Exogenous arachidonic acid was applied to overcome any limitation of supply of endogenous substrate, mediated by $cPLA_2$. After 10 min at 37 °C, the reaction was stopped with the addition of methanol (1 mL). HCl (30 μL, 1 N), prostaglandin B_1 (200 ng) and PBS (500 μL) were added and the 5-LO metabolites were extracted and analyzed by HPLC as described in the literature.[25] 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 ± SE were calculated.

4.2.3. Determination of 5-LO product formation in cell-free systems

For determination of the activity of 5-LO in S100 freshly isolated PMNL cells were resuspended in 1 mL of PBS containing 1 mM EDTA and the protease inhibitors soybean trypsin inhibitor (60 μg/mL), 1 mM phenylmethylsulfonyl fluoride, and leupeptine (10 μg/mL), cooled on ice for 10 min, and sonicated (3×10 s) at 4 °C. The whole homogenate was then centrifuged (100,000*g* for 70 min at 4 °C) to obtain the S100. For determination of 5-LO activity, S100 corresponding to 7.5×10^6 PMNL was added to 1 mL of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, and 1 mM ATP). After preincubation with the test compounds or vehicle (DMSO) for 15 min at 4 °C, the samples were prewarmed for 30 s at 37 °C, and 20 μM AA and 2 mM $CaCl_2$ were added to start the 5-LO reaction. The reaction was stopped after 10 min at 37 °C by addition of 1 mL ice-cold methanol and the formed metabolites were analyzed by HPLC as described for intact cells. Each compound was tested at least three times, and the mean ± SE were calculated.

4.3. Cytotoxicity measurements

4.3.1. Cell culture

The human leukemic monocyte cells U937 were maintained in RPMI 1660 medium containing 10% FCS, 100 μg/mL streptomycin and 100 U/mL penicillin. Cells were cultured at 37 °C in an atmosphere containing 5% CO_2.

4.3.2. In vitro cell viability assay

The WST-1 assay (Roche Diagnostic GmbH, Mannheim, Germany) was used to determine cell viability after treatment with test compounds. U937 cells were seeded in 96-well plates at a density of 10^4 cells/well and treated with increasing concentrations of test compounds for 48 h in presence of 10% FCS. Cell viability was assessed according to the distributor's protocol using a microplate reader (infinite M200, Tecan Group Ltd, Crailsheim, Germany). All experiments were performed at least three times and the mean ± SE were calculated.

4.3.3. 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 increasing concentrations of test compounds or vehicle (DMSO) for 48 h. Plates were centrifuged (250×*g*, 4 min) and an aliquot of the supernatant was transferred to a clean microplate. Cytotoxicity was assessed according to the distributor's protocol using a microplate reader (infinite M200, Tecan Group Ltd, Crailsheim, Germany). A control detergent supplied by Sigma–Aldrich (Saint Louis, Mo, USA) was used for maximum LDH release and set to 100%. Rev-5901[22] (100 μM), a non-redox-type 5-LO inhibitor, was used as a cytotoxic[23] control and was set to 100%. All experiments were tested three times and the mean ± SE were calculated.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.04.003.

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