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Characterization of the molecular mechanism of 5-lipoxygenase inhibition by 2-aminothiazoles

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ABSTRACT

5-Lipoxygenase (5-LO, EC1.13.11.34) has been implicated in the pathogenesis of inflammatory and immune diseases. Recently, aminothiazole comprising inhibitors have been discovered for this valuable target. Yet, the molecular mode of action of this class of substances is only poorly understood. Here, we present the detailed molecular mechanism of action of the compound class and the in vitro pharmacological profile of two lead compounds ST-1853 and ST-1906. Mechanistic studies with recombinant proteins as well as intact cell assays enabled us to define this class as a novel type of 5-LO inhibitors with unique characteristics. The parent compounds herein presented a certain reactivity concerning oxidation and thiol binding: Unsubstituted aminophenols bound covalently to C159 and C418 of human 5-LO. Yet, dimethyl substitution of the aminophenol prevented this reactivity and slowed down phase II metabolism. Both ST-1853 and ST-1906 confirmed their lead likeness by retaining their high potency in physiologically relevant 5-LO activity assays, high metabolic stability, high specificity and non-cytotoxicity.

1. Introduction

Inflammation is a protective response of the microcirculation to harmful stimuli. Yet, excessive inflammation is potentially harmful and a characteristic of many chronic diseases [1]. 5-Lipoxygenase (5-LO, EC1.13.11.34) contributes to the inflammatory reaction by catalyzing the conversion of arachidonic acid (AA) to potent lipid mediators, namely leukotriene (LT)B_4 and LTC_4 [2]. These mediators drive chemotaxis and leukocyte activation, or in case of LTC_4 lead to increased vasopermeability and bronchoconstriction [3]. Possible 5-LO linked indications therefore range from asthma [4] and allergic rhinitis [5], to atherosclerosis [6], Alzheimer's disease [7], leukemia [8,9] and certain types of cancer [10]. In order to widen the therapeutic armamentarium in these diseases many efforts have been made to develop anti-leukotriene drugs, which climaxed in the approval of the direct 5-LO inhibitor Zileuton [11,12] (FDA approval for the therapy of asthma [13]). Unfortunately the use of Zileuton is limited, partly due to hepatotoxic adverse effects [14]. Therefore many novel compounds were designed, resulting in various promising strategies. Given the catalytic cycle of 5-LO, direct 5-LO inhibitors can act via chelating the active site iron (iron ligand inhibitors; e.g. Zileuton) or by keeping the catalytic iron in the inactive ferrous state by reduction (redox-active inhibitors). Further on there are active site directed, non-redox type inhibitors which compete with AA for binding to 5-LO and allosteric inhibitors, which e.g. act at the regulatory C2-like domain (e.g. Hyperforin [15], EP6 [16]). Despite these rational approaches, besides Zileuton, a great number of investigated compounds subsequently failed in preclinical or clinical trials [17]. This was partly due to insufficient in vitro screening. Apart from toxicities, several compounds suffer from a lack of specificity or display only a poor efficacy in physiologically relevant conditions. The potencies of ZM 230487 or L-739,010 for example depend on the phosphorylation status of 5-LO [18]. Thus there is still a pressing need to develop novel well-characterized 5-LO inhibitors.

In a previous study we presented comprehensive structure-activity relationships (SAR) of a class of potent aminothiazole-comprising 5-LO inhibitors [19]. Based on the parent compound

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ST-1083 we developed two lead compounds (ST-1853 and ST-1906) out of this SAR with high 5-LO inhibitory potency in purified enzyme and intact cell assays (IC_{50} = 50 nM in intact human polymorphonuclear leukocytes (PMNL)) (Fig. 1) [19]. Yet, comprehensive evaluation of the lead likeness of the compounds, as well as the detailed mechanism of action of this novel 5-LO inhibitor type is still due. Therefore, we elaborated various in vitro assays to clarify these unmet questions and present a characterization of the 5-LO inhibition by *N*-phenyl-4-aryl-1,3-thiazole-2-amines with focus on the two lead compounds ST-1853 and ST-1906.

2. Materials & methods

2.1. Chemistry

2.1.1. Chemicals and analytical methods

Synthetic procedures and analytical data for compounds ST-1083, ST-1904, ST-1853, and ST-1906 were already described previously [19,20]. All chemicals and solvents were purchased from Sigma-Aldrich (Taufkirchen, Germany), Acros Organics (Geel, Belgium), Alfa Aesar (Karlsruhe, Germany), TCI Europe (Eschborn, Germany) and Apollo Scientific (Bredbury, UK) and used without further purification unless otherwise stated. Analytical thin layer chromatography (TLC) was performed with precoated TLC sheets (ALUGRAM® Xtra SIL G UV_{254}, Macherey-Nagel, Düren, Germany) visualized with ultraviolet light (254 nm). Column chromatography was performed with technical grade solvent mixtures specified in the corresponding experiment with Fluka silica gel 60 (230 – 400 mesh ASTM). ^{1}H-NMR spectra were recorded on a Bruker DPX 250 (250 MHz), AV300 (300 MHz) or AV400 (400 MHz) spectrometer (Bruker, Karlsruhe, Germany). ^{1}H-NMR data are reported in the following order: chemical shift (δ) in ppm downfield relative to tetramethylsilane: internal reference non-deuterated solvent; multiplicity (br, broad; s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; p, pseudo); number of protons; approximate coupling constant (J) in hertz (Hz). ^{13}C-NMR spectra were recorded on a Bruker DPX 250 (63 MHz) or AV300 (75 MHz) spectrometer (Bruker). ESI-MS was performed on a Fisons Instruments VG Platform II (Manchester, UK). MALDI high resolution mass spectra (HRMS) was performed on a LTQ Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany). Compound purity above 95% was ensured by the use of liquid chromatography-mass spectrometry (Shimadzu prominence LCMS 2020 (Duisburg, Germany)) with a Luna 5 µm C18(2) 100 Å, column (250 × 4.6 mm, Phenomenex Ltd, Aschaffenburg, Germany). Here, a linear gradient of acetonitrile/water/formic acid (50/50/0.1–90/10/0.1 V/V) was used for 10 min, kept constant for 5 min, and switched back to (50/50/0.1) in a 3 min linear gradient and kept constant for another 2 min. For UV-detection (254 nm and 280 nm) a SPD-20A UV/VIS-detector (Shimadzu) was used. All reported yields are unoptimized.

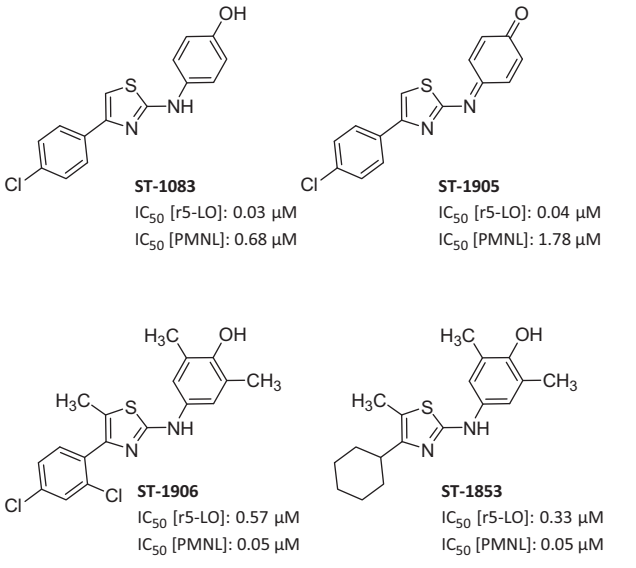

Fig. 1. Investigated 2-aminothiazole 5-LO inhibitors and corresponding IC_{50} values [19]. r5-LO: purified recombinant 5-LO; PMNL: polymorphonuclear leukocytes, for ST-1905 *cf* materials section and 3.6.

2.1.2. 4-(4-(4-Chlorophenyl)thiazol-2-ylimino)cyclohexa-2,5-dienone (ST-1905)

ST-1083 (500 mg, 1.6 mmol) was heated to 45 °C in 70 ml ethyl acetate. Lead(IV) acetate (805 mg, 1.8 mmol) was added. The solution instantly changed the color to cherry-red. After stirring for 10 min calcium carbonate (1 g, 10.0 mmol) was added. The formed sponge was filtrated. The filtrate was consequently concentrated under vacuum and purified by column chromatography (eluent: dichloromethane), yielding a brown/black solid (60 mg, 0.20 mmol, 12%). ^{1}H-NMR (250 MHz, DMSO-d_6): δ = 8.51 (1H, s, *thiazole*-5*H*), 8.43 (dd, 1H, *J* = 3.0 Hz, 10.0 Hz, *dienone*-5*H*), 8.02 (d, 2H, *J* = 8.5 Hz, *Ph*-2*H*,6*H*), 7.54 (d, 2H, *J* = 8.5 Hz, *Ph*-3*H*,5*H*), 7.35 (dd, 1H, *J* = 2.9 Hz, 10.4 Hz, *dienone*-3*H*), 6.76 (d, 2H, *J* = 10.6 Hz, *dienone*-2*H*,6*H*). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 187.06, 169.08, 157.65, 153.46, 141.82, 134.05, 133.22, 133.13, 132.19, 130.69, 128.87, 127.85, 120.113. ESI-MS: *m*/*z* = 300.9 $[M+H^+]^+$; HR-MS: calculated: *m*/*z* = 300.01241; found: *m*/*z* = 300.01295.

2.2. Biological evaluation

2.2.1. Materials

Ammonium hydrogen carbonate, calcium ionophore A23187, BWA-4C, DMSO, formic acid, hyperforin, trypan blue solution, phosphatidylcholine (egg yolk), diphenylpicrylhydrazyl (DPPH), $FeCl_3$, Ferene-S, glutathione (GSH), lipopolysaccharides, *N*-ethylmaleimide (NEM), *N*-acetylcysteine (NAC), NDGA, MK886, sodium arsenite, fMLP, glucose-6-phosphate dehydrogenase (G6PD), and MEM non-essential amino acids were purchased from Sigma-Aldrich (Taufkirchen, Germany). Alpha-cyano-hydroxycinnamic acid was purchased from Bruker Daltonics (Bremen, Germany). AA (peroxide free), Rev-5901 and Zileuton were purchased from Cayman Chemical (Ann Arbor, USA). Tween-20, and EDTA were purchased from AppliChem (Darmstadt, Germany). Pierce™ Chymotrypsin Protease (TLCK treated), MS Grade was purchased from Thermo Fisher Scientific (Bremen, Germany). NaCl, NADP, glucose 6-phosphate, acetonitrile (HPLC grade) were purchased from Carl Roth (Karlsruhe, Germany). Alamethicin, *D*-saccharic acid, and uridine 5′-diphospho-glucuronic acid (UDPGA) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Methanol (HPLC Grade) was purchased from VWR International (Darmstadt, Germany). Trifluoroacetic acid (TFA) was purchased from Merck (Darmstadt, Germany) and Carl Roth. RPMI 1660, DMEM medium (high glucose and glutamine), sodium pyruvate (100 mM), Penicillin and streptomycin, rat (Sprague Dawley) microsomes and phosphate-buffered saline (PBS) pH 7.4 were purchased from Gibco/Invitrogen (Paisley, UK). Fetal calf serum (FCS) was purchased from Biochrom AG (Berlin, Germany). Lymphocyte Separation Medium was purchased from Lonza Group (Basel, Switzerland). Human whole blood was obtained from healthy volunteers with informed consent. Fresh blood cell concentrates were kindly provided by Städtische Kliniken Frankfurt-Höchst and german red cross blood donor service Baden-Württemberg – Hessen (Frankfurt, Germany) with informed consent. ML 3000 was kindly provided by Merckle (Blaubeuren, Germany).

2.2.2. Diphenylpicrylhydrazyl (DPPH) antioxidant assay

Radical scavenging activity of the compounds was assessed by reduction of DPPH [21,22]. Compounds or vehicle control (DMSO) were incubated with 5 nmol DPPH in acetate buffered methanol (0.1 M acetate buffer pH 5.5 and methanol 40/60 V/V). After 30 min gentle shaking in the dark, absorbance was measured at 517 nm, using a microplate reader (Infinite M200, Tecan Group Ltd, Crailsheim, Germany). Each compound was tested independently three times. Compound absorbance values were normalized to vehicle controls. Ascorbic acid and NDGA (100 μM) were used as control.

2.2.3. Ferene-S assay

Iron reduction from Fe^{3+} to Fe^{2+} was measured by the specific iron(II) colorimetric detector Ferene-S [23]. Compounds or vehicle control (DMSO) were incubated with 50 μM $FeCl_3$ and 0.5 mM Ferene-S in 0.15 M NaCl with 1% Tween-20 for 1 h at 37 °C in the dark. Absorbance was measured at 594 nm, using a microplate reader (Infinite M200, Tecan Group Ltd, Crailsheim, Germany). After baseline correction with vehicle controls, absorbance values were normalized to maximal iron reduction (NDGA 100 μM). Each compound was tested independently three times. Ascorbic acid (100 μM) was used as control.

2.2.4. Isolation of intact PMNL and platelets

Human PMNL and platelets were freshly isolated from leukocyte concentrates. PMNL and platelets were isolated according to [24] and [25]. In brief, PMNL were immediately isolated by dextran sedimentation, centrifugation on lymphocyte separation medium, and hypotonic lysis of erythrocytes. Cells (purity of >96–97%) were finally resuspended in phosphate-buffered saline (PBS), pH 7.4 containing 1 mg/ml glucose (PG). For isolation of human platelets, platelet rich plasma was withdrawn and mixed with PBS buffer pH 5.9 (1:1). After centrifugation (1849×*g*, 15 min, room temperature (RT)) cell pellets were resuspended in 50 ml PBS pH 5.9/NaCl (0.9%) (1:1) and were centrifuged again (1849×*g*, 10 min, RT). Finally, platelets were resuspended in PG.

2.2.5. Determination of 5-LO, 15-LO, COX and 12-LO product formation in intact cells

Intact cell assays were performed as described in [19,20]. In brief, for 5-LO and 15-LO1 assay, freshly isolated PMNL (5×10^6 or 7.5×10^6, if stated) were resuspended in PG and 1 mM $CaCl_2$, preincubated with test compounds or vehicle control for 15 min at 37 °C and stimulated by the addition of calcium ionophore A23187 (2.5 μM) and exogenous AA (as indicated, 0–30 μM), or preincubated with 300 mM sodium chloride or 10 μM sodium arsenite 3 min prior to addition of exogenous AA (20 μM). After 10 min at 37 °C, the reaction was stopped by the addition of methanol (1 ml). To affect thiol levels (*cf* Section 3.5) either 50 μM *N*-ethylmaleimide (NEM) or 5 mM *N*-acetylcysteine (NAC) was added 20 min prior to compound preincubation.

For cyclooxygenase (COX-)1 and 12-LO product formation, 10^8 freshly isolated platelets were resuspended in 1 ml PG containing 1 mM $CaCl_2$ and subsequently preincubated with test compounds or vehicle control for 15 min at 37 °C. The reaction was started with the addition of 10 μM AA and stopped after 10 min at 37 °C by addition of 1 ml methanol.

For solid phase extraction, Prostaglandin B_1 (200 ng) was added as internal standard, as well as HCl (30 μl, 1 N) and PBS (500 μl). HPLC analysis (as described by [26,27]) comprised LTB_4, its all-*trans* isomers, and 5-hydro(pero)xy-6,8,11,14(*E,Z,Z,Z*)-eicosatetraenoic acid (5-H(P)ETE) for 5-LO product formation. 15-LO1 product formation included 15-hydro(pero)xy-5,8,11,13 (*Z,Z,Z,E*)-eicosatetraenoic acid (15-H(P)ETE), COX-1 and 12-LO product formation in human platelets included 12-(*S*)-hydroxy-5*Z*,8*E*,10*E*-heptadecatrienoic acid and 12-H(P)ETE, respectively. All values, of at least three independent measurements, were normalized to vehicle controls and either IC_{50} values and 95% confidence intervals or means ± SEM were calculated. BWA-4C (5-LO), NDGA (12-LO, 15-LO1) and acetylsalicylic acid (COX-1) were used as control.

2.2.6. Preparation of 100,000×g PMNL homogenate supernatants and measurement of 5-LO product formation

Freshly isolated PMNL were resuspended in PG containing 1 mM EDTA and sonicated 3 times for 10 s. PMNL homogenates were then centrifuged (100,000×*g*) for 70 min at 4 °C to obtain 100,000×*g* supernatants (S100). S100 corresponding to 7.5×10^6 PMNL were then added to 1 ml PBS pH 7.4 containing 1 mM EDTA, and 1 mM ATP and preincubated with test compounds, and if stated phosphatidylcholine (20 μg/ml) for 15 min at 4 °C. After 30 s prewarming at 37 °C, the reaction was started with the addition of 20 μM AA and 2 mM $CaCl_2$. After 10 min the reaction was stopped by 1 ml methanol and the formed 5-LO products were analyzed by HPLC/UV as described for intact cells. Data of at least three independent measurements were expressed as percentage of control (DMSO). Hyperforin (10 μM) was used as control.

2.2.7. Expression and purification of r5-LO

Recombinant 5-LO was purified as described by [19]. In brief, the recombinant protein (5-LO wt or cysteine mutants by [28]) was expressed and purified from a 1 L culture (*E. coli* BL21(DE3)) using a ATP affinity chromatography (5 ml ATP-agarose column) and anion exchange chromatography (ResourceQ 1 ml IEX column, GE Healthcare, Uppsala, Sweden) in an ÄKTA Xpress system (GE Healthcare).

2.2.8. Determination of 5-LO product formation in r5-LO

Test compounds or vehicle control were diluted in 1 ml reaction mix (3 μg r5-LO, PBS, 1 mM EDTA, 1 mM ATP) for 15 min preincubation time at 4 °C. After 30 s pre-warming, the reaction was started by addition of 2 mM $CaCl_2$ and 20 μM AA. After 10 min at 37 °C 5-LO product formation was stopped by 1 ml methanol. The formed metabolites (all-*trans* LTB_4, 5-H(P)ETE) were analyzed by HPLC/UV as described for intact cells. Data were normalized to vehicle control (DMSO) and means ± SEM or IC_{50} values and 95% confidence intervals of at least three independent measurements were calculated.

2.2.9. In vitro human whole blood assay

In vitro whole blood assays were performed as described by [29]. In brief, heparinized venous blood was prewarmed at 37 °C for 30 min, preincubated for 30/120 min with the test compounds and stimulated by addition of 20 μM calcium ionophore A23187 (final concentration, previously dissolved in 50 μl autologous plasma). After 15 min the reaction was stopped on ice. In case of fMLP stimulation after 30 min prewarming at 37 °C, lipopolysaccharides (10 μg/ml) were added. After 15 min test compounds were preincubated for 15 min. 5-LO reaction was started by addition of 1 μM fMLP and stopped after 1 h as described above. Plasma supernatants were taken after centrifugation at 9391×g. Liquid-liquid extraction and LC-MS/MS analysis were performed according to [25]. Data were normalized to vehicle control (DMSO) and means ± SEM or IC_{50} values and 95% confidence intervals of at least three independent measurements were calculated.

2.2.10. Sample preparation for MALDI-MS

Glutathione (GSH, 1 mM, final concentration) was diluted in PBS buffer pH 7.4 together with the compound ST-1083, ST-1905, ST-1906 (each 100 μM), or ST-1853 (300 μM) in a total volume of 2 μl. For quantitative measurements, a concentration series from

1 to 50 μM of ST-1083 and ST-1905 was prepared. The incubation was performed directly on a Thermo Scientific 384-well MALDI-MS target (Thermo Fisher Scientific, Dreieich, Germany) in a humidity chamber especially for MALDI-MS targets for 30 min at 37 °C with saturated humidity. After 30 min incubation time, the reaction was stopped by the addition of 1 μl of 3 mg/ml alpha-cyano-hydroxycinnamic acid in a mixture of acetonitrile/water/trifluoroacetic acid (TFA) (70/30/0.1 V/V). The incubation of purified 5-LO was performed following the same procedure and compound concentration (100 and 300 μM). Final 5-LO concentration was 150 ng/μl in a total volume of 2 μl. After 30 min incubation, 1 μl of trypsin, chymotrypsin or a mixture of both proteases with an enzyme concentration of 12.5 ng/μl in 25 mM NH_4HCO_3 was added to the droplets on the MALDI-target. Enzymatic digestion was performed for 1 h and stopped by the addition of the alpha-cyano-hydroxycinnamic acid with the same concentration as used for the GSH-binding experiment. After crystallization of the matrix, a chilled five percent formic acid solution was used to wash the sample spots. For sample washing, 2 μl of the formic acid solution were used to coat the matrix crystals and were immediately removed to wash away salt contaminants due to high salt concentration in PBS buffer. After sample washing, 1 μl of acetonitrile/water/TFA (80/20/0.1 V/V) was used to recrystallize the matrix for a more homogeneous crystal size distribution.

2.2.11. MALDI-MS-measurement and data-analysis

All MALDI-MS spectra were recorded with a MALDI LTQ-Orbitrap XL (Thermo Fisher Scientific). For GSH-binding, all measurements were recorded from *m/z*: 400.00 – 800.00 with a resolution of 60 000 and 50 subspectra were accumulated for data-analysis. The peptide mass fingerprint measurements of the 5-LO digests were recorded from *m/z*: 800.00 – 4000.00 with a resolution of 30 000 and 30 subspectra were accumulated for database search. MS/MS spectra were obtained using collision-induced dissociation fragmentation in combination with the ion-trap mass analyzer of the MALDI LTQ-Orbitrap XL. 100 subspectra were accumulated for analysis. The isolation width for MS/MS analysis of the GSH-bound substances was 0.5 Da and 0.7 Da for the MS/MS-analysis of the (chymo-)tryptic peptides after digestion. The laser-energy of the MALDI ion source was adjusted as needed for optimal measurement results and kept constant. All measurements were performed as quintuplicates for a reproducible MS and MS/MS analysis. For the qualitative analysis of the GSH-binding, the obtained high-resolution mass spectra were compared with a theoretical spectrum generated by the Xcalibur™ Qual browser version 2.0.4 software. For a more specific and accurate evidence of the GSH-binding, the MS/MS spectra were analyzed. A neutral loss of 129.04 Da indicated glutamic acid loss, therefore making a thiol binding more probable. Deisotoping of the obtained spectra and converting the raw-files to mascot generic files (mgf) for further data-analysis and database search were performed using an in-house programmed KNIME®-workflow. All quantitative GSH-binding measurements were analyzed by a KNIME® workflow with automatical calculation of the relative intensity using the matrix signal *m/z*: 419.32 as internal standard. The peak lists of the peptide mass fingerprint analysis were subsequently submitted to the MASCOT-database search (Mascot version 2.4). Parameters for the database search were: peptide mass tolerance: 10 ppm, enzyme for proteolysis: trypsin, missed enzyme cleavage sites: maximum 1, fixed modifications: none, variable modifications (Table 1).

Oxidized modifications were considered as a possible modification due to oxidation during the reaction. If chymotrypsin was used for proteolysis the database search parameters were as follows: peptide mass tolerance: 10 ppm, enzyme for proteolysis: chymotrypsin, missed enzyme cleavage sites: maximum 2, fixed modifications: none and variable modifications (Table 1). MS/MS database searches were also performed after conversion to mgf files with the following parameters using the MASCOT MS/MS Ion search tool: enzyme for proteolysis: trypsin; missed cleavages sites: up to one; if chymotrypsin was used then: enzyme for proteolysis: chymotrypsin and missed cleavage sites up to two; fixed modifications: the particular substance meant to bind on the peptide or none for the peptide structure verification; Peptide mass tolerance: 50 ppm; MS/MS-ion mass tolerance: 0.5 Da; peptide charge: +1.

2.2.12. Cell culture

The human leukemic monocyte cells U937 and the human liver cancer cell line Hep G2 were purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Brunswick, Germany). U973 cells were maintained in RPMI 1660. Complete culture media contained 10% FCS, 100 μg/ml streptomycin and 100 U/ml penicillin. Hep G2 cells were maintained in DMEM, containing 10% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin, 1 × MEM non-essential amino acids and 1 mM sodium pyruvate. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and discarded after passage 35.

2.2.13. Cell viability (water soluble tetrazolium-1 (WST-1)) assay

Cell viability was assessed using a WST-1 assay (Roche Diagnostics GmbH, Mannheim, Germany). U937 cells were seeded in flat-bottomed 96-well plates at a density of 10^4 cells per well. After treatment of cells with test compounds or vehicle (DMSO) 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). Absorbance values were measured after 48 h, baseline corrected with the absorbance of culture medium plus WST-1 in the absence of cells and normalized to vehicle control (DMSO). Hep G2 cells were seeded in flat-bottomed 96-well plates at a density of 3×10^4 cells per well for 48 h in presence of 10% FCS. After 24 h culture medium and test compounds were refreshed. Analysis was carried out as stated for U937 cells. Each compound was tested independently at least three times; means ± SEM were calculated. 100 μM Zileuton (cell viability U937: 88.98 ± 3.92%, Hep G2: 101.60 ± 2.55%) and Rev-5901 (3.24 ± 1.94%, 45.16 ± 7.83%) were used as control.

2.2.14. In vitro metabolism

For phase I metabolism, test compounds (10 μM) were diluted in phosphate buffer (0.1 M, pH 7.4) together with 50 μl NADPH-regenerating system (30 mM glucose-6-phosphate, 4 U/ml glucose-6-phosphate dehydrogenase, 10 mM NADP, 30 mM $MgCl_2$). After 5 min preincubation time at 37 °C the reaction was started by the addition of 13 μl rat liver microsome mix (20 mg protein/ml in 0.1 M phosphate buffer). The incubation was performed in a shaking water bath at 37 °C and subsequently stopped by the addition of 500 μl ice-cold methanol at 0, 30 and 60 min. The samples were centrifuged at 10,000×*g* for 5 min at 4 °C. The supernatants were analyzed and quantified by HPLC. Control samples were performed to ensure stability of the compounds in the reaction mixture. Controls comprise neglected NADPH, test compounds (to determine the baseline) and heat inactivated microsomes. The amounts of the test compounds were quantified by an external calibration curve and normalized to the starting (0 min) value. Phase II metabolism (glucuronidation) was determined similarly in phosphate buffer (0.1 M, pH 7.4) containing 30 mM $MgCl_2$. At variance with the terms of phase I metabolism the NADPH-regenerating system was exchanged for 5 mM uridine 5′-diphospho-glucuronic acid (UDPGA), alamethicin (25 μg/ml), and saccharic acid 1,4 lactone (5 mM). Determination of remaining parent compound is conducted as described above. Each

Table 1
Parameters for MASCOT database search.

Modification name	Mass shift (monoisotopic/average)	Oxidation possible (bound to 5-LO)	Mass shift of oxidized substance (monoisotopic/average)	Amino acid specificity for database search
ST-1083, ST-1905	300.01/300.76	Yes	297.99/298.74	C, H, K
ST-1853	316.19/316.46	Yes	314.14/314.44	C, H, K
ST-1906	376.02/377.29	Yes	374.00/377.27	C, H, K

compound was tested independently at least three times and means ± SEM were calculated. For phase I metabolism 7-ethoxycoumarin (remaining compound: 52.67 ± 1.45% after 60 min) and 7-hydroxycoumarin for phase II metabolism (below limit of detection after 60 min) were used as control compounds.

2.3. Statistics

For single measuring points, means ± SEM were calculated. IC_{50} values and 95% confidence intervals (CI) were determined using a nonlinear fit (log (inhibitor) vs. normalized response – variable slope) by GraphPad Prism software version 7.00 for Windows (GraphPad Software, La Jolla, USA). For this, five to eight increasing concentrations of each compound were measured in three to eight independent experiments. Absolute values were normalized to vehicle controls. For comparison of means, unpaired, two-tailed t-tests were performed. A p-value $\leqslant 0.05$ was considered significant.

3. Results

3.1. Aminophenolic thiazole compounds show redox activity

Since the reported compounds generally could possess antioxidant properties along with the aminophenolic moiety, we firstly tested the compounds' radical scavenging activities in a DPPH assay. Here, by measurement of the reduction of the stable radical DPPH, the reducing properties of a compound can be estimated. In comparison of the two lead compounds ST-1853 and ST-1906, we assessed the parent compound ST-1083 as well as its oxidized quinone imine form, ST-1905 (Fig. 1). Except for ST-1905 all investigated compounds showed moderate reducing properties at 10 μM (Fig. 2). Since reduction of Fe^{3+} to Fe^{2+} is a relevant mechanism of 5-LO inhibition, we further investigated the capabilities of iron reduction using a Ferene-S assay. In line with the radical scavenging activities, the compounds showed moderate iron reducing properties at 10 μM. Notably, both lead compounds showed a stronger reduction of Fe^{3+} than the parent compound ST-1083. As expected, ST-1905 displayed no reducing activities, since the compound is already in an oxidized state.

3.2. ST-1083 and ST-1905 but not the lead compounds show covalent binding to 5-LO

Previous work from our lab described several 5-LO inhibitors, like U73122 [28], nitro fatty acids [30], as well as certain quinones (submitted data, Maucher et al.) to bind covalently to 5-LO by nucleophilic attack of cysteine residues. Especially the cysteines 159, 300, 416, and 418 (located in the substrate entry channel of 5-LO) are prone to be potential targets for inhibition. As the compounds are capable of oxidation, we next investigated covalent binding by the compounds via MS analysis. For ST-1083 and ST-1905 binding both to a peptide containing C159 (Fig. 3A) and a peptide containing C416 and C418 (Fig. 3B) could be observed. To further distinguish both cysteines, the experiment was re-run, using the r5-LO C416A mutant. Here, too, the binding of ST-1083 and ST-1905 was detected (data not shown). On the contrary, both lead compounds bearing the 2,6-dimethyl substitution of the aminophenolic moiety (ST-1853, ST-1906) showed no binding to r5-LO.

3.3. Compounds show different susceptibility to GSH trapping

In order to assess this reactivity, we similarly incubated the compounds with GSH for MALDI-MS analysis and screened for the exact mass of the GSH adduct, as well as the neutral loss of

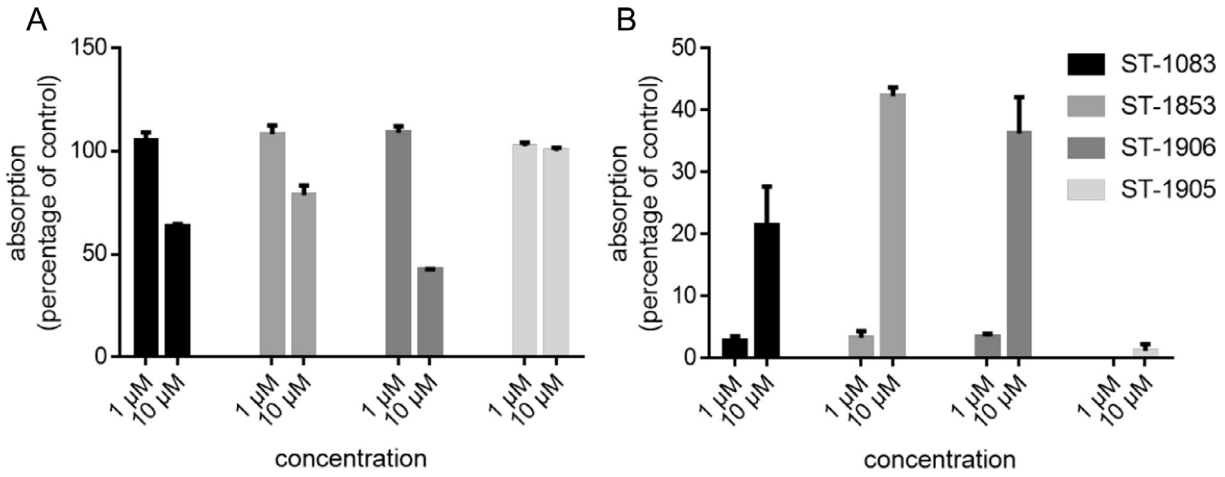

Fig. 2. Radical scavenging (A) and reducing properties (B) of the test compounds A) Compounds or vehicle control (DMSO) were incubated with 5 nmol DPPH for 30 min. Absorbance was measured at 517 nm and normalized to vehicle control. Ascorbic acid (100 μM) and NDGA (100 μM) were used as control (ascorbic acid: 3.67 ± 1.23%, NDGA 3.91 ± 1.50%), n = 3. B) Compounds or vehicle control (DMSO) were incubated with 50 μM $FeCl_3$ and 0.5 mM Ferene-S as iron(II) specific colorimetric detector. Absorbance was measured at 594 nm and normalized to NDGA (100 μM) control. Ascorbic acid (100 μM) was used as control (108.4 ± 21.75%), n = 3. Data are shown as means + SEM.

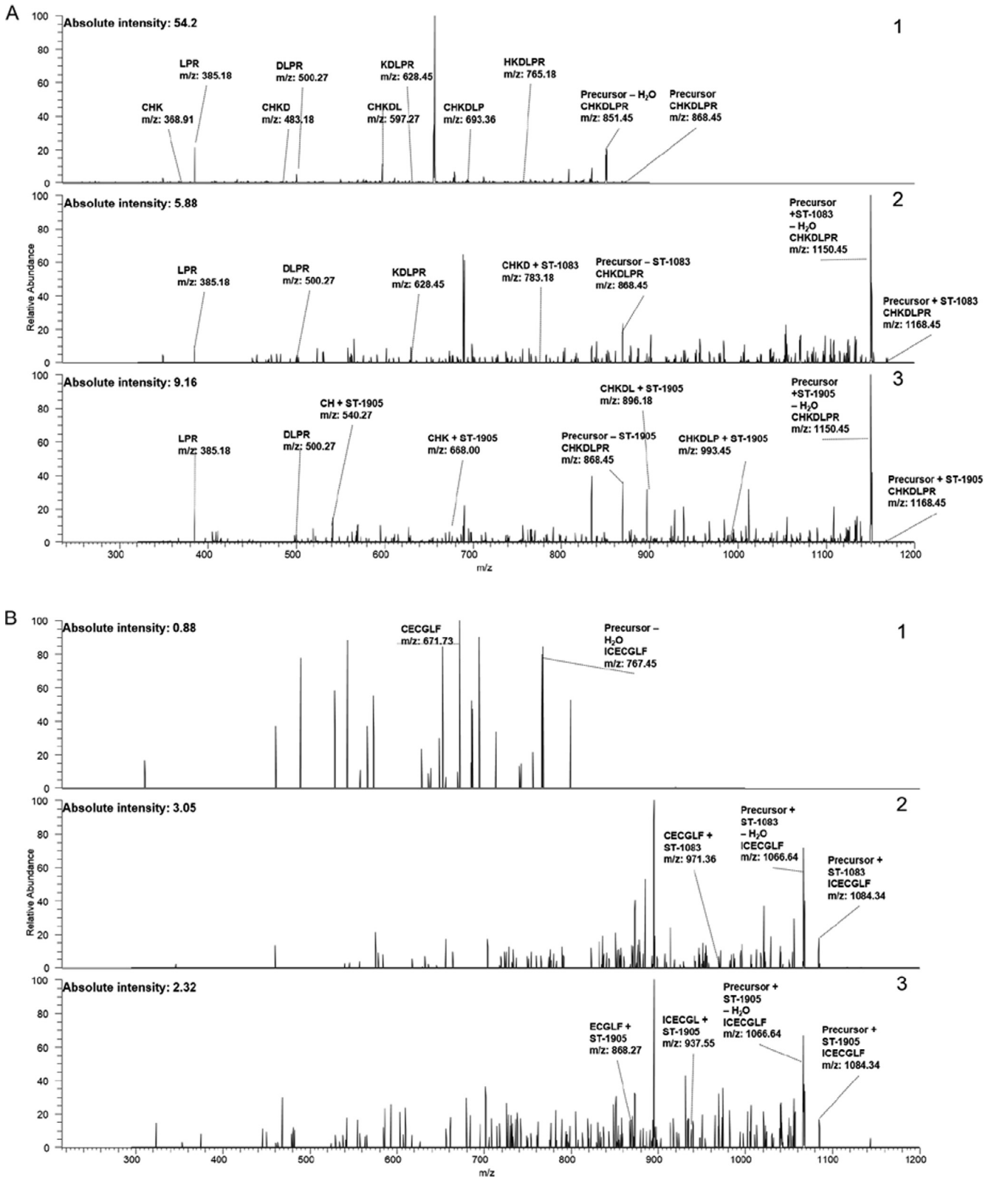

Fig. 3. MS/MS spectra of modified and unmodified cysteine containing peptides. The matched masses in the MS/MS spectra are marked with the sequence belonging to the mass. MS/MS spectra were recorded after collision induced dissociation fragmentation in the ion trap mass analyzer. A) MS/MS spectra of peptide [159–165]. 1: unmodified peptide, 2: peptide modified with ST-1083, 3: peptide modified with ST-1905. For ST-1853 and ST-1906 only the precursor mass and water loss could be detected and it was therefore no approval of the modified peptide structure. B) MS/MS spectra of peptide [415–421]. 1: unmodified peptide, 2: peptide modified with ST-1083, 3: peptide modified with ST-1905. For ST-1853 the precursor mass of the modified peptide could not be isolated and fragmented. ST-1906 showed no MS/MS spectra.

129 Da of glutamic acid. Under these conditions, all investigated compounds are capable of binding GSH (data not shown). For a quantitative analysis of this GSH binding, we further gathered relative intensity data for ST-1083 and ST-1905 by using alpha-cyano-hydroxycinnamic acid as an internal standard. Thereby it became evident that reactivity is driven by the quinone imine, since the relative intensity of GSH binding of ST-1905, the oxidized derivative, was markedly higher than the corresponding hydroquinone amine ST-1083 (Fig. 4).

3.4. Potency of ST-1083 and ST-1905, but not of the lead compounds is impaired for 5-LO cysteine mutants

To further validate the results of MS analysis, activity based 5-LO assays were conducted using purified recombinant 5-LO mutants. Here, in line with the detected covalent binding to one of the cysteines in the entry channel of 5-LO by unsubstituted aminophenols (*cf* 3.2), ST-1083 as well as the oxidized form ST-1905 showed a decreased potency using r5-LO 4C mutant (r5-LO C159S, C300S, C416S, C418S; *cf* [31]) (Fig. 5). The potency of ST-1853 and ST-1906 is hereby not impaired. In case of r5-LO 4C and C416S respectively, potency is not only not declined, but even increased (Fig. 5). Of interest, compounds with an altered aminophenolic moiety that cannot oxidize to the respective quinone imine showed no variation in inhibitory activity throughout each and every studied r5-LO mutant/wt (data not shown). This altogether reveals the cysteine dependent inhibition of unsubstituted aminophenols but not of the lead compounds.

3.5. Lead compounds show no dependency on thiol level

Since redox activity seems to be important for inhibitory potency, we next altered the cellular redox tone by increasing or decreasing the cellular thiol level. For this purpose we first added *N*-ethylmaleimide (NEM) to intact PMNL to decrease cellular thiols. In this case, thiol reactive compounds, like U73122 should show an increased potency, due to lessened GSH adducts [32]. This behavior was reflected partly by ST-1083 and its quinone imine analogue ST-1905 (Fig. 6). Substituted analogues, like ST-1853 and ST-1906, on the contrary showed no difference in inhibition. Vice versa, increase of cellular thiols via addition of *N*-acetylcysteine (NAC) should impair the potency of thiol reactive compounds, due to increased thiol trapping. Indeed, the potency of U73122 and ST-1905 was decreased, while the potency of ST-1083 and the lead compounds ST-1853 and ST-1906 was unaltered.

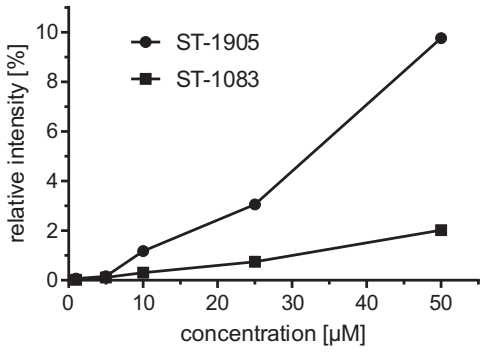

Fig. 4. Quantitative MALDI-MS based analysis of GSH binding. Compounds were incubated with 1 mM GSH for 30 min at 37 °C in a humidity chamber directly on a 384 well MALDI-MS target. The matrix signal *m/z*: 419.32 was used as internal standard. Data are displayed as means + SEM (error bars shorter than the height of the symbol), n = 5.

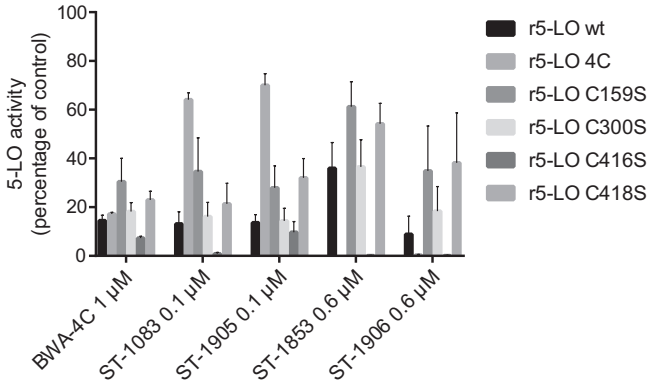

Fig. 5. Inhibition of 5-LO activity of wt and cysteine mutants by the test compounds. 3 µg r5-LO wt or cysteine mutant (C159S, C300S, C416S, C418S, and combination thereof (4C)) were incubated for 15 min at 4 °C with test compounds or vehicle control (DMSO) in 1 ml PBS pH 7.4 containing 1 mM EDTA. After pre-warming for 30 s, 5-LO reaction was started by addition of 20 µM AA and 2 mM $CaCl_2$. After 10 min the reaction was stopped by the addition of 1 ml methanol. 5-LO product formation was analyzed via HPLC/UV after solid-phase extraction. Data were normalized to respective vehicle controls and are shown as means + SEM. Absolute values of vehicle controls were 2005 ± 171.1 (wt), 1788 ± 80.93 (C159S), 1764 ± 187.9 (C300S), 803 ± 46.61 (C416S), 1629 ± 79.79 (C418S), and 1332 ± 88.28 (4C) ng/ml, n = 3–4.

3.6. Inhibitory profile of lead compounds ST-1853 and ST-1906

As we could recently show [19], both compounds exhibit direct 5-LO inhibition as seen by inhibition of r5-LO (IC_{50} [ST-1853] = 0.33 µM, IC_{50} [ST-1906] = 0.57 µM). In the intact cell system (PMNL) this high potency is even improved (IC_{50} [ST-1853, ST-1906] = 0.05 µM) [19]. These properties are in contrast to the parent compound ST-1083, and its oxidized form, ST-1905 (IC_{50} [r5-LO] = 0.04 µM (0.02 – 0.07 µM 95% CI), IC_{50} [PMNL] = 1.78 µM (1.08 – 2.92 µM 95% CI)) (Fig. 1). A plausible explanation for this positive shift of ST-1853 and ST-1906 to higher potencies in intact cells might be off-target effects concerning 5-LO upstream proteins ($cPLA_2$, FLAP). Thus, we further evaluated 5-LO product inhibition at various substrate concentrations, used alternative stimuli and a FLAP inhibitor assay (Fig. 7). FLAP inhibitors (e.g. MK886 [33]) normally present higher potency at lower substrate concentrations, while at higher exogenous substrate amounts they fail to fully inhibit 5-LO product formation (*cf* [34]). Here it was shown that ST-1853 is neither displaced by higher substrate (AA) concentrations nor considerably more potent at low AA concentrations (Fig. 7A). Furthermore, the potency of ST-1853 and ST-1906 was not impaired by 5-LO phosphorylation (mediated by hyperosmotic or genotoxic cell stress) (Fig. 7B, C). In addition, 5-LO activity of MK886-pretreated PMNL was still reduced by 0.03 µM ST-1853 (residual activity of 86.88 ± 4.82% for 7.5×10^6 PMNL and no inhibition of r5-LO), whereas incubation of two FLAP inhibitors (MK886 and ML 3000 [34]) showed no further inhibition (Fig. 7D).

To rule out additional inhibition by interference with the C2-like domain of 5-LO, 100 000 × g supernatant of PMNL homogenates were incubated in absence or presence of phosphatidylcholine. Here too, there was no decisive difference in treatment (data not shown).

In order to screen the compounds in more physiological conditions, we next conducted in vitro human whole blood assays. In detail, we analyzed 5-LO activity stimulated by calcium ionophore A23187, to acquire maximal 5-LO activation, compared to the more physiological stimulus fMLP (Fig. 8). Furthermore, we varied the preincubation time of the compounds (30 min/ 2 h) in order to get insight in plasma stability.

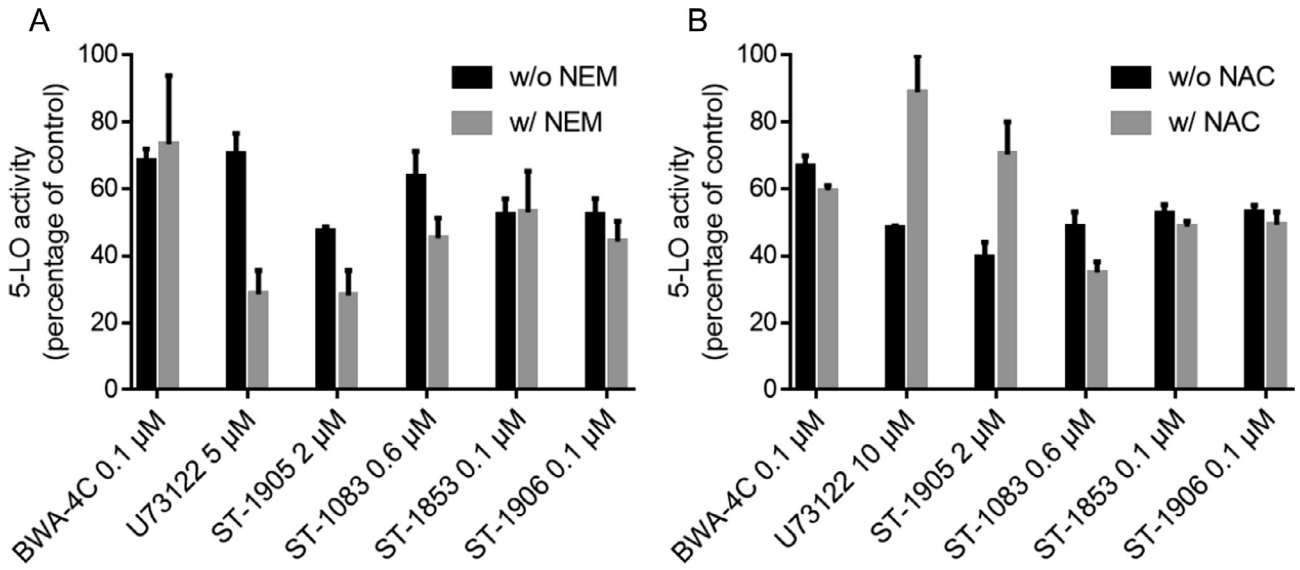

Fig. 6. Influence of thiol level on 5-LO inhibition. 5×10^6 PMNL were resuspended in PBS glucose (1 mg/ml) and 1 mM $CaCl_2$ and pretreated for 20 min at 37 °C with A) 50 µM NEM to deplete or B) 5 mM NAC to increase thiol levels. After 15 min incubation time with test compounds or vehicle controls (DMSO), 5-LO reaction was stimulated by 2.5 µM A23187 and 20 µM exogenous AA. After 10 min reaction was stopped by addition of 1 ml methanol. HPLC/UV analysis was performed after solid-phase extraction. Data were normalized to vehicle controls and are shown as means + SEM. Absolute values of vehicle controls were 18.27 ± 0.82 (with NEM), 126 ± 16.83 (without NEM) and 49.55 ± 1.90 (with NAC), and 166.8 ± 4.17 ng per 1×10^6 PMNL (without NAC), n = 3.

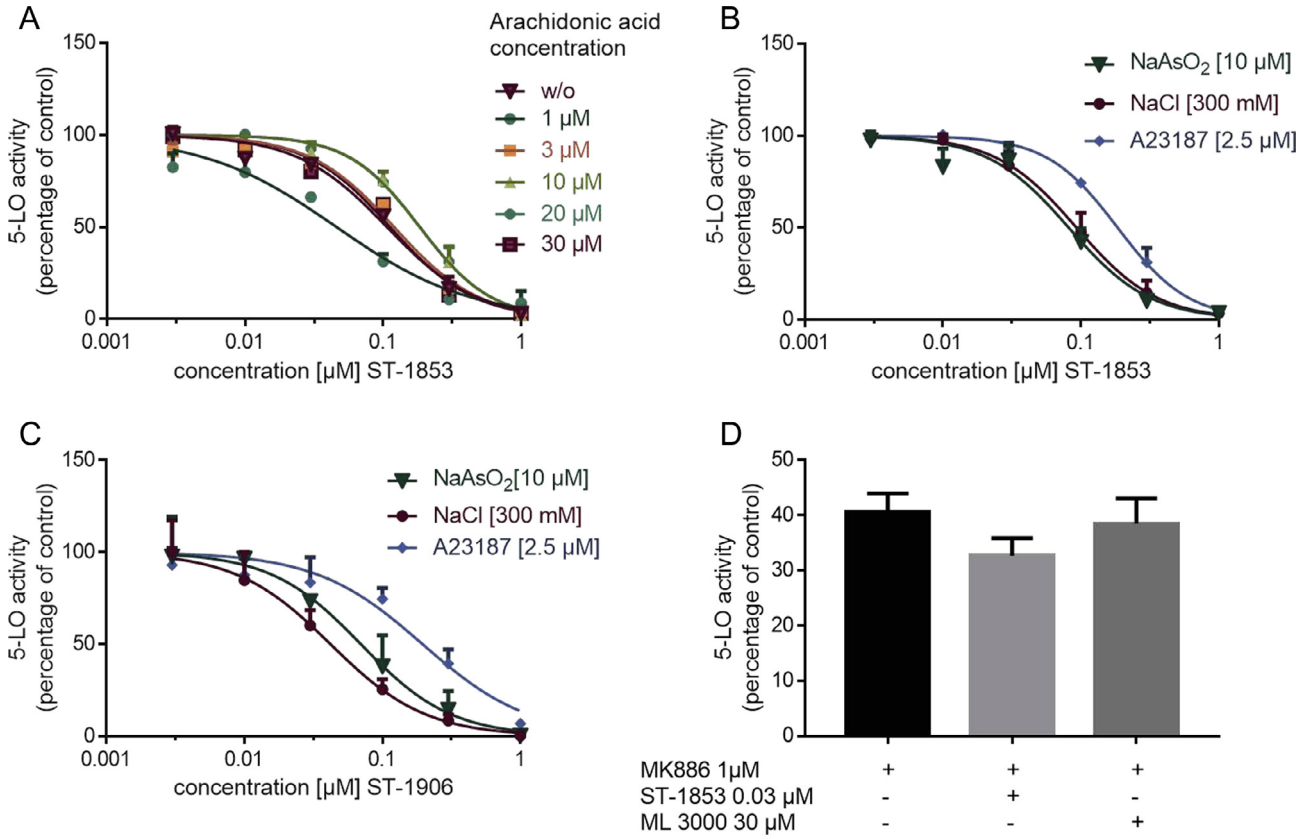

Fig. 7. Cellular screening assays for 5-LO inhibition. 7.5×10^6 PMNL were resuspended in PBS glucose (1 mg/ml) and 1 mM $CaCl_2$ and pretreated 15 min at 37 °C with test compounds or vehicle controls (DMSO). 5-LO reaction was started by A) 2.5 µM A23187 and varying concentrations of exogenous AA, B,C) 2.5 µM A23187 and 20 µM AA or 300 mM NaCl or 10 µM $NaAsO_2$ for 3 min and addition of 20 µM AA, and D) 2.5 µM A23187 and 20 µM AA. After 10 min reaction was stopped by addition of 1 ml methanol. HPLC/UV analysis was performed after solid-phase extraction. Data were normalized to vehicle controls and are shown as means + SEM, n = 3–7. Absolute values of vehicle controls were A) 43.92 ± 2.48 (w/o), 25.99 ± 1.42 (1 µM AA), 50.31 ± 1.81 (3 µM AA), 125.5 ± 5.50 (10 µM AA), 236.5 ± 10 (20 µM AA), 174.5 ± 4.45 (30 µM AA) ng per 1×10^6 PMNL, B) 66.38 ± 7.22 ($NaAsO_2$), 70.22 ± 3.30 (NaCl), and 236.5 ± 10.00 (A23187) ng per 1×10^6 PMNL, C) 270.8 ± 39.74 ($NaAsO_2$), 179.3 ± 22.40 (NaCl), 129.1 ± 9.46 (A23187) ng per 1×10^6 PMNL, D) 180.9 ± 20.89 ng per 1×10^6 PMNL.

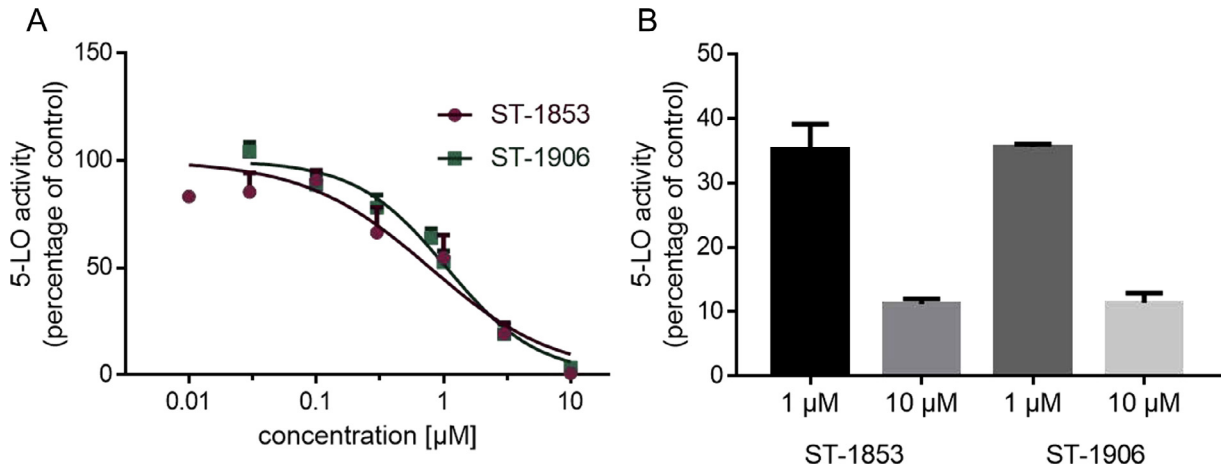

Fig. 8. Human whole blood studies. A) 450 μl venous blood was preincubated for 2 h at 37 °C with test compounds or vehicle (DMSO). 5-LO reaction was started by addition of 20 μM A23187, dissolved in autologous plasma. After 15 min, the reaction was stopped on ice. Plasma supernatants were analyzed for LTB_4 and 5-HETE by LC–MS/MS after liquid–liquid extraction. Data were normalized to vehicle controls and are shown as means + SEM. B) 450 μl venous blood was preincubated for 15 min at 37 °C with 10 μg/ml lipopolysaccharides, then, for another 15 min test compounds were added. 5-LO reaction was stimulated by addition of 1 μM fMLP and stopped on ice after 1 h. 5-LO products were measured as described in A). Absolute values of vehicle controls were A) 222.2 ± 22.03 and B) 16.48 ± 1.86 ng/ml. Zileuton (10 μM) was used as control (A: 8.64 ± 1.55%, B: 13.02 ± 1.12% residual activity), n = 3.

ST-1853 and ST-1906 both achieved a high potency with IC_{50} values of 0.78 μM (0.48–1.28 95% CI) and 1.06 μM (0.88–1.28 95% CI), respectively, when preincubated for 2 h and stimulated with calcium ionophore A23187 (Fig. 8A). This long preincubation time had no negative effect on the compounds' potencies, as a reduced (30 min) preincubation time resulted in similar inhibitory activities (data not shown). The high potency is also retained when LT formation was stimulated by the bacterial chemotactic peptide fMLP (IC_{50} below 1 μM) (Fig. 8B).

3.7. Specificity towards other eicosanoid synthesizing enzymes

Since many LO inhibitors did not present a pronounced specificity with regard to other AA metabolizing enzymes, we next screened specificity of both lead compounds on LO isoforms 12 and 15 as well as COX (Fig. 9). Here, ST-1853 and ST-1906 did not show off-target inhibition at total 5-LO product inhibition (0.3 μM). Indeed, there was an at least 100 fold threshold between IC_{50} of 5-LO and 12-LO, 15-LO, and COX inhibition. Moderate inhibition of 15-LO and COX only started at concentrations outside of this reasonable safety margin (10 μM) (residual activity 15-LO: 46.50 ± 9.21% (ST-1853) and 40.45 ± 12.04% (ST-1906); COX: 76.76 ± 4.38% (ST-1853) and 71.72 ± 2.05% (ST-1906)), whereas 12-LO was not impaired.

3.8. In vitro pharmacokinetic profile of ST-1853 and ST-1906

Given that the 5-LO inhibitory potency of both lead compounds was unaffected by prolonged incubation time in human whole blood (*cf* 3.6), we next conducted microsomal stability studies. In the course of one hour, concerning cytochrome P450 mediated phase I metabolism, both lead compounds were similarly degraded and showed a certain degree of metabolism with approximately 70% remaining parent compound (ST-1853: 70.77 ± 1.74%, ST-1906: 74.17 ± 0.51%). Further on, we validated if the 2,6-dimethyl substitution of the aminophenolic moiety is capable of reducing phase II metabolism, namely glucuronidation. Indeed, in comparison with an unsubstituted aminophenol derivative

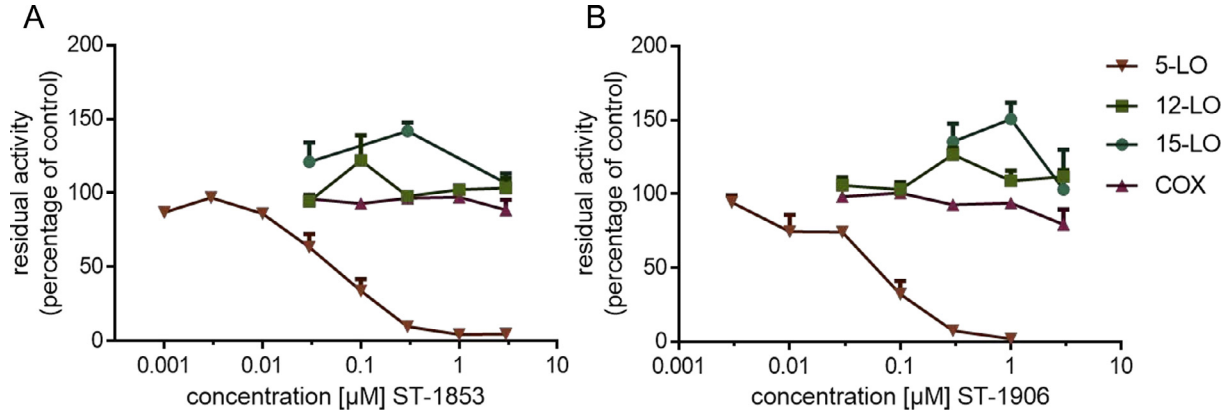

Fig. 9. Specificity of A) ST-1853 and B) ST-1906 towards LO isoforms and COX. For 5- and 15-LO, 5×10^6 PMNL were resuspended in PBS glucose (1 mg/ml) and 1 mM $CaCl_2$ and pretreated for 15 min at 37 °C with test compounds or vehicle controls (DMSO). LO reaction was started by addition of 2.5 μM A23187 and 20 μM AA. For 12-LO and COX, 10^8 platelets were resuspended in 1 ml PBS glucose (1 mg/ml) and 1 mM $CaCl_2$ and subsequently preincubated with test compounds for 15 min at 37 °C. 12-LO and COX reaction was started by addition of 10 μM AA. After 10 min reaction was stopped by addition of 1 ml methanol. HPLC/UV analysis was performed after solid-phase extraction. Data were normalized to vehicle controls and are shown as means + SEM. Absolute values of vehicle controls were 165.9 ± 21.65 (5-LO), 6.63 ± 0.81 (15-LO) ng per 1×10^6 PMNL, 1792 ± 312.7 (12-LO), 893 ± 69.84 (COX) ng per 10^8 platelets. Residual activity of control compounds was below limit of detection (15-LO, NDGA, 100 μM), 5.07 ± 2.75% (12-LO, NDGA, 100 μM) and 4.64 ± 2.32% (COX, acetylsalicylic acid, 100 μM).

(ST-1904) the substitution pattern of ST-1906 successfully slowed down glucuronidation after 1 h of incubation significantly (unpaired, two-tailed *t*-test, p value 0.0022, degrees of freedom: 7). In detail, 95.29 ± 4.71% of the lead compound was unaffected by glucuronidation, while only 69.84 ± 2.05% of ST-1904 remained unaltered.

3.9. Lead compounds show no cytotoxicity

Lastly, to rule out cytotoxicity, cell viability measurements were conducted. On this account, WST-1 assays were carried out. Previously, we could show that it is possible to disarm the observed cytotoxicity of unsubstituted aminophenols like ST-1083 via introducing the characteristic 2,6-dimethyl substitution of ST-1853 and ST-1906 using human leukemic monocytic cells (U937) [19]. To further confirm this outcome, we studied the cell viability also in an hepatic cell line, human Hep G2. Here, too, after 48 h of incubation time, ST-1853 and ST-1906 showed no impairment of cell viability in comparison to vehicle control. In both cell lines the lead compounds presented a cell viability >90% at 10 μM.

4. Discussion

Based on the results of our previous SAR study on 2-aminothiazole 5-LO inhibitors [19], we encountered a potency loss between r5-LO and intact PMNL for unsubstituted compounds and a positive shift towards a higher potency in PMNL for compounds bearing a methylated substitution motif. In the present study we demonstrated that the high potency of *N*-phenyl-4-aryl-1,3-thiazole-2-amine derivatives in the purified r5-LO assay might be due to covalent binding of the unsubstituted aminophenolic moiety to C159 and C418. We further give an explanation for the reduced activity in intact cells, as the compounds are capable of binding GSH. Corresponding previous studies have shown that compounds with tampered aminophenolic moiety show only a diminished potency [19]. Further on, compound ST-1905, bearing the quinone imine moiety, and its reduced equivalent ST-1083 presented similar potencies at the r5-LO wt (high potency) and 4C mutant assay (impaired potency) (*cf* Fig. 5). In line, ST-1905 showed a more pronounced binding of GSH in comparison with the hydroquinone amine and a lower potency in intact cells. Together with the previously deduced SAR this suggests that redox activity is one aspect of the inhibitory mode of action. While the DPPH assay attests antiradical activity of the compounds, the Ferene-S assay also approved the capability of reducing Fe^{3+}, therewith providing the basis of a partly redox active mechanism of inhibition. Both lead compounds ST-1853 and ST-1906 showed a higher potency in intact cells than with the purified recombinant enzyme. This is rather unusual, since intact cells have to be permeated and present metabolizing enzymes as well as off targets. Inhibition of 5-LO upstream enzymes ($cPLA_2$, FLAP) would be a plausible explanation for this behavior. Yet in addition to a FLAP inhibitor assay there is no impairment of potency, when analyzing 5-LO product formation with varying AA concentrations or 5-LO stimuli. Furthermore, ST-1853 and ST-1906 could, like the untypical 5-LO inhibitor Hyperforin, interfere with the membrane binding C2-like domain [15]. However, unlike Hyperforin, the potency of ST-1853 is not impaired with the presence of 20 μg/ml phosphatidylcholine, indicating a different mechanism of action. Both lead compounds bear the 2,6-dimethyl substitution pattern which may add inductive effects to enhance reductive properties of the compound. As shown for acetaminophen derivatives this substitution pattern does not impair redox cycling [35]. Yet potentially toxic protein modification by the oxidized form or certain phase II metabolism (in this study shown for glucuronidation) can be prevented by this substitution pattern. Nonetheless modification of the reducing milieu of the intact cell via alteration of the thiol level did not alter the inhibitory potency. An alternative explanation for the higher potency in intact cells could be an enrichment of compound in intact cells leading to higher concentrations at the target area. In contrast to the unsubstituted compounds, ST-1853 and ST-1906 had a higher potency using the purified 4C and C416S enzyme compared to wt 5-LO. C416 of the 5-LO wt may therefore feature negative effects on binding of the lead compounds.

Anti-LT therapy by 5-LO inhibition has been hampered by occurring liver toxicities by Zileuton or the clinical phase II compound Atreleuton [36,37] However both compounds possess a thiophene as well as an *N*-hydroxyurea moiety. These features could be linked with reactive thiophene intermediates [38,39] and redox cycling via electron transfer [40] potentially leading to the toxicity stated above. Indeed, there is no declaration of a class effect concerning liver toxicity by 5-LO inhibition *per se* [36]. The new lead compounds represent novel scaffolds with high efficacy in various in vitro assays. They circumvent bioactivation liabilities by eliminating a metabolic hot spot with the C5 methylated thiazole moiety [41,42] as well as the above-mentioned 2,6 dialkylation. Taken together, based on our in vitro data, unsubstituted aminophenolic derivatives achieve their high potency partly by covalent binding to C159 and C418 of r5-LO. The poor potency of the compounds in intact cells could be explained by GSH trapping. This thiol reactivity may also be an explanation for the observed cytotoxicity of unsubstituted compounds (*cf* [19]). Nonetheless, the identification of novel small molecules targeting C159 and C418 of 5-LO may provide the basis for novel 5-LO drug discovery approaches specifically targeting these cysteine residues. Here, basic approaches can already be seen by another study in our group (Maucher et al., submitted data). The methylation motif of the lead compounds ST-1853 and ST-1906 on the other hand led to higher potencies in intact cells. Here, we could show that the structural motif of the two lead compounds prevented covalent modification of r5-LO as well as phase II metabolism (glucuronidation) while maintaining reducing properties. ST-1853 and ST-1906 displayed a high potency in physiologically relevant assays together with high specificity against related enzymes. Adding the moderate in vitro metabolism and non-cytotoxicity, they present well-balanced, most favorable characteristics that demand for further preclinical screening as anti-inflammatory drug candidates.

Conflict of interests

The authors declare no conflict of interest.

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