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Tandem Benzophenone Amino Pyridines, Potent and Selective Inhibitors of Human Leukotriene C_4 Synthase[S]

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ABSTRACT

Cysteinyl leukotrienes (cys-LTs) are lipid mediators of inflammation. The enzyme catalyzing synthesis of cys-LTs, leukotriene C_4 synthase (LTC4S), is considered an important drug target. Here we report the synthesis and characterization of three tandem benzophenone amino pyridines as inhibitors of LTC4S in vitro and in vivo. The inhibitors were characterized in vitro using recombinant human LTC4S, MonoMac 6 cells, and a panel of peripheral human immune cells. In vivo, the compounds were tested in the Zymosan A-induced peritonitis mouse model. The molecules, denoted TK04, TK04a, and TK05, were potent and selective inhibitors of LTC4S with IC_{50} values of 116, 124, and 95 nM, respectively. Molecular docking revealed binding in a hydrophobic crevice between two enzyme monomers and interaction with two catalytic residues, Arg104 and Arg31. The TK compounds potently inhibited cys-LT biosynthesis in immune cells. In coincubations of platelets and polymorphonuclear leukocytes, inhibition of LTC4S led to shunting of LTA_4 toward anti-inflammatory lipoxin A_4, which was significantly enhanced by simultaneous inhibition of LTA4H. Finally, we found that TK05 (6 $mg \cdot kg^{-1}$·body weight) reduces LTE_4 levels in peritoneal lavage fluid by 88% and significantly decreases vascular permeability in vivo. Our findings indicate that the TK compounds are valuable experimental tools in eicosanoid research in vitro and in vivo. Their chemical structures may serve as leads for further inhibitor design. Novel drugs depleting cys-LT production could be beneficial for treatment of inflammatory diseases associated with overexpression of LTC4S.

Introduction

Cysteinyl leukotrienes (cys-LTs), namely leukotriene (LT) C_4, D_4, and E_4, are lipid mediators derived from arachidonic acid (AA), which is liberated from the nuclear membrane of leukocytes by cytosolic phospholipase A_2 ($cPLA_2$) upon cell stimulation (Haeggstrom and Funk, 2011). 5-Lipoxygenase (5-LO) translocates to the nuclear envelope and with the help of 5-LO-activating protein (FLAP), it converts AA into LTA_4. This highly unstable epoxide can either be hydrolyzed by LTA_4 hydrolase into LTB_4 or conjugated with glutathione to form LTC_4. The target protein of our study, LTC_4 synthase (LTC4S), catalyzes this coupling reaction, e.g., in mast cells, eosinophils, and monocytes. In patients suffering from asthma, the bronchial mucosa is rich in these immune cells and here synthesis and release of cys-LTs causes smooth muscle contraction and mucus secretion, contributing severely to asthmatic symptoms.

LTC4S is an 18-kDa enzyme embedded in the outer membrane of the nucleus and the endoplasmic reticulum of myeloid cells. LTC4S, together with FLAP, microsomal prostaglandin E synthase-1 (mPGES-1), and the microsomal glutathione transferases 1, 2, and 3 (MGST1-3) constitute the superfamily of membrane-associated proteins in eicosanoid and glutathione metabolism (Hebert and Jegerschöld, 2007).

The high-resolution crystal structure of LTC4S, solved by Martinez Molina et al. (2007), revealed a homotrimeric structure.

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ABBREVIATIONS: AA, arachidonic acid; CBMC, cord blood-derived mast cell; cys-LT, cysteinyl leukotrienes; DMSO, dimethylsulfoxide; EtOH, ethanol; EBD, Evans blue dye; FLAP, 5-lipoxygenase-activating protein; GSH, glutathione; 5-HETE, (5*S*, 6*E*, 8*Z*, 11*Z*, 14*Z*)-5-hydroxyeicosa-6, 8, 11, 14-eicosatetraenoic acid; LC-MS/MS, liquid chromatography tandem mass spectrometry; 5-LO, 5-lipoxygenase; LT, leukotriene; LTA_4, leukotriene A_4; LTC_4, leukotriene C_4; LTC4S, leukotriene C_4 synthase; LXA_4, lipoxin A_4; MeOH, methanol; MGST, microsomal glutathione transferase; MM6, MonoMac 6; mPGES-1, microsomal prostaglandin E synthase-1; PBMC, peripheral blood mononuclear cell; PMNL, polymorphonuclear leukocytes; SPE, solid phase extraction.

Each monomer consists of five α-helices, four of which penetrate the membrane. It has been proposed that LTC4S binds its substrate LTA_4 in a hydrophobic crevice on the surface of two adjacent monomers, indicated by a bound detergent molecule of dodecyl maltoside in the crystal structure. The second substrate glutathione (GSH) binds in a deeper pocket below LTA_4 in a unique "horseshoe"-shaped conformation.

There are two established receptors for LTC_4, D_4, and E_4, i.e., CysLT1 (Lynch et al., 1999) and CysLT2 (Heise et al., 2000), the former of which signals classic cys-LT bioactions and is targeted by the lukast class of antileukotrienes, established drugs against asthma. Unfortunately, about 40% of the patients do not respond to this medication (Malmstrom et al., 1999), which may be explained by a more complex receptor system, involving a receptor selective for LTE_4, as suggested in several studies (Kanaoka et al., 2013).

5-LO antagonists are another pharmacological approach in asthma therapy, with Zileuton Abbott Laboratories, Abbott Park, IL as the Food and Drug Administration-approved representative (Rubin et al., 1991). Inhibition of the enzyme highest upstream in LT biosynthesis decreases LTA_4 levels along with LTB_4 and cys-LTs. Despite the resulting beneficial effects of reducing all LTs, 5-LO inhibition additionally leads to reduction of anti-inflammatory and proresolving lipoxins that are also derived from LTA_4, which may lead to delayed resolution of inflammation (Rao et al., 2007).

Selective pharmacological intervention further downstream of 5-LO, at the committed step in cys-LT biosynthesis, may spare lipoxin formation as well as provide higher efficacy than CysLT1 receptor antagonists. LTC4S is thus considered to be a promising approach in the treatment of asthma and inflammation (Haeggström et al., 2010, Devi and Doble, 2012). To date, only a small number of LTC4S inhibitors have thus far been reported and none has evolved to a drug candidate.

In this study we present the synthesis and in vitro and in vivo characterization of three potent LTC4S inhibitors (Nilsson et al., 2011a), here referred to as TK04, TK04a, and TK05, that could serve as experimental tools as well as lead structures for novel inhibitors.

Materials and Methods

Materials. LTA_4 methyl ester (BIOMOL) in tetrahydrofuran was saponified with 1 M LiOH (6%, v/v) for 48 hours at 4°C. All other chemicals were obtained from common commercial sources.

Synthesis of Inhibitors TK04, TK04a, and TK05. See Supplemental Data.

Molecular Docking. Molecular docking was performed using AutoDock 4.2 Molecular Graphics Laboratory, La Jolla, CA, and the input files were prepared using AutoDockTools (Morris et al., 2009). Apo LTC4S structure (PDB ID: 2UUI) as a macromolecular target was retrieved from Research Collaboratory for Structural Bioinformatics (RCSB)-PDB. The PDB coordinates for TK04, TK04a, and TK05 were obtained using eLBOW Lawrence Berkeley Laboratory, Berkeley, CA (Moriarty et al., 2009). The input files were prepared by adding polar hydrogen atoms and Gasteiger charges using AutoDockTools. The grid box size was kept as 60, 58, 72 Å for X, Y, Z with 0.392-Å grid spacing that encompasses the active site of LTC4S. Lamarckian genetic algorithm was used with default settings, and the docking simulation yielded 20 docked conformations for each inhibitor. The resulting conformations were screened based on free energy binding (ΔG) and conservation of key interactions with LTC4S. Structure visualizations and distance measurements were done using PyMOL (Schrodinger, NY, https://www.pymol.org/).

Enzyme Inhibition Assay. The assay was performed, and samples were analyzed as described before (Niegowski et al., 2014a).

MonoMac 6 Cell Culture and Assay. MM6 cells, originally developed by Ziegler-Heitbrock et al. (1988), were cultured and differentiated as described before (Esser et al., 2011). Differentiated MM6 cells (about 2×10^6 cells) suspended in 1 ml PGC buffer (PBS containing 1 $mg \cdot ml^{-1}$ glucose and 1 mM $CaCl_2$) were treated with TK04, TK04a, and TK05 (0.2–8 μM) or vehicle [<1% dimethylsulfoxide (DMSO)] for 30 minutes at 37°C. LT production was initiated with calcium ionophore A23187 (5 μM) with or without AA (40 μM) for 10 minutes before stopping the reaction with 1 ml ice-cold MeOH.

Mast Cell Preparation and Assay. Cord blood-derived mast cells (CBMCs) were derived and then maintained as previously described by Xiang et al. (2006) and Gela et al. (2015). Before activation, cells were incubated with IL-4 (10 $ng \cdot ml^{-1}$) and IL-3 (5 $ng \cdot ml^{-1}$) for 4 days. For activation, cells were incubated with 1 $\mu g \cdot ml^{-1}$ IgE (Calbiochem, Merck Millipore, Darmstadt, Germany) overnight and activated using 2 $\mu g \cdot ml^{-1}$ α-IgE (Sigma, St Louis, MO). For inhibition studies, the TK04, TK04a, and TK05 were added 30 minutes before activation (final concentrations 0.2–8 μM). After 30 minutes of activation, cells (80,000 cells per well, equivalent to 0.4×10^6 $cells \cdot ml^{-1}$) were spun down, and supernatants were collected.

Liquid Chromatography-Mass Spectrometry Analysis of CBMC Supernatant. A stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) method was used for the quantification of lipid mediators in CBMC supernatant, previously reported by Balgoma et al. (2013). The method was modified to quantify the following lipid mediators: 5-HETE, LTC_4, and LTD_4. The detailed LC-MS platform is described in the Supplemental Data. Briefly, CBMC supernatant was mixed with a cocktail of deuterated internal standards and extracted onto Waters Oasis HLB solid phase extraction (SPE) cartridges (Waters Corporation, Milford, MA) before LC-MS/MS analysis. Samples were injected onto an Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) equipped with a BEH C18 column and analyzed on a Waters Xevo TQS-MS in negative and positive electrospray ionization mode. The calibration levels and method parameters of the analyzed compounds are provided in the Supplemental Data and Supplemental Table S1.

Primary Cells. Peripheral blood mononuclear cells (PBMCs), polymorphonuclear leukocytes (PMNL), eosinophils, and platelets were isolated from buffy coats prepared from nonallergic healthy volunteers at the Department of Clinical Immunology and Transfusion Medicine of Karolinska University Hospital Stockholm.

PBMC Preparation and Assay. Human monocytes were prepared from the PBMC fraction after Ficoll-Paque PREMIUM density centrifugation (GE Healthcare, Little Chalfont, UK). Monocytes were isolated by attachment to culture dishes for 2 hours at 37°C followed by washing off nonadherent cells with PBS. Monocytes (4×10^6 cells) in 1 ml PGC buffer (PBS containing 1 mg ml^{-1} glucose and 1 mM $CaCl_2$) were treated with TK04, TK04a, and TK05 in DMSO (1 μM) or vehicle (<0.5% DMSO) for 30 minutes at 37°C. The cells were stimulated with A23187 (5 μM) and AA (20 μM) for 10 minutes at 37°C before stopping the reaction with 1 ml ice cold MeOH.

Primary Eosinophil Assay. Cells were isolated using Ficoll-Paque PREMIUM centrifugation (GE Healthcare) followed by an eosinophil isolation kit (Milteny Biotech, Bergisch Gladbach, Germany), according to the manufacturer's description. Cells (3×10^6) in PGC buffer were incubated with TK04, TK04a, and TK05 in DMSO (1 μM) or vehicle (<0.5% DMSO) for 30 minutes at 37°C. LT production was stimulated with A23187 (5 μM) for 10 minutes at 37°C before stopping the reaction with 1 ml ice-cold MeOH.

Isolation of Platelets and Coincubations with PMNL. Platelets and neutrophils were isolated from the same buffy coat. Platelet-rich plasma was separated from the blood by centrifugation at 600 *g* for 20 minutes. The supernatant was collected and supplemented with 1/10 volume of ACD buffer (85 mM sodium citrate dehydrate, 666 mM citric acid, 111 mM glucose). For the assay, platelets were dissolved in PGC buffer supplemented with 1 $g \cdot l^{-1}$ human albumin.

Neutrophils were obtained by dextran sedimentation of the remaining blood followed by Ficoll-Paque PREMIUM density centrifugation (GE Healthcare) of the supernatant at 2100 *g* for 20 minutes (no brake applied) followed by hypotonic lysis of erythrocytes. Neutrophils were pelleted (300 *g*, 10 minutes) and resuspended in PGC buffer.

Platelets (1×10^9 cells) in 1 ml PGC buffer were preincubated with 10 μM TK04, TK04a, TK05, or vehicle (<0.5% DMSO) for 30 minutes at 37°C followed by addition of 10 μM LTA_4 for 5 minutes at 37°C before stopping the reaction with 1 ml cold MeOH.

For the coincubations, 1×10^9 platelets were allowed to equilibrate with 10×10^6 neutrophils in 1 ml PGC buffer for 5 minutes at 37°C. Next, they were treated with either TK05 in DMSO (final concentrations 0.5–3 μM), with or without additional 0.2 μM SC 57461A in DMSO, or vehicle (<0.5% DMSO) for 30 minutes at 37°C followed by A23187 stimulation (5 μM) for 30 minutes. The reaction was stopped with 1 ml ice cold MeOH.

Analysis of LTs, 5-HETE, and Lipoxins. LTs, 5-HETE, and lipoxins were obtained from the aliquots (2 ml) of the stopped reaction mixtures by SPE before reversed-phase HPLC as described before (Basavarajappa et al., 2014). Prostaglandin B_2 (100 pmol) and 17-OH-C22:4 (100 pmol) were added as internal standards, kind gifts from Mats Hamberg (Karolinska Institutet).

mPGES-1 Activity Assay. The effect of the inhibitors on mPGES-1 was determined using a protocol described by Hammarberg et al. (2009). Reaction mixtures were pretreated with vehicle (0.1% DMSO) or TK04, TK04a, and TK05 (10 μM) for 30 minutes.

MGST2 Activity Assay. The affinity of the inhibitors toward MGST2 was determined essentially as described by Ahmad et al. (2013). Purified recombinant enzyme (0.5 μM) supplemented with 5 μM GSH was incubated with vehicle (2% DMSO) or TK04, TK04a, or TK05 (10 μM) for 30 minutes. The reaction was started by addition of 0.5 mM CDNB. Initial reaction rates were analyzed spectrophotometrically at 340 nm over 1–2 minutes.

5-LO Activity Assay. Purified human recombinant stable 5-LO (5.6 nM) in 100 μl of the reaction buffer (50 mM Tris HCl, 1 mM $MgCl_2$, 0.25 μg 13-HpODE, 25 μg PC, pH 7.5) was preincubated with vehicle (DMSO <1%) or TK04, TK04a, or TK05 (10 μM) for 30 minutes. AA (40 μM) was added, and the initial reaction rate was monitored spectrophotometrically at 234 nm for 2 minutes.

Animals. All animal protocols were carried out as approved by the regional ethical committee for animal experimentation. The study included male wild-type C57BL/6 mice (Harlan, The Netherlands), weighing 20–25 g (n = 18, age 7–8 weeks). Animals were kept in a temperature-controlled room (21 ± 2°C), exposed to light-dark cycles of 12 hours each, and were allowed ad libitum access to water and food.

Zymosan A Induced Peritonitis Mouse Model. Mice were divided into five groups (n = 3–4) to receive intraperitoneal injections of either 1) control (0.25% ethanol in PBS, 500 μl); 2) Zymosan (1 $mg \cdot ml^{-1}$ in 0.25% ethanol in PBS, 500 μl); 3) Zymosan + low dose TK05 (1 $mg \cdot ml^{-1}$ + 10 μM, respectively, in 0.25% EtOH in PBS, 500 μl); 4) Zymosan + high dose TK05 (1 $mg \cdot ml^{-1}$ + 500 μM, respectively, in 0.25% EtOH in PBS, 500 μl), or 5) high-dose TK05 (500 μM in 0.25% EtOH in PBS, 500 μl). The low and high dose of TK05 corresponds to exposure of 0.12 and 6 $mg \cdot kg^{-1}$ body weight, respectively. Evans blue dye (EBD, 1% in PBS, 50 $mg \cdot kg^{-1}$) was injected into the tail vein followed by an intraperitoneal injection of Zymosan and/or compound as described above. After 60 minutes, mice were euthanized and peritoneal exudates were collected using 4 ml PBS.

Detection of EBD in Lavage Fluid. The collected lavage fluid was spun at 3000 rpm for 10 minutes. Lavage fluid (200 μl) was subjected to absorbance spectrophotometry (Multiskan FC Microplate Photometer, Thermo Fisher Scientific, Waltham, MA) at 620 nm.

Analysis of LTs in Mouse Peritoneal Lavage Fluid. LTE_4, the only cys-LT detected in lavage fluid, was analyzed by SPE followed by HPLC as described above. LTB_4 levels were analyzed using an LTB_4 EIA Kit (Cayman, Ann Arbor, MI).

Statistical Analysis. GraphPad Prism 6 (La Jolla, CA) was used to calculate the kinetic parameters IC_{50} and K_i. IC_{50} was determined using the equation Y = bottom + (top − bottom)/$(1+10^{X\text{-LogIC50}})$, and K_i was determined using the Michaelis-Menten equation edited for competitive inhibition.

Results

Synthesis of inhibitors TK04, TK04a, and TK05. In this study, we present a detailed chemical synthesis of the compounds TK04, TK04a, and TK05 in 8, 8, and 10 steps, respectively. We based our synthesis on a procedure published by Nilsson et al. (2011a). Our protocol involves the use of aldehydes instead of carboxylic acid chlorides in the first step of the synthesis. Moreover, we chose a convergent approach, as opposed to the original linear strategy, to increase the efficiency of the reactions including three crucial steps. A Grignard reaction of an aromatic iodide with an aryl aldehyde on the one hand and a Buchwald coupling of a monoalkylated arylamine and an aromatic bromide on the other led to two bis-aromatic building blocks. Those were coupled in the second Grignard reaction. The cyclopropyl moiety demanded two extra steps in the synthesis of TK05 before the Buchwald coupling: a condensation of cyclopropanecarbaldehyde and 4-chloroaniline followed by the reduction of the resulting imine to the corresponding secondary amine. The entire synthesis is shown in Supplemental Fig. S1, and the chemical structures of the three inhibitors are depicted in Fig. 1A.

Molecular Docking. The docked conformation for each ligand was chosen based on the lowest predicted free energy of binding. All three inhibitors have taken up an extended conformation as a favored mode of binding at the active site of LTC4S. The inhibitors prefer the same location in the hydrophobic cleft of the active site at the dimer interface (Fig. 1B), the same region where a bound dodecyl maltoside molecule was observed in the crystal structure of LTC4S (PDB ID: 2UUH), possibly mimicking the LTA_4 substrate. The binding site of the compounds in this docking study supports the competitive mode of inhibition for all three inhibitors observed in the present study (see below). Additionally, we observed that these inhibitors are accommodated within the active site in alignment with Trp-116, which has been suggested to act as a molecular ruler for the positioning of LTA_4 substrate but also as a lid involved in product release from the active site (Martinez Molina et al., 2007, Niegowski et al., 2014b). The orientation of all three inhibitors is consistent by facing its chlorine end toward Trp-116 and the methyl end pointing opposite to the hydrophobic pocket.

At this docked conformation, all three inhibitors exhibit three key ionic interactions with two arginine residues of LTC4S. The carbonyl oxygen between rings b and c of the inhibitor establishes the interaction with Arg-104 and Arg-31 from the adjacent monomer interacts with the oxygen of the carboxyl group on ring b. The predicted docked conformations were further strengthened by a third interaction created by the carbonyl oxygen between rings a and b with Arg-31 (Fig. 1, C–E). Because of the presence of a methoxy group on ring a, TK04a and TK05 were further stabilized by interaction with Ser-36 (Fig. 1, D and E).

Inhibition of Human Recombinant LTC4S. We found that TK04, TK04a, and TK05 are nanomolar inhibitors of the isolated human enzyme in agreement with Nilsson et al. (2011a). At constant substrate concentrations, we determined the IC_{50} values to 116 ± 18 nM (TK04), 124 ± 10 nM (TK04a),

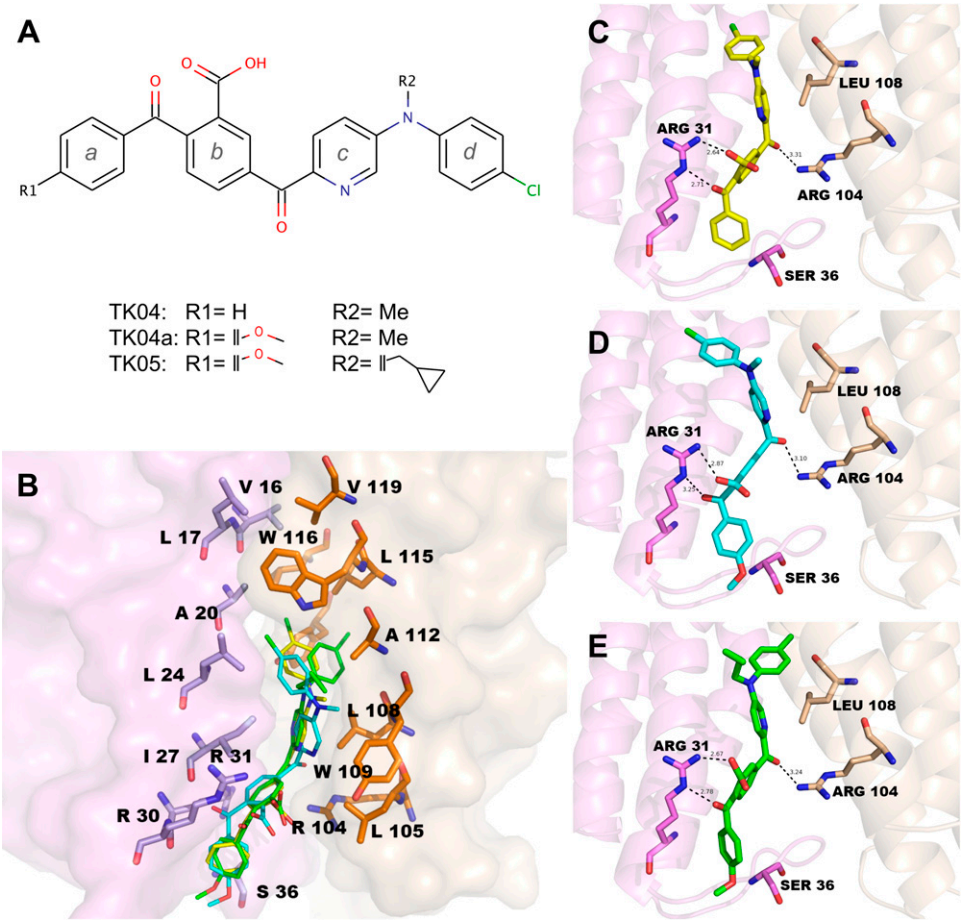

Fig. 1. Chemical structure of the TK inhibitors and molecular docking. (A) Chemical structure of TK04, TK04a, and TK05. (B) Superposition of docked inhibitors TK04 (yellow), TK04a (cyan), and TK05 (green) located at the hydrophobic cleft of the LTC4S active site. (C) Interaction of docked inhibitor TK04 (yellow), TK04a (cyan), and TK05 (green) with Arg-104 (wheat) and Arg-31 (violet).

and 95 ± 25 nM (TK05). When varying the amount of the substrate LTA_4, we observed a competitive mode of inhibition, consistent with the docking data. K_i values were calculated to 13 ± 7 nM (TK04), 53 ± 15 nM (TK04a), and 6 ± 5 nM (TK05). Interestingly, LTC4S inhibition was time dependent, with a maximum effect after 30 minutes on both the isolated and the cellular enzyme (Supplemental Fig. S2).

Specificity of TK04, TK04a, and TK05 for LTC4S. To assess their specificity, we treated the membrane-associated proteins in eicosanoid and glutathione metabolism enzymes mPGES-1 and MGST2 as well as the upstream biosynthetic enzyme 5-LO with the TK inhibitors. We found that TK04, TK04a, and TK05 had no significant inhibitory effect on mPGES-1, MGST2, or 5-LO at a concentration of 10 μM (Fig. 2).

Inhibition of LTC4S in Differentiated MM6 Cells. To study the cellular activity of the inhibitors, we chose differentiated MM6 cells, a human monocytic cell line with high expression of 5-LO and LTC4S and low expression of LTA_4 hydrolase. We found that all three compounds reduced LTC_4 production in a dose-dependent manner with IC_{50} values of 666 ± 79 nM (TK04) and 1820 ± 208 nM (TK04a), and 318 ± 60 nM (TK05) as shown in Fig. 3A. Consistent with the isolated enzyme assay, TK05 and TK04a were found to be the strongest and weakest inhibitor, respectively. To further elucidate the compound's effect on the LT pathway in MM6 cells, we additionally measured formation of 5-HETE, the reduced hydroxyl derivative of the hydroperoxide 5-HpETE, an intermediate during LTA_4 production and thus an indicator of effects on the 5-LO/FLAP complex. We also determined effects on other LTA_4-derived products, namely LTB_4, as well as the nonenzymatic degradation products all-*trans*-LTB_4 and 12-epi all-*trans*-LTB_4. When treated with the TK compounds, we observed increased levels of LTB_4 and its all-*trans* isomers peaking at 1 μM inhibitor concentration. In contrast, 5-HETE was reduced to 40% by 8 μM TK04a and to 40% and 20% by 3 and 8 μM TK05, respectively (Fig. 3B).

When challenged with additional 40 μM AA, the IC_{50} values of TK04, TK04a, and TK05 slightly increased to 1289 ± 391 nM, 2217 ± 659 nM, and 446 ± 150 nM, respectively.

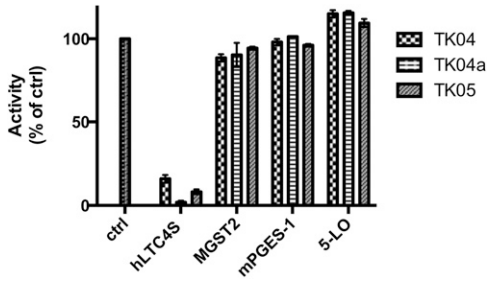

Fig. 2. Specificity of TK inhibitors for LTC4S. The activity of MGST2, mPGES-1 and stable 5-LO was not affected by 10 μM TK04, TK04a, or TK05. Data are presented as percentage of control (ctrl; 100%), mean ± S.E.M. (n = 3).

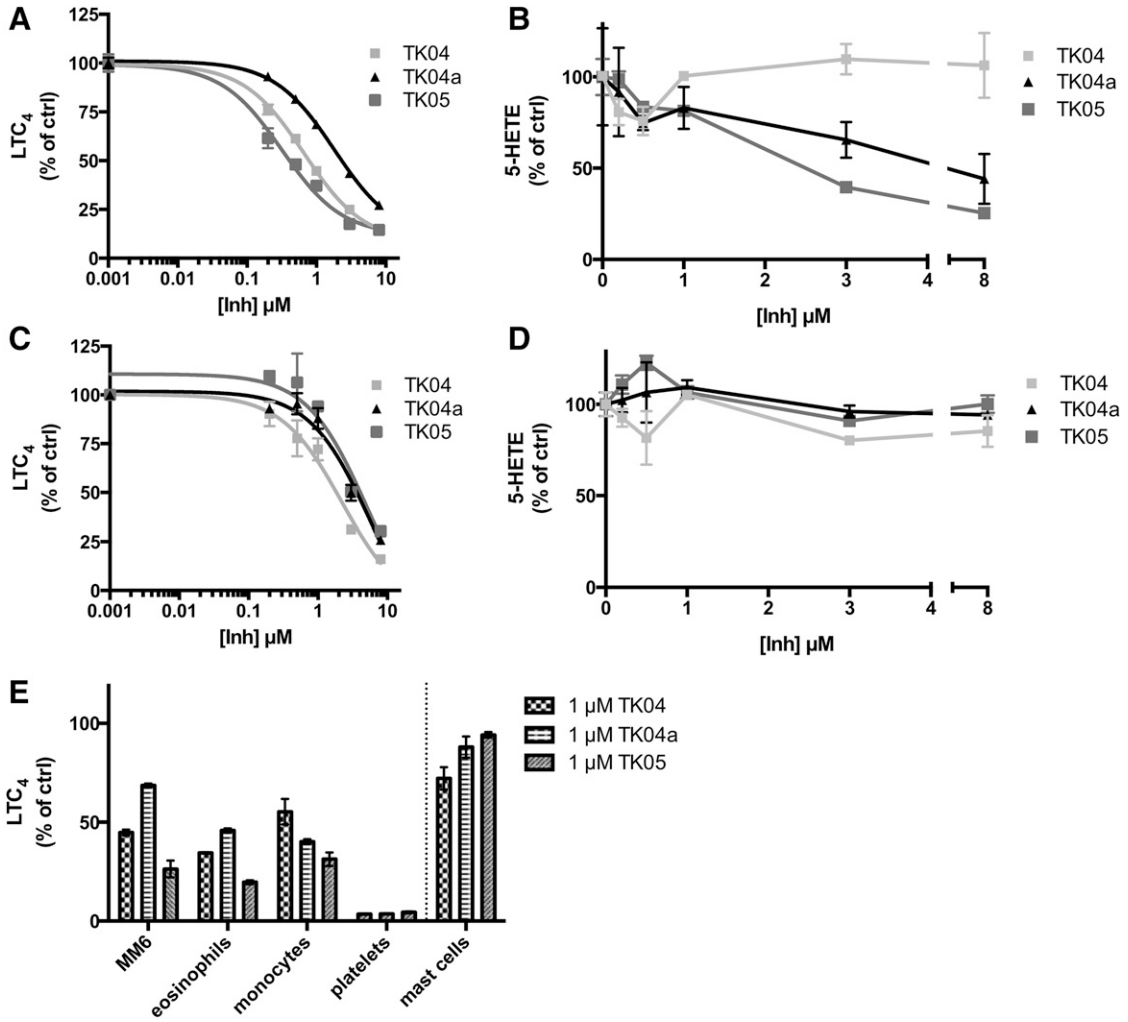

Fig. 3. Inhibition of LTC_4 formation in the cell line MM6 and peripheral immune cells. (A) Differentiated MM6 cells (1×10^6 cells·ml^{-1}) were preincubated with TK inhibitors (0.2–8 μM) or vehicle (<1% DMSO) for 30 minutes at 37°C. LT formation was initiated with A23187 (5 μM) for 10 minutes, and LTC_4 was analyzed with HPLC. Data are presented as percentage of ctrl (100%), mean ± S.E.M. (n = 3), and each observation represents one out of two or three independent experiments. LTC_4 concentrations of ctrl samples were 30.4 ± 6.0 pmol·10^6 cells. (B) The effect of TK inhibitors on 5-HETE formation in MM6 cells. (C) CBMCs were incubated with 10 ng·ml^{-1} IL-4 and 5 ng·ml^{-1} IL-3 for 4 days followed by IgE treatment (1 μg) overnight. Cells were incubated with TK04, TK04a, and TK05 (0.2–8 μM) for 30 minutes and then activated using 2 μg·ml^{-1} α-IgE for 30 minutes. Data are presented as percentage of ctrl (100%), mean ± S.E.M. (n = 4), ctrl samples had a LTC_4 concentration of 1.46 ± 0.22 ng·ml cell supernatant. (D) Effect of TK04, TK04a, and TK05 on 5-HETE formation in mast cells. (E) Effect of the TK inhibitors (1 μM) on LTC4S in MM6, eosinophils, PBMCs, platelets, and CBMCs. In contrast to the other cell types, CBMCs were stimulated with IgE and LTC_4 formation analyzed with LC-MS. Data are presented as percentage of ctrl (100%), mean ± S.E.M. (n = 3) and represents one out of two independent experiments.

Inhibition of LTC4S in Human Cord Blood Derived Mast Cells. Cord blood-derived mast cells (CBMCs) were treated with the same concentrations of the inhibitors as the MM6 cells. Surprisingly, we found an increase in the IC_{50} values for all compounds with 2194 nM (TK04), 5644 nM (TK04a), and 5098 nM (TK05) (Fig. 3C). LTD_4 was inhibited in a similar fashion, whereas 5-HETE levels were not affected by the inhibitors (Fig. 3D).

Inhibition of LTC4S in Human Monocytes. To verify our MM6 cell data with primary cells, we treated human monocytes with our inhibitors. We observed 45, 60, and 69% inhibition of LTC_4 production when treated with 1 μM TK04, TK04a, and TK05, whereas 5-HETE, LTB_4, and its two all-*trans* isomers, nonenzymatic hydrolysis products of LTA_4, were not affected (data not shown).

Inhibition of LTC4S in Human Eosinophils. We conducted the same experiments with isolated human eosinophils, another type of inflammatory cells that express LTC4S. One micromole of TK04, TK04a, and TK05 reduced LTC_4 formation by 65, 49, and 80%, respectively, compared with control cells.

The effects of the TK inhibitors (1 μM) on LTC4S in all tested cell types are compared in Fig. 3E.

Inhibition of LTC4 in Isolated Human Platelets and Coincubations with Neutrophils. Human platelets express not only LTC4S but also 12-lipoxygenase, allowing them to produce both inflammatory LTC_4 and anti-inflammatory lipoxins. On the HPLC-UV system, LXA_4, LXB_4, as well as their all-*trans* isomers, could be detected (Serhan and Sheppard, 1990). First, we incubated untreated platelets and platelets treated with 1 μM of TK04, TK04a, and TK05 with 10 μM exogenous LTA_4 and found reduced LTC_4 production by >95% for all three inhibitors along with a significant increase in the overall lipoxin production and

LXA_4 alone by TK04 and TK05 (Supplemental Fig. S3). In a second experimental setting, we coincubated platelets with neutrophils producing LTA_4, allowing transcellular LT metabolism. Upon LTC4S inhibition with TK05 (Fig. 4A), we again observed significantly increased levels of LXA_4 to 1.6-fold of control levels for inhibitor concentrations of 0.5 μM, whereas overall lipoxin formation was increased, but not statistically significant. Formation of LTB_4 was increased in a similar fashion to about 1.4-fold upon LTC4S inhibition, whereas 5-HETE levels did not differ from controls (Fig. 4B). Moreover, we observed that this effect was further boosted with the addition of an LTA_4 hydrolase inhibitor (SC 57461A). Incubations of platelets together with neutrophils with both 0.2 μM SC 57461A and 1 μM TK05 resulted in more than threefold increased lipoxin and LXA_4 levels compared with control cells (Fig. 4, C and D).

Inhibition of LTC4S In Vivo. Zymosan A-induced peritoneal inflammation in mice is driven by cys-LTs (Kanaoka et al., 2001). Using this model, we found that 0.12 $mg \cdot kg^{-1}$ of TK05 significantly reduces LTE_4 levels, whereas 6 $mg \cdot kg^{-1}$ body weight of the compound resulted in almost complete inhibition compared with mice treated with only Zymosan A. LTE_4 could neither be detected in the control mice nor in animals treated with only 6 $mg \cdot kg^{-1}$ (Fig. 5A). Cys-LT production leads to increased permeability of small blood vessels at the sites of inflammation. The leakage of intravenously injected EBD serves as a marker for the extravasation of plasma proteins. The lower dose of TK05 slightly reduced plasma exudation evoked by Zymosan, whereas the effect of the high dose was significant (Fig. 5B). LTB_4 levels were not significantly altered by either doses of TK05 compared with the Zymosan group (Fig. 5C).

Discussion

Cys-LTs are involved in many inflammatory conditions, especially asthma and allergic rhinitis, and inhibitors of 5-LO and CysLT1 receptor antagonists are established medications for these diseases. However, there is a need for more effective and safe treatments. Drugs that inhibit the committed step in cys-LT biosynthesis, i.e., the enzyme LTC4S, may have fewer side effects than 5-LO inhibitors because they do not interfere with prostaglandin or lipoxin metabolism. Additionally, responsiveness may increase in comparison with receptor antagonist, because all cys-LT signaling will be interrupted.

As a membrane protein, LTC4S is a difficult target for inhibitor design. Potential inhibitors require both hydrophilic and lipophilic features to enter and bind well to the catalytic site. The proposed hydrophobic binding site for LTA_4 provides few amino acid residues for strong hydrogen or electrostatic bonds. Inhibition has to occur mainly via weaker van der Waals and hydrophobic forces. This has made drug development challenging (Devi and Doble, 2012).

In this study we synthesized three potent inhibitors of LTC4S, denoted TK04, TK04a, and TK05, and characterized them both in vitro against purified enzyme and immune cells as well as in vivo in a mouse model of peritonitis.

Previous attempts to develop LTC4S inhibitors have led to several inhibitors with IC_{50} values in the micromolar range, e.g., LL-699.333 and MK886 from Merck (Kenilworth, NJ), which, however, are unselective (Gupta et al., 1997; Sala et al., 1997). The crystal structure of LTC4S, published in 2007, allowed new efforts in the field. Apart from the series of bis-aromatic compounds that include our TK inhibitors, libraries of methylenebisphenyl, bis-aryl, and indole molecules

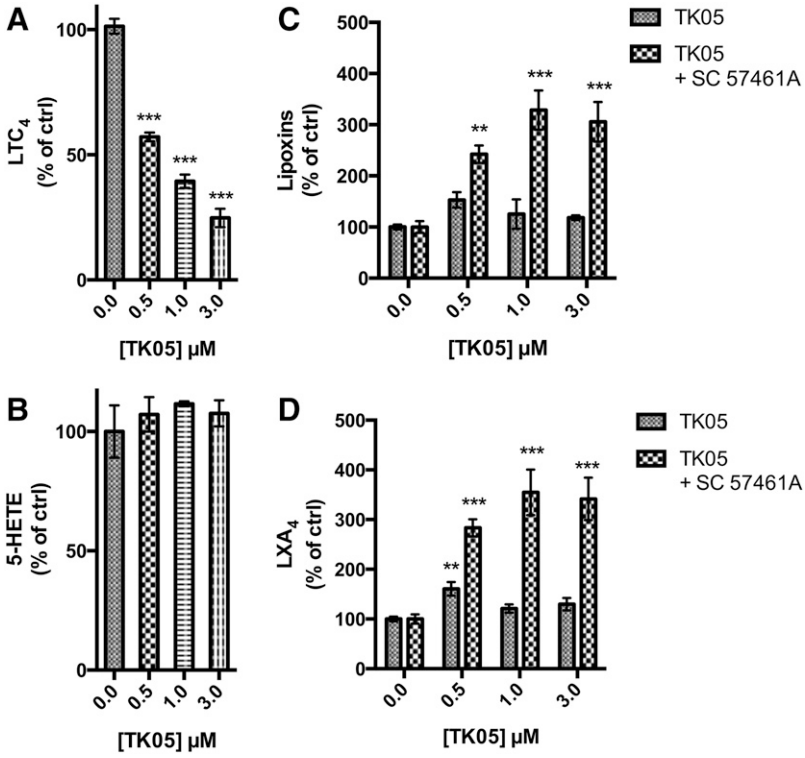

Fig. 4. Effect on LTC_4 and lipoxin formation in coincubations of platelets and neutrophils. 1×10^9 platelets and 10×10^6 PMNL were mixed for equilibration for 5 minutes at 37°C. TK05 (0.5–3 μM) or vehicle (<0.5% DMSO) was added for 30 minutes with or without addition of the LTA4H inhibitor SC 57461A (0.2 μM) at 37°C followed by A23187 (5 μM) stimulation for 30 minutes at 37°C. Effect of TK05 on the biosynthesis of LTC_4 (A), 5-HETE (B), and overall lipoxin levels (C) and LXA_4 (D) specifically. Data are presented as percentage of ctrl (100%), mean ± S.E.M. (n = 3, $^{**}P < 0.01$, $^{***}P < 0.001$) and represents one out of two independent experiments.

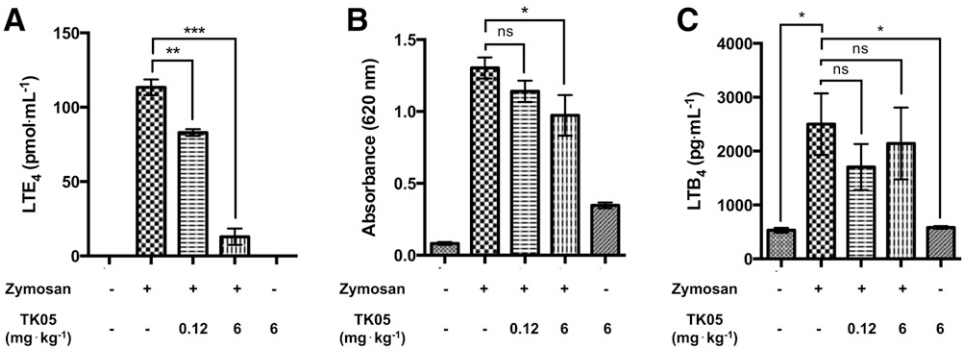

Fig. 5. Effect of TK05 on Zymosan A-induced peritonitis in mice. Eighteen male wild-type mice received a tail vein injection of 1% EBD, immediately followed by a peritoneal injection of PBS + 0.5% EtOH (ctrl), Zymosan, Zymosan and 0.12 mg·kg^{-1} TK05, Zymosan and 6 mg·kg^{-1} TK05, or 6 mg·kg^{-1} TK05. The Peritoneal lavage fluid was analyzed for LTE_4 formation (A), leakage of EBD (B), and LTB_4 formation (C). Data are presented as mean ± S.E.M. (n = 3–4, *$P < 0.05$, **$P < 0.01$, ***$P <$ 0.001, ns = not significant).

have been developed and patented (Pelcman and Nilsson, 2008, Nilsson, 2010, Nilsson et al., 2011b). Some of those exhibit low nM IC_{50} values, but to our knowledge, they have not been further studied. More recently, Ago et al. (2013) published an in silico screening focused on the GSH binding site of LTC4S, which resulted in a series of 5-(5-methylene-4-oxo-4,5-dihydrothiazol-2-ylamino) isophthalic acid compounds. The strongest candidate (IC_{50} 1.9 μM) was active in vitro and in cell assays but was shown to be a dual inhibitor of LTC4S and 5-LO.

TK04, TK04a, and TK05 are hydrophobic compounds with a backbone of four aromatic rings connected by two benzophenone moieties and an alkylated nitrogen connecting rings c and d. Addition of a cyclopropyl ring to that nitrogen in TK05 increases its activity. Our results show that our TK compounds potently inhibit LTC4S in cell free and whole cell assays. We did not observe any cell toxicity during incubations of MM6 cells with 10 μM compound for 24 hours (data not shown). Furthermore, TK04, TK04a, and TK05 are specific toward LTC4S without interacting with mPGES-1, MGST-2, or 5-LO. However, the effect of TK04a and TK05 on 5-HETE production may suggest a weak interference with FLAP at higher concentrations.

The results of the molecular docking study suggest that TK04, TK04a, and TK05 inhibit the action of LTC4S competitively, by occupying the proposed binding site for LTA_4 and interacting with the key catalytic arginine residues.

Several cys-LT producing cells are notorious for their role in inflammatory and allergic disease, recently reviewed by Liu and Yokomizo (2015). Allergic reactions are largely driven by IgE-mediated degranulation of mast cells. In nasal allergy, cys-LTs are released during both the early- and late-phase response to allergens (Haberal and Corey, 2003) and result in reinforced symptoms, e.g., sneezing, rhinorrhea, and nasal obstruction (Miadonna et al., 1987). Mast cells are also a key player in the pathogenesis of abdominal aortic aneurysm (Swedenborg et al., 2011). Elevated levels of LTC4S expression along with production of cys-LTs were discovered in human abdominal aortic aneurysm thrombus and wall tissue (Di Gennaro et al., 2010). In our hands, TK04, TK04a, and TK05 inhibited cys-LT formation with IC_{50} values in the low micromolar range in human mast cells activated by cross-linking of the IgE receptor, indicating that these compounds are able to target cys-LT biosynthesis during mast cell dependent inflammation.

Eosinophil infiltration is a well-known characteristic of allergic asthma (Beasley et al., 1989), and the cell number correlates with the severity of the disease (Bousquet et al., 1990). They are believed to contribute to asthmatic symptoms by secreting cytotoxic agents and bronchoconstrictive LTs (Nakagome and Nagata, 2011) and it has been shown that eosinophils are the predominant source of cys-LTs in patients suffering from aspirin-intolerant asthma (Cowburn et al., 1998). Our data show that 1 μM of our compound can reduce eosinophil-derived LTC_4 by up to 80%.

Monocytes are another type of immune cells capable of cys-LT production. TK04, TK04a, and TK05 were comparably effective as in the monocytic cell line MM6. Monocytes migrate from the blood stream to the site of infection or tissue damage, where they eliminate noxious molecules and cells with phagocytosis or differentiate into macrophages or dendritic cells. Their recruitment is associated with inflammatory conditions, e.g., atherosclerosis (Shi and Pamer, 2011).

As a result, targeting LTC_4 production of resident mast cells and recruited eosinophils may be a successful approach in the clinical management of many acute and chronic inflammatory and allergic diseases. Our inhibitors were less potent against mast cells compared with monocytes, eosinophils, platelets, and MM6 cells. A possible explanation for this may be different degrees of LTC4S phosphorylation among cell types, which in turn may influence binding of the inhibitors, a subject that is currently under investigation in our laboratory.

Platelets play a fundamental role in primary hemostasis, but more recent work has also implicated these corpuscles in inflammation (Gros et al., 2014). Overproduction of cys-LTs by platelet-adherent leukocytes has been shown to amplify tissue inflammation in aspirin-exacerbated respiratory disease (Laidlaw et al., 2012) as well as contribute to pathologic vascular events (Cerletti et al., 2010). We found that LTC_4 production in isolated platelets incubated with exogenous LTA_4 is almost completely blocked by TK04, TK04a, and TK05 in vitro. In parallel, we observed a significant increase in lipoxin formation. In vivo, platelet-dependent LTC_4 and LXA_4 biosynthesis requires a neighboring leukocyte capable of donating LTA_4 through transcellular routes. Therefore we tested our LTC4S inhibitors in mixed platelet neutrophil suspensions. In this setting, we found that TK05 decreased LTC_4 synthesis, whereas LXA_4 formation was significantly increased at a TK05 concentration of 0.5 μM. Furthermore, we show that this effect became more pronounced upon simultaneous inhibition of both LTC4S and LTA4H, presumably reflecting increased export of LTA_4 from the neutrophils when conversion into LTB_4 is blocked in agreement with Lehmann et al. (submitted manuscript). These data suggest that specific LTC4S inhibition may contribute to resolution of airway inflammation in two ways: by reducing the formation of proinflammatory cys-LTs and by boosting generation of

anti-inflammatory lipoxins (Levy et al., 2002). Moreover, a dual LTC4S and LTA4H inhibitor will most likely have an even stronger effect due to additional inhibition of proinflammatory LTB_4 formation together with enhanced lipoxin production.

For in vivo testing we used Zymosan A-induced peritonitis in the mouse, an inflammation model in which cys-LTs contribute to extravasation of plasma, as demonstrated with LTC4S-deficient mice (Kanaoka et al., 2001). In our hands, a dose of 6 $mg \cdot kg^{-1}$ LTC4S inhibitor TK05 administered intraperitoneally reduced amounts of LTE_4 in lavage fluid by almost 90%. Additionally, we observed decreased plasma protein extravasation as judged by measurements of EBD in lavage fluid. Together with earlier data showing that purified human and mouse LTC4S are both inhibited by the TK compounds (Niegowski et al., 2014a), the results of the present study supports the notion that mouse models will be valuable tools to assess the efficacy of LTC4S inhibitors.

There is a distinct need for new and improved medications against asthma and LTC4S is one promising target. Pharmacological intervention of cys-LT production by blocking LTC4S would be an approach that circumvents multiple receptors and intervention with the lipoxin metabolism and has not yet been explored. Our findings demonstrate that the LTC4S inhibitors TK04, TK04a, and TK05 are potent and selective inhibitors of human LTC4S, which block LTC_4 and cys-LT synthesis both in vitro and in vivo. Thus we now have useful experimental tools in leukotriene research as well as lead structures for further LTC4S inhibitor design. Finally, our data suggest that a combination of LTC4S and LTA4H inhibitors would perhaps be an even better approach that will block both arms of the leukotriene signaling pathways, whereas boosting synthesis of anti-inflammatory and proresolving lipoxins.

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Authorship Contributions

Participated in research design: Kleinschmidt, Haraldsson, Basavarajappa, Kahnt, Lindbom, Haeggström.

Conducted experiments: Kleinschmidt, Lundeberg, Thulasingam, Ekoff, Fauland, Lehmann.

Contributed new reagents or analytic tools: Kleinschmidt.

Performed data analysis: Kleinschmidt, Lundeberg, Thulasingam, Fauland.

Wrote or contributed to the writing of the manuscript: Kleinschmidt, Haraldsson, Lundeberg, Thulasingam, Ekoff, Fauland, Haeggström.

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