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RESEARCH PAPER

Pharmacological characterization of the first potent and selective antagonist at the cysteinyl leukotriene 2 ($CysLT_2$) receptor

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Background and purpose: Cysteinyl leukotrienes (CysLTs) have been implicated in the pathophysiology of inflammatory and cardiovascular disorders. Their actions are mediated by $CysLT_1$ and $CysLT_2$ receptors. Here we report the discovery of 3-({[(1S,3S)-3-carboxycyclohexyl]amino}carbonyl)-4-(3-{4-[4-(cyclo-hexyloxy)butoxy]phenyl}propoxy) benzoic acid (HAMI3379), the first potent and selective $CysLT_2$ receptor antagonist.
Experimental approach: Pharmacological characterization of HAMI3379 was performed using stably transfected $CysLT_1$ and $CysLT_2$ receptor cell lines, and isolated, Langendorff-perfused, guinea pig hearts.
Key results: In a $CysLT_2$ receptor reporter cell line, HAMI3379 antagonized leukotriene D_4- (LTD_4-) and leukotriene C_4- (LTC_4-) induced intracellular calcium mobilization with IC_{50} values of 3.8 nM and 4.4 nM respectively. In contrast, HAMI3379 exhibited very low potency on a recombinant $CysLT_1$ receptor cell line (IC_{50} > 10 000 nM). In addition, HAMI3379 did not exhibit any agonistic activity on both CysLT receptor cell lines. In binding studies using membranes from the $CysLT_2$ and $CysLT_1$ receptor cell lines, HAMI3379 inhibited $[^3H]$-LTD_4 binding with IC_{50} values of 38 nM and >10 000 nM respectively. In isolated Langendorff-perfused guinea pig hearts HAMI3379 concentration-dependently inhibited and reversed the LTC_4-induced perfusion pressure increase and contractility decrease. The selective $CysLT_1$ receptor antagonist zafirlukast was found to be inactive in this experimental setting.
Conclusions and implications: HAMI3379 was identified as a potent and selective $CysLT_2$ receptor antagonist, which was devoid of CysLT receptor agonism. Using this compound, we showed that the cardiac effects of CysLTs are predominantly mediated by the $CysLT_2$ receptor.
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Keywords: $CysLT_2$ receptor antagonist; cysteinyl leukotrienes; $CysLT_1$ receptor; $CysLT_2$ receptor

Abbreviations: CysLT, cysteinyl leukotriene; FLAP, 5-lipoxygenase activating protein; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4

Introduction

Cysteinyl leukotrienes (CysLTs), namely leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4), are the products of the 5-lipoxygenase pathway in arachidonic acid metabolism, and are predominantly synthesized by inflammatory cells, such as polymorphonuclear leukocytes, macrophages and mast cells (Funk, 2001). CysLTs have been implicated in a number of pathological inflammatory diseases, including asthma and allergic rhinitis (Drazen *et al.*, 1999; Sharma and Mohammed, 2006; Capra *et al.*, 2007; Riccioni *et al.*, 2007). In addition, CysLTs are also involved in the pathophysiology of different cardiovascular disorders, including atherosclerosis, unstable angina pectoris and acute myocardial infarction. CysLTs reduce coronary blood flow, myocardial contractility and cardiac output without affecting the heart rate. They also increase vascular permeability, which ultimately leads to oedema formation (Burke *et al.*, 1982; Letts, 1987; Fauler and Frölich, 1989; Funk, 2005; Bäck, 2009). In patients with ischemic heart disease and acute myocardial infarction, elevated LTC_4 plasma levels and increased urinary LTE_4 excretion have been observed (Carry *et al.*, 1992; Takase

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et al., 1996). Human atherosclerotic coronary arteries have been shown to be hyperreactive in response to CysLTs, and all components of the 5-lipoxygenase pathway have been detected in the arterial walls of patients with various stages of atherosclerosis (Allen *et al*., 1993, 1998; Spanbroek *et al*., 2003). Variants of the gene encoding 5-lipoxygenase activating protein (FLAP), the key regulator of the CysLT synthetic pathway, have recently been linked to increased leukotriene production and to the pathogenesis of myocardial infarction (Helgadottir *et al*., 2004).

CysLT effects are mediated by at least two different G protein-coupled receptors (GPCRs), the $CysLT_1$ and the $CysLT_2$ receptor (nomenclature follows Alexander *et al*., 2009). These receptors were originally defined pharmacologically, based on their sensitivity to $CysLT_1$ receptor-specific antagonists (Coleman *et al*., 1995). Both receptors have been isolated and functionally characterized. Activation of both receptors is coupled to intracellular calcium mobilization. Therefore, the Ca^{2+}-sensitive photoprotein aequorin and fluorescent calcium indicator dyes have been used to study functional receptor activation in mammalian cell lines *in vitro* (Lynch *et al*., 1999; Sarau *et al*., 1999; Heise *et al*., 2000; Nothacker *et al*., 2000; Takasaki *et al*., 2000; Hui *et al*., 2001; Ogasawara *et al*., 2002). Recently, a third putative CysLT receptor subtype, formerly GPR17, has been identified (Ciana *et al*., 2006). In addition, a CysLT receptor subtype preferentially activated by LTE_4 has also been described (Maekawa *et al*., 2008).

The most studied classes of CysLT receptor antagonists are $CysLT_1$ receptor selective antagonists such as montelukast (Singulair™, Merck & Co, Whitehouse Station, NJ, USA), zafirlukast (Accolate™, AstraZeneca, London, UK) and pranlukast (Onon™, Ono Pharmaceutical, Osaka, Japan), which are used clinically for the treatment of bronchial asthma and allergic rhinitis (Drazen *et al*., 1999; Riccioni *et al*., 2007). The only $CysLT_2$ receptor antagonist identified so far is the LTE_4 analogue BAY u9773. However, BAY u9773 has been reported as a dual $CysLT_1$ and $CysLT_2$ receptor antagonist and as a partial agonist at the $CysLT_2$ receptor (Labat *et al*., 1992; Tudhope *et al*., 1994).

$CysLT_2$ receptors are strongly expressed in cardiovascular tissues. Cardiac expression of the $CysLT_2$ receptor could be detected throughout the entire human heart, including ventricles, atrium, septum, apex and Purkinje fiber cells, as well as in vascular endothelial and smooth muscle cells (Heise *et al*., 2000; Nothacker *et al*., 2000; Takasaki *et al*., 2000; Hui *et al*., 2001; Kamohara *et al*., 2001; Moos *et al*., 2008). In contrast, expression of the $CysLT_1$ receptor within the cardiovascular system is barely detectable (Lynch *et al*., 1999; Sarau *et al*., 1999; Kamohara *et al*., 2001). As $CysLT_2$ receptors are highly expressed in heart and blood vessels, a role for this receptor in the pathogenesis of various cardiovascular diseases might be anticipated. In a study using $CysLT_2$ receptor-deficient mice, a function for the $CysLT_2$ receptor in inflammatory responses and pulmonary fibrosis could be demonstrated (Beller *et al*., 2004). However, the cardiovascular phenotype of these knockout mice was not further characterized. Using $CysLT_2$ receptor transgenic, as well as knockout mice, a role for the $CysLT_2$ receptor in vascular permeability and myocardial ischemia/reperfusion injury has recently been shown (Hui *et al*., 2004; Jiang *et al*., 2008; Moos *et al*., 2008).

Therefore, $CysLT_2$ receptor antagonists are very desirable as both pharmacological tools to probe the CysLT pathway and as potential therapeutic agents. However, the characterization of the (patho)physiological role of the $CysLT_2$ receptor is currently hampered by the fact that no selective $CysLT_2$ receptor antagonists are available. We report here the identification of HAMI3379, a potent and selective $CysLT_2$ receptor antagonist that does not exhibit agonistic activity at CysLT receptors. Using this compound, we showed that the effects of CysLTs on myocardial contractility and coronary blood flow were mediated by the $CysLT_2$ receptor.

Methods

Characterization of recombinant $CysLT_1$ and $CysLT_2$ receptor cell lines

Full-length DNAs encoding human $CysLT_1$ and $CysLT_2$ receptors were amplified from human genomic DNA by PCR. A Kozak consensus sequence was artificially introduced, both PCR fragments were cloned into mammalian expression vectors (Invitrogen, Carlsbad, CA, USA), and receptor sequences were verified by sequencing. Both constructs were transfected by electroporation into recombinant apoaequorin-expressing Chinese hamster ovary (CHO) cells. After selection with geneticin (G418), positive clones were identified by stimulation with LTD_4 and purified twice by the limited dilution technique.

Cells were cultured at 37°C and 5% CO_2 in DMEM/F12 with Glutamax supplemented with 10% (v/v) inactivated fetal calf serum, 20 mM HEPES, 1.4 mM sodium pyruvate, 1.8 mM sodium bicarbonate, 50 $U \cdot mL^{-1}$ penicillin, 50 $\mu g \cdot mL^{-1}$ streptomycin and 1 $mg \cdot mL^{-1}$ geneticin. Confluent cultures were passaged using trypsin. All cell culture reagents were obtained from Invitrogen.

Measurement of agonist-induced calcium mobilization was performed according to Heise *et al*. (2000). Agonist testing was performed on opaque 384 well microtiter plates (MTP). Two thousand five hundred cells per well were cultured to confluence for 2 days. After removal of the cell culture medium, cells were loaded for 3 h at 37°C and 5% CO_2 with 5 $\mu g \cdot mL^{-1}$ coelenterazine in Ca^{2+}-free Tyrode (130 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 4.8 mM $NaHCO_3$, pH 7.4). Test compounds were applied for 5 min in Ca^{2+}-free Tyrode. Measurement of the aequorin luminescence was started for 1 min immediately before adding Ca^{2+} ions (3 mM final concentration).

Characterization of CysLT receptor antagonists by luminescence measurements

For the characterization of receptor antagonists, cells were loaded with coelenterazine in Tyrode containing 2 mM $CaCl_2$ for 3 h. Cells were incubated with test compounds for 5 min at room temperature prior to the addition of 10 nM LTD_4 ($CysLT_1$ and $CysLT_2$) or 10 nM LTC_4 ($CysLT_2$). Immediately before agonist addition, measurement of the aequorin luminescence was started for 1 min by using a charge-coupled device (CCD) camera (Hamamatsu Corporation, Shizuoka, Japan) in a light tight box.

Radioligand binding studies
CHO cells stably expressing the human $CysLT_1$ or $CysLT_2$ receptor were harvested after reaching 80% confluence. The cells were dispersed in buffer A containing 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 10 $\mu g \cdot mL^{-1}$ leupeptin, 10 $\mu g \cdot mL^{-1}$ pepstatin, 1 $\mu g \cdot mL^{-1}$ aprotinin, 1 $mg \cdot mL^{-1}$ pefabloc and 0.5 $mg \cdot mL^{-1}$ EDTA and subsequently homogenized by using an Ultra Turrax homogenizer (IKA, Staufen, Germany) for 10 min at 4°C. After centrifugation of the homogenate at 1000× *g* for 10 min at 4°C, the resulting supernatant was again centrifuged at 20 000× *g* for 30 min at 4°C. The supernatant was discarded, and the pellet was resuspended in binding buffer (20 mM HEPES, pH 7.4, 20 mM $CaCl_2$, 1 mM cysteine, 0.1% BSA) at a protein concentration of 1 mg mL^{-1}. For the competition studies, membrane preparations (300 μg mL^{-1}) were incubated with 200 pM $[^3H]$-LTD_4 in binding buffer together with the test compounds at room temperature for 1 h. The specific activity of $[^3H]$-LTD4 used for the binding assay was 180 Ci $mmol^{-1}$. The $[^3H]$-LTD_4 concentration of 200 pM was chosen according to Heise *et al*. (2000) and Frey *et al*. (1993). Under these experimental conditions, approximately 5–10% of $[^3H]$-LTD_4 added to the incubation was bound. Non-specific binding of $[^3H]$-LTD_4 was determined in the presence of 1 μM unlabelled LTD_4. Specific binding was linear with respect to radioligand and protein concentration, and represented 85–90% of the total $[^3H]$-LTD_4 binding to the membranes. After termination of the reaction by addition of washing buffer (10 mM HEPES, pH 7.4, 0.01% BSA), the mixture was centrifuged at 10 000× *g* for 10 min at 4°C. The resulting supernatant was removed and the remaining radioactivity in the pellet was measured by liquid scintillation counting.

Characterization of guinea pig $CysLT_1$ and $CysLT_2$ receptor expression by quantitative real-time PCR
Quantitative TaqMan analysis was performed using the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Guinea pig tissue mRNA probes were obtained from male guinea pigs (Crl:HA, Charles River, Sulzfeld, Germany) weighing 325–375 g. Due to the limited tissue size, coronary arteries from three animals were pooled. Genomic DNA was removed by DNAse digestion, and mRNAs were reverse transcribed using random hexamers. Comparable probe efficencies were assured by titration of genomic DNA. Normalization was performed using the house-keeping gene L32 as control, and relative expression was calculated using the formula: relative expression = $2^{(18-(Ct(probe)-Ct(L32)))}$. The parameter Ct is defined as the threshold cycle number at which the amplification plot passed a fixed threshold above baseline. The resulting expression is given in arbitrary units.

The following primers and fluorescent probes were used: $CysLT_1$: forward primer: 5′-TGGCTGATCTATTGTGTGTGTG-3′; probe: 5′-(FAM)ACACTGCCTCTCCGTGTGGCCTATT(TAMRA)-3′; reverse primer: 5′-AGCCAAATGCCTTTGTGAAC-3′; $CysLT_2$: forward primer: 5′-TGCTGAGTGTGGTGCGTTTT-3′; probe: 5′-(FAM)TGGCTACTGTTCACCCCTTCCGGCT(TAMRA)-3′; reverse primer: 5′-CTGAAGCTGGTGACGTGGAG-3′; L32: forward primer: 5′-TGCTCACAATGTCTCCTCCA-3′; probe: 5′-(FAM)CAAGGCCATTGTGGAACGAGCAG(TAMRA)-3′; reverse primer: 5′-GTGACTCGGATGGCTAGCTG-3′

Effects of LTC_4, HAMI3379 and zafirlukast on Langendorff-perfused guinea pig hearts
All animal care and experimental protocols complied with the German Law for the Protection of Laboratory animals and were approved by the local Laboratory Animals Science and Welfare Council. Hearts from male guinea pigs (Dunkin Hartley, 200–250 g, from Charles River) were Langendorff perfused according to Letts and Piper (1982) at 37°C with a non-recirculating system. The perfusion medium was a filtered Krebs–Henseleit solution containing 10 mM glucose and 1.8 mM $CaCl_2$, equilibrated with $O_2 + CO_2$ (95% + 5%), to give a pH of 7.4 and a pO_2 of 650–700 mmHg. Perfusion was performed at a constant flow rate of 10 $mL \cdot min^{-1}$. A latex balloon filled with saline and connected to a pressure transducer (TBD-1222, FMI GmbH, Seeheim, Germany) was inserted into the left ventricular cavity to measure the isovolumetric contractions of the left ventricle. Perfusion pressure was recorded by a second pressure transducer at the aortic cannula. Drug solutions were infused into the aortic cannula at a rate of 1% of the total flow rate. After an equilibration period, LTC_4 was infused with increasing concentration steps for 20 min. For the characterization of receptor antagonists, 10 nM LTC_4 was given continuously for the duration of the experiment. The antagonists were additionally infused with increasing concentration steps for 20 min.

Data analysis
The GraphPad Prism Software (version 4.02, GraphPad Software Inc., San Diego, CA, USA) was used for curve fitting and calculation of the half-maximal inhibitory concentration (IC_{50}) and of the half-maximal effective concentration (EC_{50}). The IC_{50} and EC_{50} values from the calcium mobilization assay were determined from four to six independent experiments performed in quadruplicate. The data from radioligand binding studies were derived from three to five independent experiments. IC_{50} and EC_{50} values are given as mean ± SEM.

Materials
HAMI3379 (3-({[(*1S,3S*)-3-carboxycyclohexyl]amino}carbonyl)-4-(3-{4-[4-(cyclo-hexyloxy)butoxy]phenyl}propoxy) benzoic acid) was synthesized by the chemistry department of Bayer Schering Pharma AG (Wuppertal, Germany) as described (Härter *et al*., 2004). BAY u9773 (4-({(*1R,2E,4E,6Z,9Z*)-1-[(*1S*)-4-carboxy-1-hydroxybutyl] pentadeca-2,4,6,9-tetraen-1-yl}thio) benzoic acid), LTC_4 and LTD_4 were purchased from Biomol (Hamburg, Germany). Zafirlukast (cyclopentyl{3-[2-methoxy-4-({[(2-methylphenyl)sulfonyl]amino}carbonyl)benzyl]-1-methyl-*1H*-indol-5-yl}carbamate) was eluted and purified from commercially available tablets.

Results

Characterization of recombinant $CysLT_1$ and $CysLT_2$ receptor cell lines
In the search for antagonists of the CysLT pathway, we identified 3-({[(*1S,3S*)-3-carboxycyclohexyl]amino}carbonyl)-4-(3-{4-[4-(cyclo-hexyloxy)butoxy]phenyl}propoxy) benzoic acid

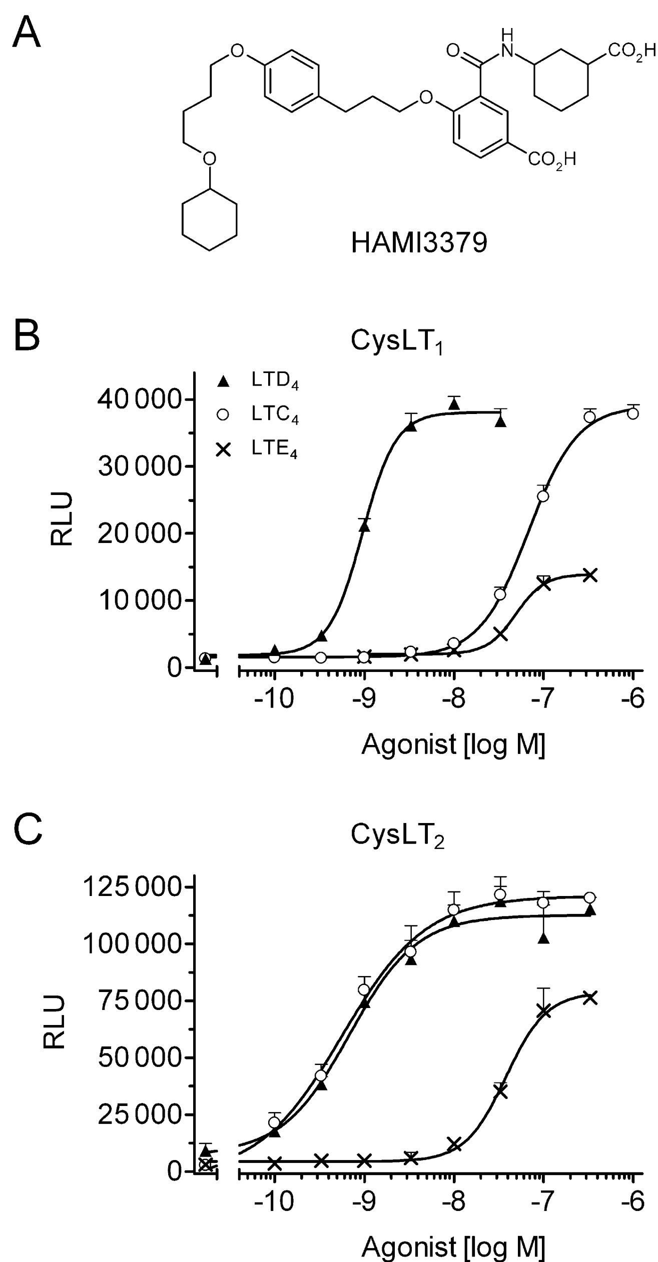

Figure 1 Characterization of recombinant $CysLT_1$ and $CysLT_2$ receptor cell lines. (A) Structure of HAMI3379. (B, C) Agonistic activities of LTC_4, LTD_4 and LTE_4 on reporter cell lines stably transfected with (B) human $CysLT_1$ or (C) human $CysLT_2$ receptors. Results are expressed as relative light units (RLU), and data are presented as mean ± SEM (n = 4).

(HAMI3379) as a potent $CysLT_2$ receptor antagonist (Figure 1A).

In order to assess the potency and selectivity of HAMI3379, CHO cells stably transfected with either the human $CysLT_1$, or the human $CysLT_2$ receptor were generated and intracellular calcium mobilization was monitored by aequorin luminescence measurements, according to Heise *et al*. (2000). For the characterization of our newly generated $CysLT_1$ and $CysLT_2$ receptor reporter cell lines, we used the natural receptor ligands LTC_4, LTD_4 and LTE_4. Both recombinant CysLT receptor cell lines responded in a concentration-dependent manner to the agonists. The rank order for the activation of the $CysLT_1$ receptor cell line was $LTD_4 > LTC_4 > LTE_4$, with respective EC_{50} values of 1.12 ± 0.16 nM, 51.2 ± 9.0 nM and 55.9 ± 6.5 nM (Figure 1B). LTE_4-mediated luminescence signals reached only ~30–50% of the maximal stimulation obtained with the full agonists LTD_4 and LTC_4. The rank order for the activation of the $CysLT_2$ receptor cell line was LTD_4~LTC_4 > LTE_4. LTD_4 and LTC_4 stimulated intracellular calcium mobilization in the recombinant $CysLT_2$ receptor cell line with EC_{50} values of 0.99 ± 0.22 nM and 0.81 ± 0.13 nM respectively (Figure 1C). LTE_4 stimulated the $CysLT_2$ receptor cell line with lower potency (EC_{50} = 59.0 ± 6.2 nM; Figure 1C). In addition, LTE_4-mediated luminescence signals reached ~40–70% of the maximal stimulation obtained with the full agonists LTD_4 and LTC_4.

Characterization of CysLT receptor antagonists by luminescence measurements

Preincubation with HAMI3379 only weakly inhibited 10 nM LTD_4-induced (~EC_{80-90}) calcium release in the reporter cell line expressing the human CysLT1 receptor (IC_{50} > 10 000 nM; Figure 2A). In contrast, HAMI3379 was found to cause potent inhibition of 10 nM LTD_4- and LTC_4-induced (~EC_{80-90}) intracellular calcium release in the $CysLT_2$ receptor cell line, with IC_{50} values of 3.8 ± 0.6 nM and 4.4 ± 0.7 nM respectively (Figure 2B,C). The structurally distinct CysLT1 receptor antagonist zafirlukast (Krell *et al*., 1990) potently inhibited LTD_4-induced calcium mobilization in the $CysLT_1$ receptor cell line (IC_{50} = 20.6 ± 4.1 nM; Figure 2A). However, zafirlukast was only weakly active on the $CysLT_2$ receptor cell line (IC_{50}~7000 nM; Figure 2B,C). The dual $CysLT_1$/$CysLT_2$ receptor antagonist and partial $CysLT_2$ receptor agonist BAY u9773 (Labat *et al*., 1992; Tudhope *et al*., 1994) was also tested. In the $CysLT_1$ receptor cell line, BAY u9773 inhibited 10 nM LTD_4-induced luminescence signals with an IC_{50} value of ~5000 nM (Figure 2A). In the $CysLT_2$ receptor cell line, BAY u9773 inhibited 10 nM LTD_4- and LTC_4-induced calcium signals with IC_{50} values of 18.3 ± 1.1 nM and 19.5 ± 3.8 nM respectively (Figure 2B,C).

Next, we tested if HAMI3379, zafirlukast and BAY u9773 were able to activate $CysLT_1$ and $CysLT_2$ receptors and stimulate intracellular calcium mobilization in the absence of LTC_4 or LTD_4. HAMI3379 was added to both the $CysLT_1$ (Figure 3A) and the $CysLT_2$ (Figure 3B) receptor cell lines and did not induce any intracellular calcium mobilization when tested up to 10 000 nM. The same results were obtained with the selective $CysLT_1$ receptor antagonist zafirlukast (data not shown). BAY u9773 did not stimulate calcium mobilization when added to the CysLT1 receptor cell line (Figure 3A). In contrast, BAY u9773 stimulated luminescence signals in the $CysLT_2$ receptor cell line with an EC_{50} value of 69.5 ± 8.3 nM (Figure 3B). The response to BAY u9773 was concentration dependent and reached ~40–50% of the maximal luminescence signals obtained by stimulation with the full agonists LTC_4 and LTD_4. In addition, HAMI3379 (1 μM) effectively abolished the luminescence signals stimulated by BAY u9773 (Figure 3B).

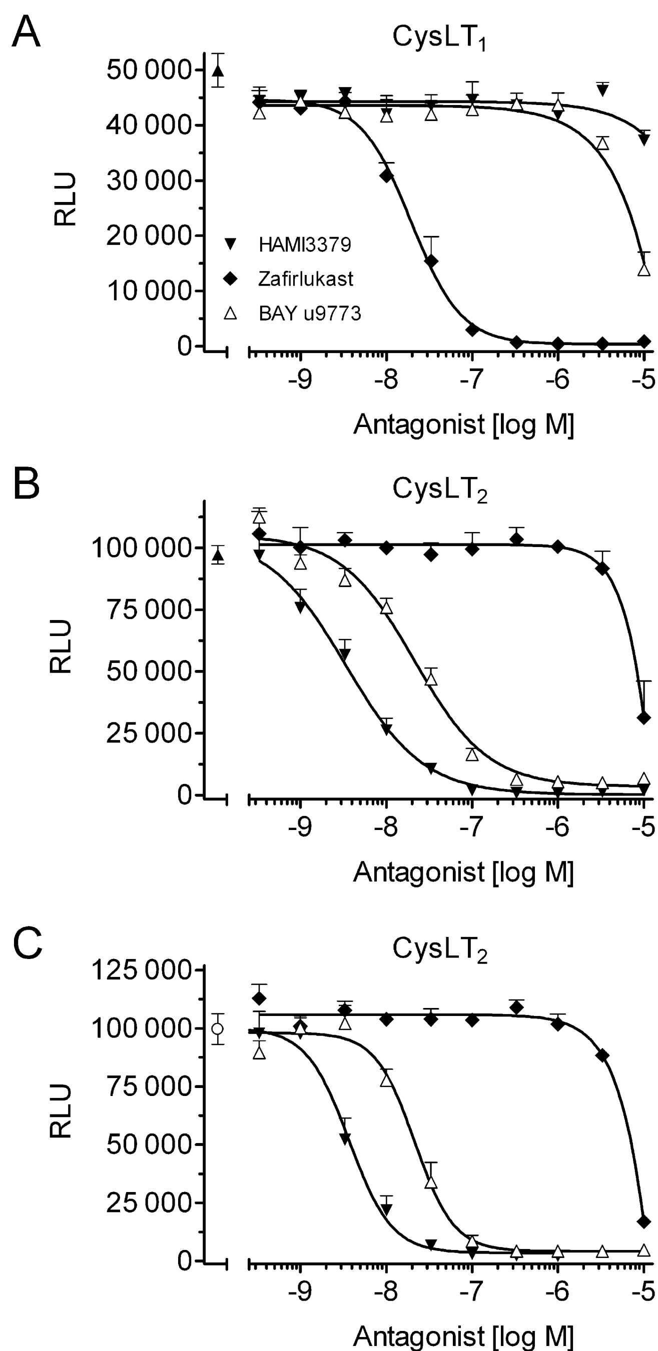

Figure 2 Characterization of CysLT receptor antagonists by luminescence measurements. (A) Inhibition curves of HAMI3379, zafirlukast and BAY u9773 using stably transfected CysLT$_1$ receptor reporter cells challenged with 10 nM LTD$_4$. (B, C) Corresponding inhibition curves using CysLT$_2$ receptor reporter cells challenged with (B) 10 nM LTD$_4$ or (C) 10 nM LTC$_4$. Data are presented as mean ± SEM (n = 4).

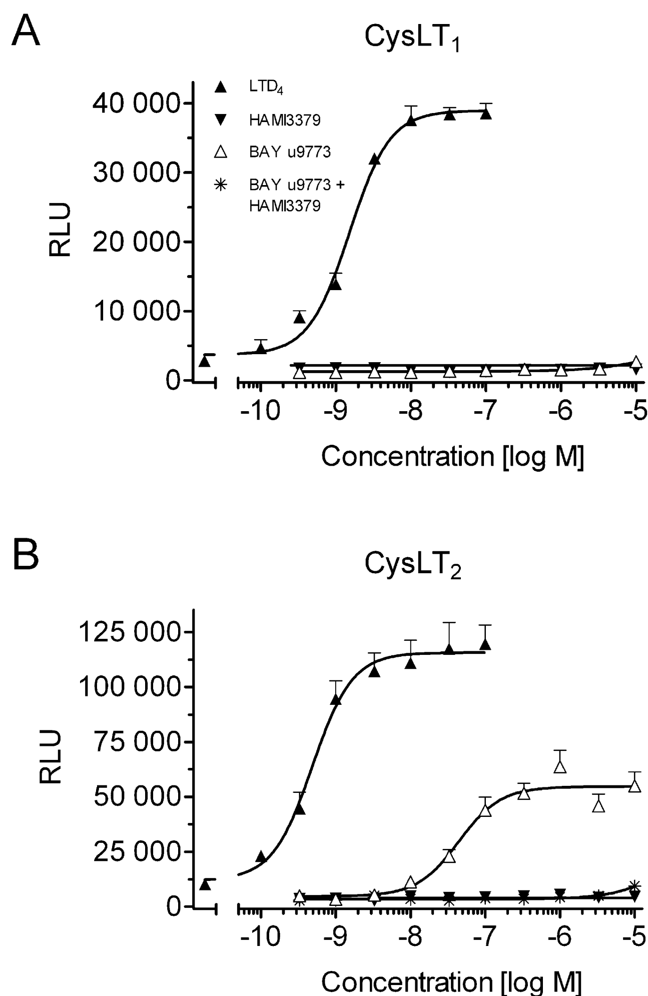

Figure 3 Characterization of CysLT receptor antagonists by luminescence measurements. Intracellular calcium mobilization induced by LTD$_4$, HAMI3379, BAY u9773, and BAY u9773 in the presence of 1 μM HAMI3379 on reporter cell lines stably transfected with (A) human CysLT$_1$ or (B) human CysLT$_2$ receptors. Data are presented as mean ± SEM (n = 4).

Radioligand binding studies

We next performed saturation analysis of [^{3}H]-LTD$_4$ binding to membranes prepared from our recombinant CysLT$_1$ and CysLT$_2$ receptor cell lines. Membranes from CysLT receptor transfected CHO cells specifically bound [^{3}H]-LTD$_4$, whereas membranes prepared from non-transfected control cells did not exhibit specific [^{3}H]-LTD$_4$ binding (data not shown). This effect could be inhibited by increasing concentrations of unlabelled LTD$_4$ (Figure 4A) with IC$_{50}$ values of 2.9 ± 0.7 nM (CysLT$_1$) and 1.3 ± 0.2 nM (CysLT$_2$) respectively. HAMI3379 displaced [^{3}H]-LTD$_4$ bound to the CysLT$_2$ receptor with an IC$_{50}$ value of 37.9 ± 14.7 nM (Figure 4B). In contrast, HAMI3379 showed very low affinity to the CysLT$_1$ receptor (IC$_{50}$ > 30 000 nM).

The CysLT$_1$ receptor selective antagonist zafirlukast displaced [^{3}H]-LTD$_4$ with an IC$_{50}$ value of 45.4 ± 14.8 nM at the CysLT$_1$ receptor (Figure 4C), but was found to be inactive at the CysLT$_2$ receptor (IC$_{50}$ > 10 000 nM). In addition, BAY u9773 inhibited radioligand binding at the CysLT$_2$ receptor with an IC$_{50}$ value of 496 ± 173 nM. However, BAY u9773 showed only very low activity (IC$_{50}$ value > 10 000 nM) against membranes prepared from the human CysLT$_1$ receptor cell line (Figure 4D).

Figure 4 Radioligand binding studies. Effects of (A) LTD_4, (B) HAMI3379, (C) zafirlukast and (D) BAY u9773 on competitive binding of $[^3H]$-LTD_4 to membranes prepared from the recombinant $CysLT_1$ and $CysLT_2$ receptor cell lines. Data are presented as mean ± SEM (n = 3–5).

Characterization of guinea pig $CysLT_1$ and $CysLT_2$ receptor expression by quantitative real-time RT-PCR

We next studied the expression of $CysLT_1$ and $CysLT_2$ receptors in guinea pig lung and heart tissue by quantitative real-time RT-PCR. As shown in Figure 5, we confirmed high expression levels of the $CysLT_1$ receptor and medium expression levels of the $CysLT_2$ receptor in guinea pig lung. In addition, we were able to detect high expression levels of the $CysLT_2$ receptor in left and right heart ventricle and coronary artery. $CysLT_1$ receptor expression was also detected in guinea pig heart ventricles and coronary artery, however, at significantly lower levels compared to the $CysLT_2$ receptor.

Effects of LTC_4, HAMI3379 and zafirlukast on Langendorff-perfused guinea pig hearts

The effects of LTC_4, HAMI3379 and zafirlukast on isolated, Langendorff-perfused, guinea pig hearts were examined under constant flow conditions. Langendorff-perfused hearts were stimulated with LTC_4 to preferentially stimulate the $CysLT_2$ receptor and to monitor $CysLT_2$ receptor-mediated cardiac effects. Continuous perfusion of the hearts with increasing concentrations (10^{-10}–10^{-7} M) of LTC_4 resulted in a significant increase in coronary perfusion pressure, a decrease of the left

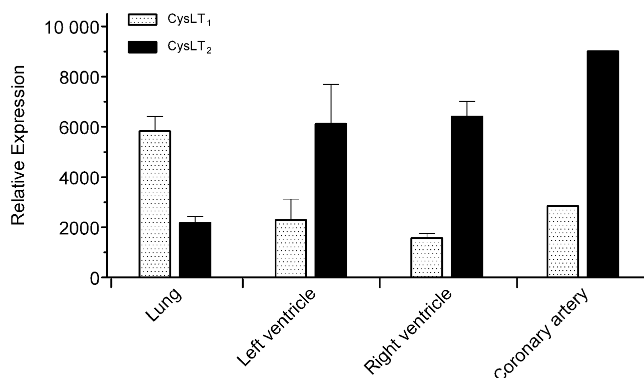

Figure 5 Characterization of $CysLT_1$ and $CysLT_2$ receptor expression in guinea pig tissues by quantitative real-time RT-PCR. Analysis was performed on guinea pig lung and heart tissue cDNAs using specific oligonucleotide probes. Expression levels were normalized to the house-keeping gene L32. Data are presented as mean ± SEM (n = 3).

ventricular contractility (dp/dt_{max}) and of the left ventricular developed pressure (LVDP), whereas the heart rate remained unaffected (Figure 6). These effects persisted for the time of LTC_4 perfusion if no antagonist was added. For the characterization of receptor antagonists, 10 nM LTC_4 was given

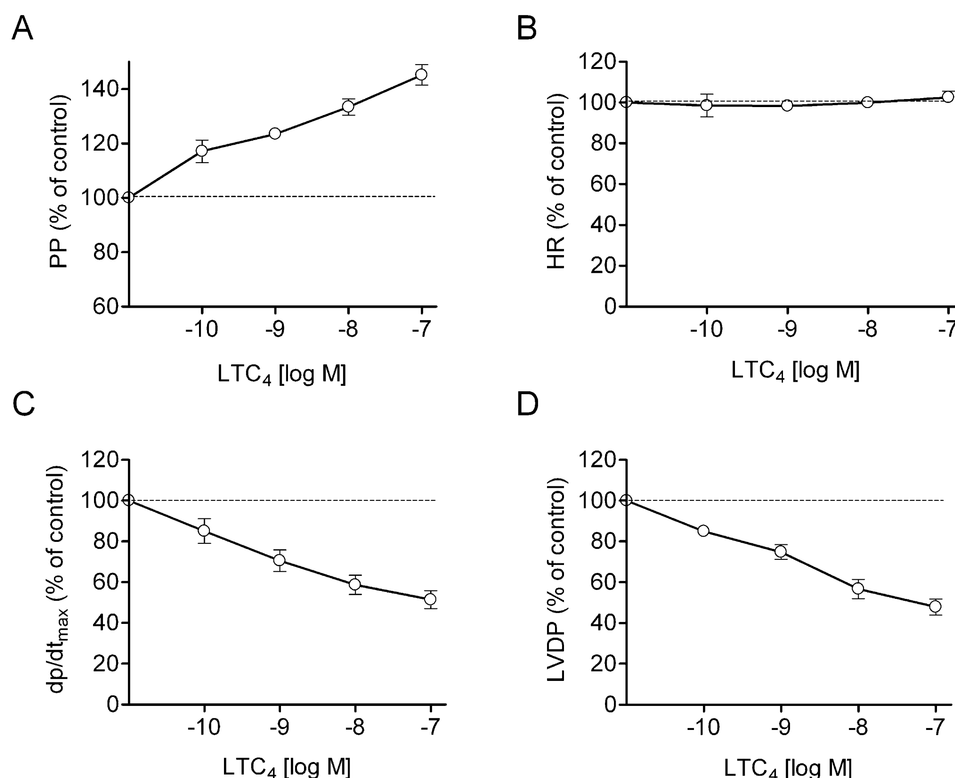

Figure 6 Effects of LTC_4 in Langendorff-perfused guinea pig hearts. Effects of LTC_4 on (A) perfusion pressure (PP), (B) heart rate (HR), (C) contractility (dp/dt_{max}) and (D) left ventricular developed pressure (LVDP). Hearts were perfused with increasing concentrations of LTC_4. Data are presented as mean ± SEM (*n* = 16).

continuously for the duration of the experiment. Addition of HAMI3379 concentration-dependently antagonized the LTC_4-induced perfusion pressure increase and the negative effects of LTC_4 on contractility (Figure 7). Perfusion with HAMI3379 (10^{-8}–10^{-6} M) in the absence of LTC_4 had no influence on perfusion pressure, heart rate or contractility (data not shown). In contrast to HAMI3379, the $CysLT_1$ receptor antagonist zafirlukast (10^{-8}–10^{-6} M) was not able to antagonize the cardiac effects induced by 10^{-8} M LTC_4 (Figure 8).

Discussion

CysLT effects are mediated by at least two different G protein-coupled receptors, the $CysLT_1$ and the $CysLT_2$ receptor, respectively. Both receptors have been cloned, and their tissue distribution has been characterized. Whereas the relevance of the $CysLT_1$ receptor in inflammatory diseases such as asthma has been extensively investigated, an in-depth characterization of the (patho)physiological role of the $CysLT_2$ receptor has been hindered by the lack of selective $CysLT_2$ receptor antagonists. In this report, we describe the identification and initial pharmacological characterization of HAMI3379, the first potent and selective $CysLT_2$ receptor antagonist, which is devoid of CysLT receptor agonism.

We have characterized the antagonistic and agonistic properties of HAMI3379 on human $CysLT_1$ and $CysLT_2$ receptors, respectively. The results show that HAMI3379 potently and selectively antagonizes LTD_4- and LTC_4-induced intracellular calcium mobilization and $[^3H]$-LTD_4 binding using a recombinant human $CysLT_2$ receptor cell line. The potency of HAMI3379 on the human $CysLT_1$ receptor cell line was found to be very low. In addition, the compound was tested for its ability to activate the two human CysLT receptors. In contrast to the partial $CysLT_2$ receptor agonist BAY u9773 (Labat *et al*., 1992; Tudhope *et al*., 1994), HAMI3379 did not induce intracellular calcium mobilization. This is an important finding that clearly differentiates HAMI3379 from BAY u9773. A putative CysLT receptor agonism of HAMI3379 would preclude the use of this compound for the treatment of cardiovascular indications.

We also tried to characterize the activity of HAMI3379 on GPR17, recently identified as a CysLT receptor (Ciana *et al*., 2006). Unfortunately, we were not able to reproduce GPR17 activation by LTC_4 or LTD_4 in different expression systems (F. Wunder, unpubl. data). Similar observations have recently been made by other groups (Maekawa *et al*., 2009).

The LTE_4 analogue BAY u9773 antagonized LTD_4- and LTC_4-mediated luminescence signals and acted as a partial agonist on $CysLT_2$ receptor cells with comparable potencies. Interestingly, BAY u9773 was relatively weakly active on the human recombinant $CysLT_1$ receptor cell line, in both the functional as well as the binding assay. The weak potency of BAY u9773 at the human $CysLT_1$ receptor might be species related, as antagonistic activities against both CysLT receptors were observed in tissues from experimental animals (Tudhope *et al*., 1994). Similar differences in the antagonistic potency have been reported for pranlukast on human and murine

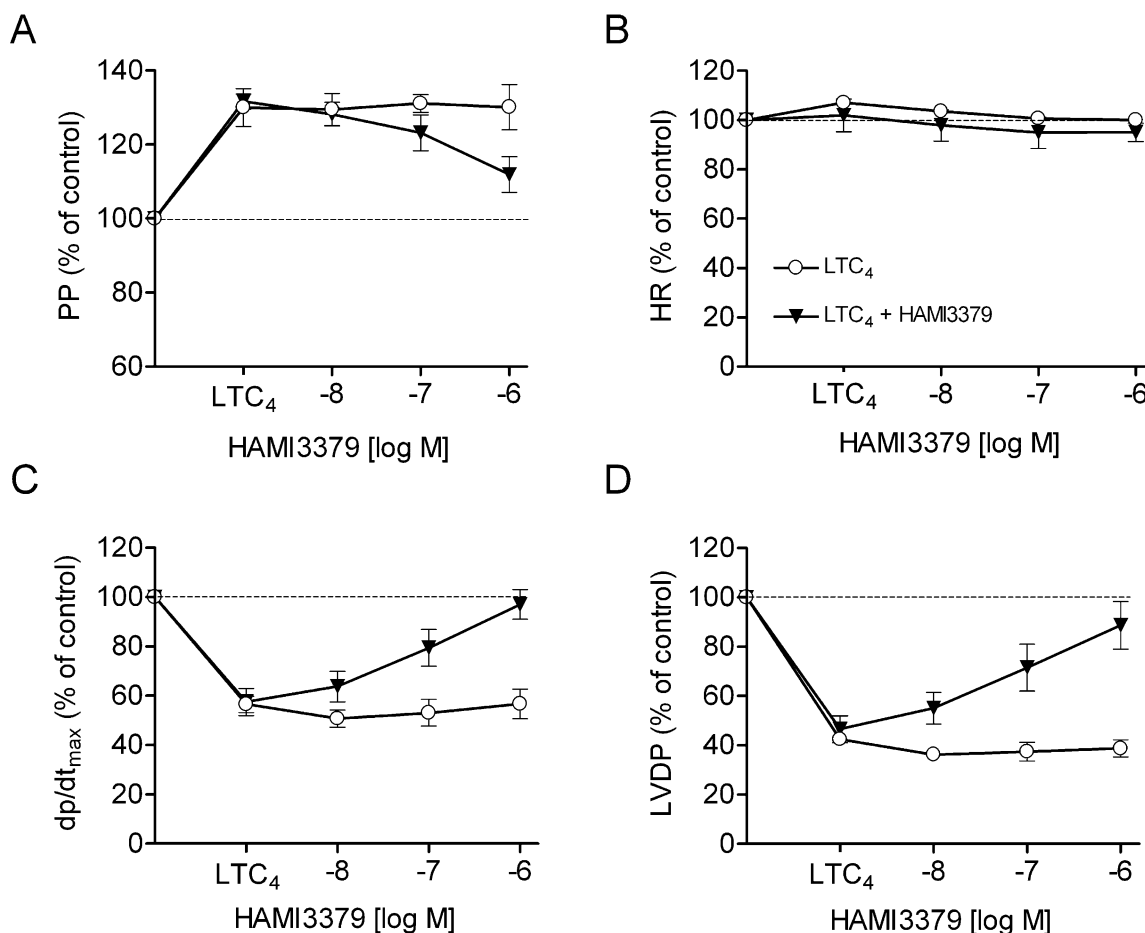

Figure 7 Effects of HAMI3379 in Langendorff-perfused guinea pig hearts. Effects of HAMI3379 on (A) perfusion pressure (PP), (B) heart rate (HR), (C) contractility (dp/dt_{max}) and (D) left ventricular developed pressure (LVDP) in the presence of LTC_4. Hearts were constantly perfused either with 10 nM LTC_4 alone or with LTC_4 in the presence of increasing concentrations of HAMI3379. Data are presented as mean ± SEM (n = 10).

$CysLT_2$ receptors (Ogasawara *et al*., 2002). In addition, tissues with predominant $CysLT_1$ receptor expression might also express certain (low) levels of the $CysLT_2$ receptor. This would lead to mixed $CysLT_1$/$CysLT_2$ receptor populations and could interfere with the pharmacological characterization of CysLT receptor antagonists. Therefore, cell lines with recombinant receptors offer a valuable method for the pharmacological characterization of isolated $CysLT_1$ and $CysLT_2$ receptors and their respective ligands.

CysLTs have been implicated in the pathophysiology of atherosclerosis and coronary heart disease (Letts, 1987; Fauler and Frölich, 1989; Carry *et al*., 1992; Allen *et al*., 1993, 1998; Folco *et al*., 2000; Funk, 2005). However, the precise roles of CysLTs and their respective receptors in cardiovascular physiology remain largely to be explored. The tissue distribution of the $CysLT_2$ receptor suggests an important function for this receptor in the cardiovascular system. High levels of $CysLT_2$ receptor expression have been detected in the human myocardium and conduction system, as well as in human coronary artery smooth muscle cells and vascular endothelial cells. In contrast, expression of the $CysLT_1$ receptor mRNA is barely detectable in human heart and coronary arteries (Heise *et al*., 2000; Nothacker *et al*., 2000; Takasaki *et al*., 2000; Hui *et al*., 2001; Kamohara *et al*., 2001; Sjöström *et al*., 2003). These published data are in good agreement to the results of our quantitative real-time RT-PCR experiments using guinea pig tissues. We could detect very high expression of the $CysLT_2$ receptor in guinea pig heart ventricles and coronary artery. In contrast, expression of the $CysLT_1$ receptor in these tissues was found to be significantly lower.

According to the expression data, the $CysLT_2$ receptor may mediate cardiovascular CysLT effects, including coronary vasoconstriction, negative inotropy and increased vascular permeability. Therefore, we characterized the effects of the CysLT antagonists HAMI3379 and zafirlukast on LTC_4-treated, Langendorff-perfused, guinea pig hearts. The results presented in this report show that HAMI3379 is an effective antagonist of the cardiac effects of LTC_4. Since HAMI3379 blocked most (~90–95%) of the cardiac LTC_4 effects, and the $CysLT_1$ receptor selective antagonist zafirlukast was found to be inactive in this experimental setting, the results imply that the cardiac CysLT effects are predominantly mediated by $CysLT_2$ receptors and therefore are in good agreement to the high expression of the $CysLT_2$ receptor in heart and blood vessels. However, according to the available data, an additional contribution of the $CysLT_1$ receptor to cardiac CysLT effects

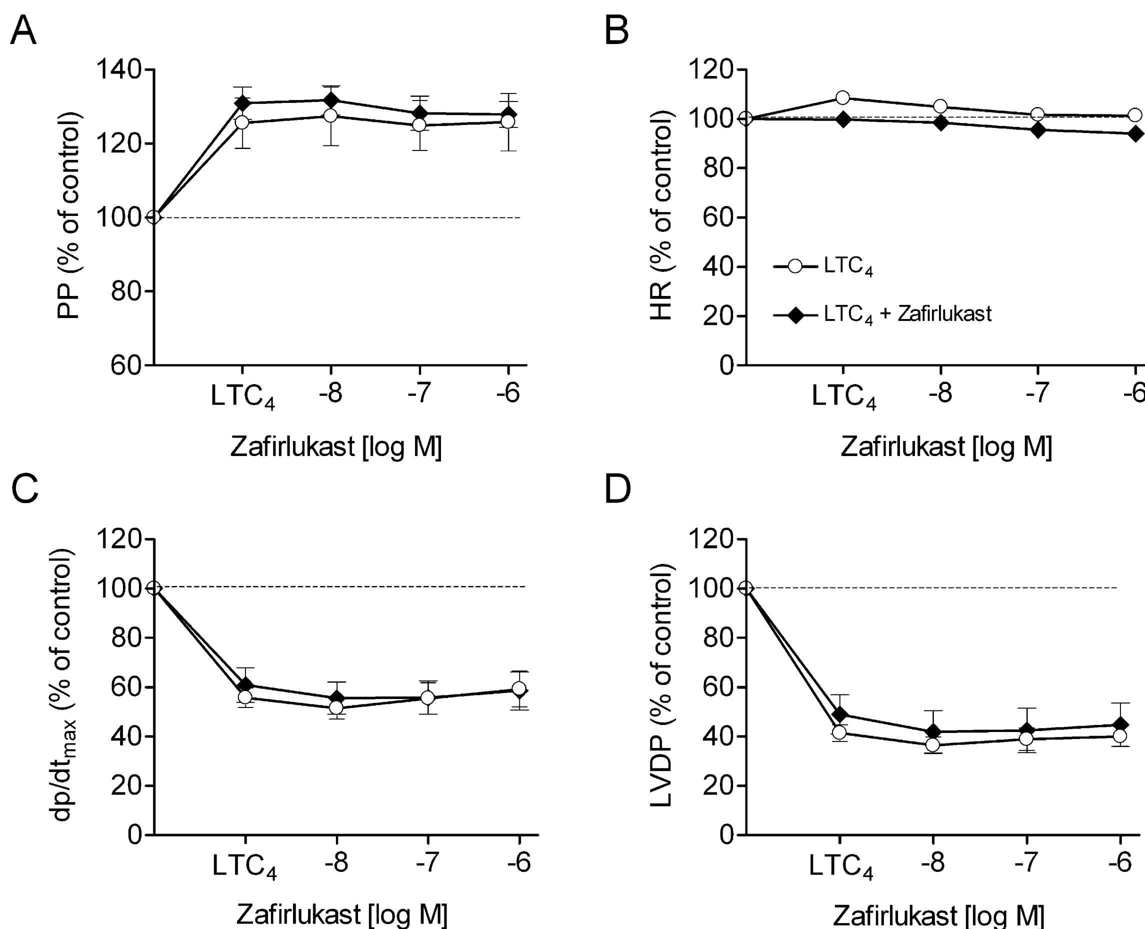

Figure 8 Effects of zafirlukast in Langendorff-perfused guinea pig hearts. Effects of zafirlukast on (A) perfusion pressure (PP), (B) heart rate (HR), (C) contractility (dp/dt_{max}) and (D) left ventricular developed pressure (LVDP) in the presence of LTC_4. Hearts were constantly perfused either with 10 nM LTC_4 alone or with LTC_4 in the presence of increasing concentrations of zafirlukast. Data are presented as mean ± SEM (n = 5).

cannot be completely ruled out. In previous studies, it has been shown that $CysLT_1$ receptor antagonists were also able to antagonize cardiac CysLT effects (Burke *et al*., 1982; McLeod and Piper, 1991; Allen *et al*., 1993). Therefore, further studies are required to clarify whether cardiac CysLT effects are mediated by the $CysLT_2$ receptor only.

It is tempting to speculate that HAMI3379 might have beneficial effects in various cardiovascular indications, for example during myocardial infarction, by antagonizing the negative inotropy and coronary constriction induced by endogenously synthesized CysLTs. HAMI3379 might also reduce the plasma leakage under these conditions, since the $CysLT_2$ receptor is highly expressed in vascular endothelial cells and has been linked to increased vascular permeability (Sjöström *et al*., 2003; Beller *et al*., 2004; Jiang *et al*., 2008; Moos *et al*., 2008).

Besides being widely expressed in cardiovascular tissues, the $CysLT_2$ receptor has also been detected in peripheral blood leukocytes, macrophages, mast cells, eosinophils, lymph nodes and spleen, which implies a role for the $CysLT_2$ receptor in immune and inflammatory responses, as well (Heise *et al*., 2000; Nothacker *et al*., 2000; Takasaki *et al*., 2000; Kanaoka and Boyce, 2004).

In summary, we have identified the first potent and selective $CysLT_2$ receptor antagonist, HAMI3379. Using this compound, we could clearly show that the $CysLT_2$ receptor mediates cardiovascular CysLT effects in rodents. HAMI3379 will certainly help to elucidate further the (patho)physiological relevance of the $CysLT_2$ receptor and may offer a novel therapeutic approach for the treatment of cardiovascular and inflammatory diseases.

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Conflicts of interest

None. All authors are employees of Bayer Schering Pharma AG.

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