A Selective Cysteiny1 Leukotriene Receptor 2 Antagonist Blocks Myocardial
Ischemia/Reperfusion Injury and Vascular Permeability in Mice

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Running Title: CysLT2R antagonism blocks ischemia/reperfusion injury

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Number of Text Pages: 41
Number of Tables: 1
Number of Figures: 6
Number of References: 35
Number of Words (Abstract): 245
Number of Words (Introduction): 637
Number of Words (Discussion): 994

List of Non-Standard Abbreviations:
5-LO: 5-lipoxygenase
BayCysLT2: 3-(((3-carboxycyclohexyl)amino)carbonyl)-4-((3-(4-(4-phenoxybutoxy)phenyl)propoxy)benzoic acid
Bay-u9773: 4-(((1R,2E,4E,6Z,9Z)-1-((1S)-4-Carboxy-1-hydroxybutyl)-2,4,6,9-pentadecatetraen-1-yl)thio)benzoic acid
CysLT: cysteinyl leukotriene
CysLT₁R: cysteiny1 leukotriene receptor 1
CysLT₂R: cysteiny1 leukotriene receptor 2
EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EtOAc: ethyl acetate
FLAP: 5-lipoxygenase-activating protein
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
HAMI3379: 3-(((3-carboxycyclohexyl)amino)carbonyl)-4-(3-(4-(4-(cyclohexyloxy)butoxy)phenyl)propoxy)-benzoic acid
hEC-CysLT₂R: human endothelial cell-specific cysteiny1 leukotriene 2 receptor overexpressor
HEK: human embryonic kidney
HOBt: hydroxybenzotriazole
ICAM: intracellular adhesion molecule
LAD: left anterior descending coronary artery
LT: leukotriene
MI: myocardial infarction
MK-571: (E)-3-((3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)((3-(dimethylamino)-3-oxopropyl)thio)methyl)thio)-propanoic acid
MPO: myeloperoxidase
N-Methyl-LTC₄: N-methyl-5S-hydroxy-6R-(S-glutathionyl)-7E,9E,11Z,14Z-eicosatetraenoic acid; NMLTC₄
ROS: reactive oxygen species
THF: tetrahydrofuran
TLC: thin layer chromatography
TTC: 2,3,5-triphenyltetrazolium chloride
VCAM: vascular cell adhesion molecule

**Recommended Section Assignment:** Cardiovascular
Abstract

Cysteinyl leukotrienes are potent inflammatory mediators that predominantly exert their effects by binding to cysteinyl leukotriene receptors of the G protein-coupled receptor family. The cysteinyl leukotriene receptor 2 (CysLT2R), expressed in endothelial cells of some vascular beds, has been implicated in a variety of cardiovascular functions. Endothelium-specific overexpression of human CysLT2R in transgenic mice (hEC-CysLT2R) greatly increases myocardial infarction damage. Investigation of this receptor, however, has been hindered by the lack of selective pharmacological antagonists. Here, we describe the characterization of 3-(((3-carboxycyclohexyl)amino)carbonyl)-4-(3-(4-(4-phenoxybutoxy)phenyl)-propoxy)benzoic acid (BayCysLT2), and explore the selective effects of this compound in attenuating myocardial ischemia/reperfusion damage and vascular leakage. Using a recently developed β-galactosidase–β-arrestin complementation assay for CysLT2R activity (Yan et al., 2011), we determined BayCysLT2 to be ~20-fold more potent than the non-selective dual cysteinyl leukotriene receptor 1 (CysLT1R)/CysLT2R antagonist Bay-u9773 (IC50: 274 nM vs 4.6 μM). Intracellular calcium mobilization in response to cysteinyl leukotriene administration showed that BayCysLT2 was >500-fold more selective for CysLT2R as compared to CysLT1R. Intraperitoneal injection of BayCysLT2 in mice significantly attenuated leukotriene D4-induced Evans blue dye leakage in the murine ear vasculature. Finally, BayCysLT2 administration either prior to or post ischemia/reperfusion attenuated the aforementioned increased myocardial infarction damage in hEC-CysLT2R mice. Finally, decreased neutrophil infiltration and leukocyte adhesion molecule mRNA expression was observed in mice treated with antagonist compared to untreated controls. In conclusion, we present the characterization of a potent and selective antagonist for CysLT2R that is useful for discerning biological activities of this receptor.
Introduction

Myocardial infarction (MI) is the leading killer of both males and females in the industrialized world (Yellon and Hausenloy, 2007). The condition is characterized by a cessation of oxygen/nutrient supply to the myocardium, usually caused by a blockage of the coronary circulation. While the only remedy to this problem is timely restoration of blood flow (“reperfusion”), this results in the en masse formation of reactive oxygen species (ROS) creating oxidative stress (Zweier, 1988). ROS formation, in tandem with chemoattractant release during MI, invokes the innate inflammatory response, resulting in adhesion protein upregulation, leukocyte infiltration, and extracellular matrix degradation (Vinten-Johansen, 2004). It is these post-reperfusion events that contribute the majority of myocardial infarction-induced damage (Yellon and Hausenloy, 2007).

The cysteinyl leukotrienes (CysLTs) – so termed due to the common cysteine moiety in their structures – are a group of pro-inflammatory arachidonic acid metabolites consisting of leukotriene (LT) C₄, LTD₄, and LTE₄. CysLTs exert their effects primarily by binding two characterized GPCRs: the cysteinyl leukotriene receptor 1 (CysLT₁R) (Lynch et al., 1999) and receptor 2 (CysLT₂R) (Heise et al., 2000). CysLTs have also been shown to interact with the putative cysteinyl leukotriene E receptor (Maekawa et al., 2008), the P2Y₁₂ receptor (Nonaka et al., 2005), and GPR17 (Ciana et al., 2006; Maekawa et al., 2009). Of these receptors, the CysLT₁R is the most extensively studied (Moos and Funk, 2008), and has been shown to mediate bronchoconstriction and airway inflammation (Funk, 2005). The CysLT₂R has not received as much attention as the CysLT₁R, although there has been a wave of interest concerning this receptor in cardiovascular disease (Funk, 2005). The CysLT₂R is expressed in multiple organs in
the mouse, including the heart (Jiang et al., 2008), brain, cremaster muscle vasculature, (Moos et al., 2008), and myenteric neurons (Barajas-Espinosa et al., 2011). In humans, it is found in umbilical cord endothelial cells (Uzonyi et al., 2006; Lotzer et al., 2003), myocardium, and coronary vessels (Kamohara et al., 2001). CysLT2R mediates vascular endothelium permeability via a vesicular transcytotic mechanism (Hui et al., 2004; Moos et al., 2008) as well as by transcriptional activation of chemokine secretion (Thompson et al., 2008).

The role of enzymes involved in leukotriene biosynthesis and the CysLT receptors in the cardiovascular context is being studied extensively. Research has shown that single nucleotide polymorphisms in the genes encoding 5-lipoxygenase (5-LO) and leukotriene A4 hydrolase are linked to increased risk of myocardial infarction (Helgadottir et al., 2006; Helgadottir et al., 2004). Some studies have also shown that the 5-LO pathway is involved in atherosclerosis (Spanbroek et al., 2003; Di Gennaro et al., 2010; Qiu et al., 2006; Funk, 2005). As well, plasma LTC4 levels (Takase et al., 1996) and both CysLT1R and CysLT2R expression (Jiang et al., 2008) have been shown to increase following myocardial infarction. Previous work by our group has shown that endothelial-specific overexpression of human CysLT2R in transgenic mice resulted in significantly greater myocardial infarcts following coronary vessel ligation, which was attenuated by the non-selective CysLT receptor antagonist Bay-u9773 (4-(((1R,2E,4E,6Z,9Z)-1-((1S)-4-Carboxy-1-hydroxybutyl)-2,4,6,9-pentadecatetraen-1-yl)thio)benzoic acid). The same study also showed that overexpression of CysLT2R resulted in enhanced adhesion molecule upregulation, as well as leukocyte infiltration (Jiang et al., 2008). However, due to the absence of a CysLT2R-selective antagonist (Moos and Funk, 2008), the precise pathological contributions of CysLT1R and CysLT2R, respectively, could not be dissected (Jiang et al., 2008).
Recent breakthroughs in CysLT2R-related pharmacology have allowed the characterization of both a CysLT2R-selective agonist (N-methyl LTC₄, NMLTC₄) (Yan et al., 2011) and antagonist (HAMI3379) (Wunder et al., 2010). In this manuscript, we characterize the pharmacological properties of another CysLT2R-selective antagonist 3-(((3-carboxycyclohexyl)amino)carbonyl)-4-(3-(4-(4-phenoxybutoxy)phenyl)-propoxy)benzoic acid (BayCysLT2) and use it to investigate the specific role of CysLT2R in myocardial injury and vascular leakage. We show that BayCysLT₂ fully attenuates the exacerbation of MI injury seen in mice overexpressing CysLT2R, and that it accomplishes this by reducing leukocyte infiltration.
Methods

BayCysLT₂ Synthesis

All reagents were purchased from commercial sources and used without further purification. Column chromatography was carried out using Silica-P Flash silica gel (60 Å 40-63 μm, 500 m²/g) from Silicycle (Quebec City, Canada). \(^1\)H (and \(^{13}\)C) NMR spectra were recorded at 25 °C on a Bruker Avance Spectrometer (Bruker, Billerica, MA) at 300 (75) MHz or 400 (100) MHz. The desired compound, 3-(((3-carboxycyclohexyl)amino)carbonyl)-4-(3-(4-(4-phenoxybutoxy)phenyl)-propoxy)benzoic acid (BayCysLT₂) is a patented antagonist from Bayer pharmaceuticals (Haerter et al., 2009) and was synthesized according to the route in Figure 1 from four commercially available starting materials. 3-(4-Hydroxyphenyl) propionic acid (1), 4-hydroxyisophthalic acid (7) and 3-aminocyclohexane-1-carboxylic acid (10) were purchased from TCI America (Portland, OR) while 4-phenoxy-1-butyl bromide (3) was from Alfa Aesar (Ward Hill, MA).

Methyl 3-(4-hydroxyphenyl) propanoate (2). After 3 h at room temperature, a solution of 1 (1.5 g, 9.02 mmol) in 15 ml acidified MeOH (5% final volume with HCl) gave its corresponding methyl ester (2). The solvent was evaporated, and the residue was dissolved in EtOAc. The solution was washed with saturated NaHCO₃, dried over MgSO₄, and concentrated \textit{in vacuo} to give a yellow oil in quantitative yield. Spectroscopic characterization was consistent with that reported in the literature (Lewin et al., 2005).

Methyl-3-(4-(4-phenoxybutoxy)-phenyl) propanoate (4). A solution of 2 (1 g, 6.012 mmol) and 3 (1.5 g, 6.55 mmol) in 15 ml acetonitrile was mixed with K₂CO₃ (2.5 g, 2 mmol) and heated to
reflux for 15 h. Solvent was removed and the residue taken up in EtOAc/H2O for work up. The aqueous phase was washed with EtOAc and combined. The organic extracts were washed with brine, dried over MgSO4, filtered through a silica plug, and concentrated in vacuo. The residue was purified with flash chromatography using cyclohexane:EtOAc (19:1) to give 1.5 g of 4 (81% yield). Spectroscopic characterization was consistent with that reported in the literature (Haerter et al., 2009).

**3-(4-(Phenoxybutoxy)phenyl)-1-propanol (5).** LiAlH4 (1 M in 5 ml THF) was introduced dropwise, while stirring, to a solution of 4 (1.3 g, 4.16 mmol) in minimal (~5 ml) THF. The solution was stirred at room temperature for 30 min. MeOH (1-2 ml) was cautiously added to the suspension in order to destroy excess LiAlH4. The mixture was then added to 1M HCl and extracted with EtOAc. The organic phase was washed with brine, dried over Na2SO4 and concentrated to give 5 in quantitative yield. Spectroscopic characterization was consistent with that reported in the literature (Haerter et al., 2009).

**1-(3-bromopropyl)-4-(4-phenoxybutoxy)benzene (6).** Alcohol 5 (1.4 g, 4.86 mmol) was dissolved in 10 ml THF, to which was added triphenylphosphine (1 g, 4 mmol) and tetrabromomethane (1.2 g, 4 mmol) with stirring. The reaction became cloudy after 5 min, and was complete after 4 h as judged by TLC. The precipitate was filtered off, the filtrate was concentrated in vacuo, and the residue subjected to flash chromatography on silica gel with cyclohexane:EtOAc (19:1) to yield 1.23 g (3.39 mmol) of 6 in 69% yield. Spectroscopic characterization was consistent with that reported in the literature (Haerter et al., 2009).
**2-Hydroxy-5-(methoxycarbonyl)benzoic acid (8).** 4-Hydroxyisophthalic acid 7 (1 g, 5.5 mmol) was refluxed in MeOH (10 ml) containing 5% concentrated sulfuric acid (by volume) for 6 h. The mixture was carefully poured into ice water. NaHCO₃ was added to adjust the pH to 8, and the mixture was filtered retaining both the precipitate and filtrate. The precipitate was dissolved in EtOAc and purified by silica gel flash chromatography with 19:1 cyclohexane:EtOAc and trace amounts of glacial acetic acid to separate the desired product from the a diester dimethyl 4-hydroxyisophthalate (9), which was the major product at 0.6 g (2.75 mmol). The undesired diester 9 could be converted to the desired monoester by hydrolysis in wet pyridine (10 ml) at reflux for 18 h. The reaction progress was monitored by TLC, and purified by silica gel flash chromatography (19:1 Cyclohexane:EtOAc) after acidification with HCl, extraction with chloroform, washing with water and NaHCO₃ and concentrated under reduced pressure. The combined yield of mono-ester was 80%. Spectroscopic characterization was consistent with that reported in the literature (Haerter et al., 2009).

**Methyl 3-aminocyclohexane-carboxylate hydrochloride (11).** 3-Aminocyclohexane-1-carboxylic acid hydrochloride (10) (1 g, 7.0 mmol) was stirred together with trimethylsilyl chloride (2 ml, 0.012 mmol) in 15 ml of MeOH at room temperature overnight to give a viscous oil, which under vacuum became 0.4 g of a spongy foam in 36% yield. The product was used immediately in the next synthetic step.

**Methyl 4-hydroxy-3-((3-(methoxycarbonyl)cyclohexyl)amino)carbonyl benzoate (12).** The entire yield of 11, EDC (1 g, 6 mmol) and HOBt (0.5 g, 4 mmol) were dissolved in 15 ml of Et₃N; this mixture was added dropwise to a solution of 8 in 10 ml of CH₂Cl₂. After stirring for 15
h at room temperature, 25 ml of H2O was added to the reaction mixture, and the organic phase was extracted. Combined extracts were washed with brine, dried over MgSO4, and concentrated in vacuo. The crude product was then purified by flash chromatography on silica gel with cyclohexane:EtOAc (4:1) to yield the product in 63 % yield. Spectroscopic characterization was consistent with that reported in the literature (Haerter et al., 2009).

**Methyl 3-(((3-(methoxycarbonyl)cyclohexyl)amino)carbonyl)-4-(3-(4-(4-phenoxybutoxy)phenyl)propoxy) benzoate (13).** The coupling product **12** (0.5 g, 1.50 mmol) was dissolved in butylnitrile (25 ml), to which K2CO3 (5 g, 36 mmol) was added and the mixture heated to reflux. The alkyl bromide **6** (0.6 g, 1.7 mmol) was added over 15 hours until **12** was consumed as judged by TLC. A 5 % solution of NaH2PO4 (10 ml) was then added to the reaction mixture, the organics were extracted with EtOAc, washed with brine, dried with MgSO4 and purified by flash chromatography on silica gel with cyclohexane:EtOAc (2:1). Purification yielded 17 %. Spectroscopic characterization was consistent with that reported in the literature (Haerter et al., 2009).

**BayCysLT2.** Methyl ester **13** (0.15 g, 2.43 mmol) was dissolved in 15 ml of THF and 8 ml of a 1:1 solution of MeOH and 2 M NaOH. The mixture was heated to 60 °C for 4 h and then acidified with concentrated HCl, extracted with EtOAc, and precipitated with diethyl ether to obtain the product in quantitative yield. Spectroscopic characterization was consistent with that reported in the literature (Haerter et al., 2009). Stock solution (100 mg/ml) was prepared in DMSO for use in all experiments.
β-Galactosidase–β-Arrestin Assay

The β-galactosidase-β-arrestin complementation assay for CysLT₂R activation was recently described (Yan et al., 2011). Briefly, Bay-u9773 (Cayman Chemical Co., Ann Arbor, MI) or BayCysLT₂ (10 pM to 10 μM final concentration) were preincubated for 15 min with C2C12 myoblast cells expressing modified hCysLT₂R and β-arrestin-2 (25,000 cells/well in 96-well plates; 100 μl) at 37 °C. Cells were stimulated with LTD₄ (30 or 300 nM, correlating approximately to the EC₅₀ and 10x EC₅₀ values respectively) (Cayman Chemical Co.) for 1 h after which 50 μL of Tropix Gal-Screen (Applied Biosystems, Foster City, CA) luminescent reagent mix was added to each well. The luminescent readout (relative light units, RLU) was recorded after 1 h incubation at 28°C.

In separate assays, C2C12 cells expressing modified hCysLT₂R and β-arrestin-2 were preincubated with BayCysLT₂ at a fixed concentration (100 nM) for 15 min, then stimulated with varying concentrations of LTD₄ (10 pM to 10 μM) for 1 h. The RLU was recorded after 1 h incubation at 28°C.

Measurement of Agonist-induced Intracellular Calcium Mobilization

The coding regions for CysLT₁R and CysLT₂R were subcloned into pcDNA3 vectors (Invitrogen, Carlsbad, CA) and HEK293 cells were stably-transfected with Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s instructions. Stably-transfected cells were selected with G418 (2 mg/ml; Invitrogen) in Dulbecco’s modified Eagle’s medium (Millipore, Billerica, MA) containing 10 % fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin/100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5 % CO₂.
For confirmation of antagonist specificity, HEK293 cells expressing either CysLT1R or CysLT2R were plated onto poly-D-lysine-treated 3 cm clear bottom plates at a density of 25,000 cells/plate. Cells were loaded with 3 mM Fura-2-acetoxymethyl ester dye (Invitrogen) and 0.03% Pluronic F-127 solution (Invitrogen) and incubated for 45 min at 37 °C. The cells were then washed twice with GIBCO® Hank’s Buffered Saline Solution (Invitrogen) and incubated at 37°C for 15 min in the presence or absence of 300 nM BayCysLT2. All cells were then stimulated with 300 nM LTD4 (Cayman Chemical Co.) and fluorescence at the 340 nm and 380 nm wavelengths as measured using a Nikon Eclipse TS-100 microscope (Nikon Instruments Inc., Melville, NY) and analyzed using Northern Eclipse 7.0 software (EMPIX Imaging Inc., Mississauga, Canada). At the end of the assay period, non-selective calcium release indicating cell viability was confirmed via stimulation with 20 μM cyclopiazonic acid (Sigma Aldrich, St. Louis, MO).

For generation of an antagonist dose response curve, HEK293 cells were plated onto poly-D-lysine-coated 96-well plates at a density of 25,000 cells/well. The next day, cells were incubated for 1 h with Fluo-4 Direct calcium assay reagent (Invitrogen) with 2.5 mM probenecid (Invitrogen). Cells were then incubated in varying concentrations of BayCysLT2 for 15 min and stimulated with LTD4 (Cayman Chemical Co.). Maximum fluorescence at 516 nm, indicating the changes in intracellular calcium concentrations, was then measured using a SpectraMax Gemini XS Microplate Spectrofluorometer (Molecular Devices, Sunnyvale, CA).

Animal Models
Transgenic endothelium-specific human CysLT\(_2\)R overexpressing mice (hEC-CysLT\(_2\)R) (Hui et al., 2004) were previously described. The transgenic mice express seven copies of the human CysLT2R gene under the control of the Tie2 promoter/enhancer, integrated in a gene-sparse region of chromosome 6. Hemizygous mice were continuously backcrossed with C57BL/6 mice to generate equal numbers of transgenic and littermate wildtype (WT) controls.

**Vascular Ear Permeability Assay**

A vascular ear permeability assay was conducted as previously described (Hui et al., 2004). Briefly, mice received either: 1) 3 mg/kg BayCysLT\(_2\) diluted in 2.5 % DMSO and 97.5% 1x phosphate buffered saline (PBS) via intraperitoneal injection; or 2) no injection 15 min prior to anaesthesia. Mice were anesthetized i.p with ketamine/xylazine (150 mg/kg ketamine (Vetalar®) and 10 mg/kg xylazine (Rompun®)) and received 200 µL of 2 % Evans blue dye in PBS via i.v. injection through the tail vein once sedated. After 15 min, the right ear was injected intradermally with 5 ng NMLTC\(_4\) or 5 ng LTD\(_4\), both diluted in saline (0.5% EtOH final concentration). The left ear was injected with vehicle (0.5% EtOH in saline). Animals were euthanized 15 min later. A 6 mm biopsy was removed from both ears and soaked in formamide (750 µL) overnight (~18 h) at 55°C, and absorbance of the extracted Evans blue dye at 610 nm was measured with a Varian Cary 50 spectrophotometer (Agilent Technologies, Santa Clara, CA). Readouts were averaged for each experimental group and the relative change was calculated by comparing ears injected with NMLTC\(_4\) or LTD\(_4\) with vehicle injected ears.

**Ischemia/Reperfusion Surgical Model**
Ischemia was induced via ligation of the left anterior descending coronary artery (LAD). Briefly, mice 14-18 weeks of age were administered 20 mg/kg of tramadol (Ultram®) at least 1 h prior to surgery. Mice were then anesthetized with 5% isoflurane, intubated, and constantly ventilated (150 breaths/min) with 1-5% isoflurane throughout the procedure. An incision was made at the fourth intercostal space, with 50 μl 50% lidocaine/50% bupivicaine injected subcutaneously along the incision line as an analgesic. The intercostal muscles were cut and the pericardium and heart were exposed. The pericardium was pulled apart and 6-0 silk suture (Ethicon, Somerville, NJ) was passed underneath the LAD and surrounding myocardium. 30 min of ischemia was then induced by tightening the suture against a piece of PE-10 tubing placed on top of the LAD, and confirmed by visible paling of the affected myocardium. Following ischemia, the ligature was loosened to allow reperfusion. The ribs, muscle, and skin layers were closed, isoflurane delivery stopped, and animals were extubated as soon as they exhibited signs of consciousness. Extubated animals were given 0.5-1.0 ml warm lactated Ringer’s solution (Baxter, Mississauga, ON) and returned to their cages once fully mobile. The entire procedure was performed on a heated pad.

Mice were divided into three groups: no injection, vehicle injection, and drug injection. BayCysLT2 (3 mg/kg) in DMSO was diluted in 1x PBS (final concentration 2.5% DMSO), and was delivered to the mice via intraperitoneal injection either 45 min prior to ischemia or 45 min post-reperfusion. All surgical procedures and treatment regimens were approved by the University Animal Care Committee at Queen’s University and adhered to the guidelines of the Canadian Council of Animal Care.

**Analysis of Infarct Volume**
Mice were euthanized 48 h post-surgery. The heart was fully exposed, the ligature was re-tightened, and the coronary circulation was perfused with 150-200 μl phthalocyanine blue ink (Liquitex, Cincinnati, OH) dye via the aorta in order to mark the non-risk area. The heart was then rinsed in ice-cold PBS, blotted dry, wrapped in Saran™ wrap, frozen for 15 min at -20 °C, and cut transversely along the longitudinal axis into six sections using a 1.0 mm Mouse Heart Slicer Matrix (Zivic Instruments, Pittsburgh, PA). These sections were then immersed in 1 % 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Aldrich) for 15 min at 37 °C to demarcate viable and necrotic tissue. Sections were then removed, blotted dry, pressed flat to 1 mm thickness between two pieces of glass, and immersed in water. Sections were then photographed on both sides using a digital camera (Q-Color5; Olympus Corp., Tokyo, Japan).

The infarct area (pale white), the area at risk (brick red), the non-risk area (blue), and the total left ventricular area were calculated for both sides of each section using ImageJ software (National Institutes of Health, Bethesda, MD) The areas for each slice were multiplied by the thickness of the slice to obtain a measure of volume. The cumulative volume for all sections for each heart was used for comparisons. As previously described (Jiang et al., 2008), infarct size was calculated as the ratio of the infarct volume to the volume of the risk area. Animals with risk volume in the 35 to 70 % range of total LV volume were used as inclusion criteria in the study.

**RNA Extraction and Quantitative Real-Time PCR**

Total RNA from the risk area of the left ventricle was obtained using guanidinium thiocyanate-phenol-chloroform extraction (Jiang et al., 2008). Briefly, tissue was manually homogenized while immersed in TRI Reagent (Sigma Aldrich). Chloroform (Fisher Scientific, Ottawa, ON)
was then added, and the suspension was separated into three phases via centrifugation (10 min, 13,000 x g, 4 °C). The clear upper aqueous layer was isolated and RNA was precipitated using isopropanol, pelleted and resuspended in DEPC-treated ddH₂O (Invitrogen). RNA quality and quantity were assessed using an Agilent Bioanalyzer (Agilent Technologies) and Nanodrop N-1000 spectrophotometer (Nanodrop, Wilmington, DE), respectively. Total RNA was reverse-transcribed to cDNA using the iScript kit (BioRad, Berkeley, CA) according to the manufacturer’s protocol. Quantitative real-time PCR was performed using a thermal cycler (Applied Biosystems Model 7500) with SYBR Green PCR master mix (BioRad). GAPDH was used as a control housekeeping gene. Murine Cysltr2 primers were acquired from Invitrogen, while all other primers were acquired from Eurofins MWG Operon (Huntsville, AL). All primer sequences are listed in Table 1. Data were calculated using the 2^ΔΔCT method and are presented as fold-induction of transcripts for target genes normalized to Gapdh, with respect to controls.

Myeloperoxidase Assay

Neutrophil quantification was indirectly assessed by myeloperoxidase (MPO) activity assay. Briefly, mice were subjected to 30 min of ischemia and 48 h of reperfusion as detailed above. Some mice were administered 3 mg/kg BayCysLT₂ 45 min post reperfusion. At endpoint, the ventricles were isolated, flash frozen, and stored at -80 °C. All mice were heparinized prior to sacrifice. Samples were thawed on ice and placed into 20 mM potassium phosphate (Fisher Scientific) buffer (pH 7.4) at a 1 mg:20 μl ratio. Samples were then homogenized (PowerGen Model 125, Fisher Scientific) and centrifuged at 10,000 x g for 5 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 50 mM potassium phosphate (Fisher Scientific) buffer (pH 6.0) with 0.5% hexadecyltrimethylammounium bromide (Sigma Aldrich), sonicated
(Ultrasonic Dismembrator Model 500, Fisher Scientific), and immersed in a water bath at 60 °C for 2 h. Samples were then centrifuged at 10,000 x g for 5 min at 4 °C, and the supernatant assayed in a 96-well plate by adding 7 μl resuspended heart tissue to 200 μl O-dianosidine buffer (0.5 mM O-dianosidine dihydrochloride (Sigma Aldrich); 89.95 % ddH₂O; 10 % 50 mM potassium phosphate buffer, pH 6.0; 0.05 % H₂O₂ (Sigma Aldrich)). The change in absorbance at 460 nm was measured every 30 seconds for 5 min in a 96-well plate reader (FLUOStar Optima, BMG Tech GmbH, Offenburg, Germany).

**Statistical Analysis**

Dose response curve data were analyzed by non-linear regression using GraphPad 5.0 Prism software (GraphPad Software Inc, La Jolla, CA). Two-tailed unpaired Student’s t-tests were performed using GraphPad 5.0 Prism software. All values are given as mean ± standard error of the mean. A p value less than 0.05 indicated statistical significance.
Results

Synthesis and Chemistry

3-(((3-carboxycyclohexyl)amino)carbonyl)-4-(3-(4-(4-phenoxybutoxy)phenyl)-propoxy)benzoic acid, termed BayCysLT\textsubscript{2}\textsuperscript{2}, was synthesized (as outlined in Figure 1) using a similar protocol to that used by Haerter et al. (2009). In lieu of purchasing it, we synthesized compound \textbf{2} via esterification of \textbf{1} as performed by Lewin et al. (2005). Furthermore, in lieu of the literature (Haerter et al., 2009), where \textbf{8} was purchased, we generated compound \textbf{8} by pyridine hydrolysis of the intermediate diester \textbf{9} according to Coutts et al. (1981). \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR and high resolution mass spectra, as well as melting point characterization of our BayCysLT\textsubscript{2} were consistent with the literature report (Haerter et al., 2009) and are provided as supplementary material.

\textit{BayCysLT\textsubscript{2} is More Potent than Bay-u9773}

A \β\textsubscript{-}galactosidase/\β\textsubscript{-}arrestin assay was used to determine the relative potencies of BayCysLT\textsubscript{2} and the dual CysLT\textsubscript{1R}/CysLT\textsubscript{2R} antagonist Bay-u9773. Cells were preincubated for 15 min prior to stimulation with either 30 nM or 300 nM LTD\textsubscript{4}. The IC\textsubscript{50} value for BayCysLT\textsubscript{2} was determined to be 53 nM when cells were stimulated with 30 nM LTD\textsubscript{4} (≈EC\textsubscript{50} for LTD\textsubscript{4} in this assay). Increasing the stimulus concentration 10-fold to 300 nM resulted in an increase in the IC\textsubscript{50} to 274 nM. Comparatively, Bay-u9773 was found to have an IC\textsubscript{50} of 4.6 μM when stimulated with 300 nM LTD\textsubscript{4} (Figure 2A). Thus, BayCysLT\textsubscript{2} is an approximately 20-fold more potent CysLT\textsubscript{2R} inhibitor, as tested via the \β-arrestin assay.
We then assessed the competitive nature of BayCysLT2 inhibition by holding its concentration constant at 100 nM and stimulating the cells with 10 pM to 10 μM LTD4. BayCysLT2 treatment resulted in a rightward shift in the dose-response curve. However, the agonist concentrations employed were not potent enough to elicit a plateau response in antagonist-treated cells (preventing the generation of a Schild plot). LTD4 stimulation-elicited luminescence increased in a dose-dependent manner in the presence of BayCysLT2 (Figure 2B).

**BayCysLT2 is Selective for the CysLT2R In Vitro and In Vivo**

We sought to confirm the BayCysLT2 selectivity for CysLT2R compared to CysLT1R. We examined intracellular calcium mobilization in HEK293 cells stably transfected with either CysLT1R or CysLT2R in response to LTD4 stimulation, and whether BayCysLT2 could abrogate this response. Both CysLT1R and CysLT2R transfected cells responded robustly to challenge with 300 nM LTD4, and while the CysLT1R response was transient, the CysLT2R response was relatively sustained (Figure 3A and 3C respectively). Application of 100 nM BayCysLT2 for 15 min prior to leukotriene stimulation failed to affect intracellular calcium mobilization in CysLT1R-transfected cells (Figure 3B), but virtually eliminated responses in CysLT2R-transfected cells (Figure 3D). Cyclopiazonic acid stimulation at the end of each experiment demonstrated cell viability. Antagonist dose-response curves showed that BayCysLT2 is ≥500-fold more selective for CysLT2R than CysLT1R in HEK cells stimulated with 300 nM LTD4 (Figure 3E).

The selectivity of BayCysLT2 was examined *in vivo* by examination of vascular leakage in the ear following agonist stimulation. Evans blue dye was injected into the circulation of
anaesthetized WT (C57BL/6) and hEC-CysLT2R transgenic mice, and vascular permeability was assessed by the extravasation of blue dye into the aural tissue in response to CysLT challenge, as measured by absorbance at 610 nm. In WT mice injected with the CysLT1R antagonist MK-571 ((E)-3-(((3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)((3-(dimethylamino)-3-oxopropyl)thio)methyl)thio)-propanoic acid), stimulation LTD4 resulted in significantly greater dye extravasation than vehicle-injected controls (Figure 4A). Mice injected with BayCysLT2 showed significantly less dye extravasation in both LTD4 and NMLTC4 treated ears compared to mice treated with MK-571 (Figure 4A). In hEC-CysLT2R mice, LTD4 injection resulted in significantly greater dye extravasation compared to vehicle control, a phenomenon that was almost eradicated when mice were treated with BayCysLT2 (Figure 4B).

BayCysLT2 Attenuates Exacerbation of Myocardial Damage in hEC-CysLT2R Mice

Given our previous findings (Jiang et al., 2008) using the non-selective CysLT receptor antagonist Bay-u9773 in a myocardial ischemia/reperfusion injury model, as well as more recent in vitro data with a CysLT2R specific antagonist (Wunder et al., 2010), we sought to determine whether the exacerbation of myocardial damage following ischemia/reperfusion found in hEC-CysLT2R transgenic mice was CysLT2R-dependent. In accordance with previous work, there was no difference in risk volumes between any of the experimental groups (Figure 5B). Infarct volume in hEC-CysLT2R transgenic mice was significantly greater than in WT controls. However, treatment with BayCysLT2 (3 mg/kg) in transgenic mice 45 min prior to ischemia attenuated infarct damage. Mice subjected to vehicle injections did not differ in infarction volume compared to sham counterparts. WT mice injected with BayCysLT2 did not differ in infarction volume compared to vehicle or sham groups (Figure 5C).
Given that selective CysLT2R inhibition in this study and non-selective CysLT2R inhibition in a previous study (Jiang et al., 2008) did not affect infarct volume in wildtype mice and that myocardial infarction damage occurs in two distinct phases: one consisting of ischemia/reperfusion injury, the second mediated by the inflammatory response to the initial injury, we propose that CysLT2R is involved in the second phase. To evaluate this hypothesis, we injected hEC-CysLT2R transgenic mice with 3 mg/kg BayCysLT2 one h after reperfusion. Risk volumes did not differ significantly between experimental groups (Figure 5B). However, infarct volume in this group was significantly reduced compared to transgenic mice that did not receive BayCysLT2 (Figure 5C).

**Upregulation of Adhesion Molecule Gene Expression is Attenuated by BayCysLT2**

ICAM-1 and VCAM-1 are essential proteins for leukocyte adhesion and subsequent diapedesis. Indeed, previous work has found both ICAM-1 and VCAM-1 mRNA levels to be significantly elevated in hEC-CysLT2R mice 3 h following ischemia/reperfusion (I/R) compared to basal levels (Jiang et al., 2008). We report here that ICAM-1 mRNA expression is significantly upregulated in hEC-CysLT2R transgenic mice at 48 h post-I/R relative to basal levels, and that application of BayCysLT2 45 min post-reperfusion attenuated this response (Figure 6A). Concordantly, VCAM-1 mRNA is also significantly upregulated in transgenic mice at 48 h post-I/R relative to basal levels. Application of BayCysLT2 45 min post-reperfusion also attenuated this response (Figure 6B).

**BayCysLT2 Attenuates Neutrophil Infiltration in hEC-CysLT2R Mice**
MPO activity was quantified as an indirect assessment of neutrophil presence within the ventricles of mice 48 h post-ischemia/reperfusion. MPO activity was significantly increased in hEC-CysLT₂R transgenic mice after I/R compared with wildtype counterparts. However, transgenic mice treated with BayCysLT₂ 45 min post-reperfusion showed significantly less MPO activity than untreated transgenic mice (Figure 6C).
Discussion

We hereby demonstrate the in vitro and in vivo characterization of a CysLT2R-selective pharmacological antagonist, and establish its utility in both cell assays and attenuation of post-myocardial infarction inflammatory response and vascular leakage, respectively.

BayCysLT2 is one of a new family of CysLT2R-selective antagonists to be characterized that also includes HAMI3379 (Wunder et al., 2010). BayCysLT2 differs only in one ring structure from HAMI3379 (benzyl versus cyclohexyl ring). BayCysLT2 is \( \approx 10 \) -fold less potent than HAMI3379 (IC\(_{50}\): 50 nM to 4.3 nM respectively in response to 30 nM LTD\(_4\) (Wunder et al., 2010)), but is \( \approx 20 \) -fold more potent than the dual antagonist Bay-u9773. In addition, unlike Bay-u9773, BayCysLT2 does not show partial agonist properties, as intracellular calcium mobilization was not observed during antagonist incubation (data not shown). Both BayCysLT2 and HAMI3379 are highly selective for CysLT2R, with HAMI3379 showing greater than 10,000-fold affinity for the CysLT2R versus CysLT1R (Wunder et al., 2010). Our data indicate that BayCysLT2 is at least 500-fold more selective for CysLT2R and appears to show competitive inhibition, as does HAMI3379 (Wunder et al., 2010).

Previous work has shown that the CysLTs mediate vascular permeability in peripheral vasculature and that this effect is exacerbated in transgenic CysLT2R overexpressing mice and abolished in CysLT2R knockout mice (Hui et al., 2004; Moos et al., 2008). We have confirmed, pharmacologically, that CysLT2R mediates this vascular permeability response, as BayCysLT2 treatment attenuated Evans blue dye extravasation in both WT and hEC-CysLT2R transgenic animals. The mechanism appears to result from calcium signaling, which in turn regulates...
transendothelial endocytosis/exocytosis of caveolae-generated vesicles (Moos and Funk, 2008).

It was also noted that CysLT2R blockage with Bay-u9773 resulted in attenuation of exocytosis but not endocytosis in the vascular endothelium, although this phenomenon may potentially be attributed to the partial agonist properties of Bay-u9773 (Moos et al., 2008).

Transgenic overexpression of CysLT2R in endothelium has been shown to result in a significant exacerbation of damage following myocardial infarction, as well as increased CD45+ cell infiltration, intermyofibrilar erythrocyte accumulation, and fluid extravasation (Jiang et al., 2008). Previous work has also noted that basal left ventricular function is unaffected by overexpression of CysLT2R in the uninjured myocardium, but post-infarction (2 weeks) remodeling was accelerated in the TG heart (Jiang et al., 2008). In this study, we determined that selective blockade of CysLT2R with BayCysLT2 is capable of abrogating the damage observed in hEC-CysLT2R transgenic mice. While the mechanism underlying this effect is not fully clear, our data indicate that it likely involves alterations in the post-reperfusion inflammatory response.

Myocardial infarction damage can be loosely separated into three phases: ischemic, reperfusion, and inflammatory (Yellon and Hausenloy, 2007). Ischemic damage occurs during the presence of the coronary circulation blockage, and is generally characterized by cellular necrosis. Reperfusion damage occurs at the immediate time when circulation is restored to the myocardium, and is characterized by oxidative stress, triggering of pro-apoptotic pathways, and myocardial stunning (Yellon and Hausenloy, 2007). Finally, the damage to the tissue caused by the aforementioned two phases initiates the innate immune response, characterized by increased
expression of cell-adhesion molecules, as well as leukocyte recruitment and migration (Yellon and Hausenloy, 2007).

Previous studies from our laboratory observed no reduction in cardiac damage following infarction in mice lacking the CysLT$_2$R or mice lacking 5-lipoxygenase, the enzyme responsible for leukotriene synthesis, compared to wildtype controls (Jiang et al., 2008). In the current study, BayCysLT$_2$ treatment attenuated increased infarction damage when applied either before ischemia or after reperfusion. Finally, treatment with BayCysLT$_2$ prevented the increases in cell-adhesion molecule gene expression and leukocyte infiltration (as measured by MPO assay) into the myocardium that is characteristic of the inflammatory response following acute myocardial infarction. These findings indicate that the CysLTs and their receptors do not play a role in the initial ischemia and/or reperfusion components of the acute myocardial infarction injury response, but rather, mediate post-I/R inflammation.

Given this evidence, we propose a pathological mechanism where CysLT$_2$R activation results in heightened facilitation of diapedesis, which in turn enhances the magnitude of the inflammatory response and results in additional damage to the site of injury. CysLTs required for CysLT$_2$R activation can be produced via a transcellular mechanism (Zarini et al., 2009). In the myocardium, CysLT production also likely occurs transcellularly with neutrophils (the first inflammatory cells arriving after reperfusion) being the main LTA$_4$ source and other cell types (e.g. endothelial cells) converting neutrophil-donated LTA$_4$ into CysLTs. As such, ischemia/reperfusion tissue damage may result in a positive feedback loop resulting in uncontrolled inflammation. Certainly, endothelial or heterogeneous CysLT$_2$R activation has been
shown to result in activation of transcription factors and chemokine genes (Thompson et al., 2008; Uzonyi et al., 2006). Prevention of CysLT2R activation via pharmacological antagonism would thus be protective by preventing this positive feedback.

Although we have proposed a potential mechanism for CysLT2R-mediated exacerbation of the inflammatory response here and from our past studies (Jiang et al., 2008), many questions remain to be investigated and there are limitations to our findings. Precisely how does activation of CysLT2R in endothelial cells facilitate this upregulation of pro-leukocyte adhesion genes? What is the exact interplay between endothelium and infiltrating leukocytes to CysLT generation and activation of the receptor? Although our preliminary experiments have shown that overexpression of CysLT2R does not result in basal changes to superoxide levels (unpublished observations), it is possible that other reactive oxygen species are altered under stress conditions upon CysLT2R activation. How does the overexpression of CysLT2R in the transgenic model used here translate to human myocardial infarction injury? Future endeavors should be made to determine the long-term effects of CysLT2R blockade on cardiac function and remodeling post-infarction in our model and to move beyond mice to man.

In summary, we have characterized, both in vitro and in vivo, a CysLT2R-selective pharmacological antagonist, BayCysLT2. Along with NMLTC4, a novel CysLT2R agonist (Yan et al., 2011) and HAMI3379 (Wunder et al., 2010), we now have the required pharmacological tools to fully exploit CysLT2R biological signaling in health and disease.
Acknowledgments

The authors would like to thank Mr. Matthew Crerar and Ms. Christie DeVille for their assistance with the calcium imaging experiments, Mr. Graham E. Garrett for assistance with the NMR and mass spectrometry analysis, Dr. Yiqun Hui for providing hEC-CysLT2R mice, Dr. Alastair V. Ferguson for use of his calcium imaging equipment, and Dr. Michael E. Nesheim for use of his plate reader.
Authorship Contributions

Participated in research design: Ni, Yan, Barajas-Espinosa, Pratt, Funk

Conducted experiments: Ni, Yan, Ballantyne, Barajas-Espinosa, St. Amand

Contributed new reagents or analytic tools: Yan, Pratt

Performed data analysis: Ni, Yan

Wrote or contributed to the writing of the manuscript: Ni, Yan, Pratt, Funk
Reference List


Footnotes

This work was supported by Canadian Institutes of Health Research grants [MOP-79459 and MOP-93689] to C.D.F. and a Natural Sciences and Engineering Research Council grant to D.A.P. C.D.F. holds a Tier I Canada Research Chair in Molecular, Cellular and Physiological Medicine and is recipient of a Career Investigator award from the Heart and Stroke Foundation of Ontario. D.A.P. holds a Tier II Canada Research Chair in Free Radical Chemistry.

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1These authors contributed equally to the work.

2A compound termed BayCysLT2 has previously been used by Dr. Erqing Wei’s group at Zhejiang University (Qi et al., 2011; Xu et al., 2010; Yuan et al., 2009), but no details were given on the chemical structure or specific identification of the compound except to state that it was received from Amira Pharmaceuticals courtesy of Dr. T. Jon Seiders. However, in discussions with Dr. Jilly F. Evans (personal communication), co-founder of Amira Pharmaceuticals, we believe that the compound synthesized here is identical to the BayCysLT2 given to the Zhejiang University team. As such, we will use this designation throughout the text.
Figure Legends:

Figure 1: Chemical synthesis of BayCysLT2. Compounds are indicated by bold numbers and are described under *Materials and Methods*. Reagent and conditions are indicated by letters as follows: (a) MeOH, HCl (5 %), rt, 3 h, 100 %; (b) K2CO3, MeCN, reflux, 15 h, 81 %; (c) LiAlH4, THF, rt, 30 min, 100 %; (d) Ph3P, CBr4, THF, rt, 4 h, 69 %; (e) MeOH, HCl (5 %), reflux, 6 h, 30 % : 70 %; (f) C5H5N(aq), reflux, 18 h, 80 %; (g) (CH3)3SiCl, MeOH, rt, 18 h, 36 %; (h) EDC, HOBT, Et3N, CH2Cl2, rt, 15 h, 63 %; (i) K2CO3, C5H10N, reflux, 15 h, 17 %; (j) NaOH, MeOH, THF, 60 °C, 4 h, 100 %.

Figure 2: BayCysLT2 is a more potent antagonist of the human CysLT2 receptor than Bay-u9773. (A) Relative luminescence unit (RLU) response by C2C12 cells (n = 4) expressing modified hCysLT2R and β-arrestin-2 to 30 nM or 300 nM LTD4 stimulation in the presence of varying concentrations of BayCysLT2 or Bay-u9773. IC50 values were determined to be 53 nM (30 nM LTD4) and 274 nM (300 nM LTD4) for BayCysLT2, and 4.6 μM (300 nM LTD4) for Bay-u9773. (B) A constant concentration (100 nM) of BayCysLT2 was incubated with increasing concentrations of LTD4. Response to LTD4 increased in a dose dependent manner in the presence of BayCysLT2, but did not reach a plateau phase (n = 4). Means of duplicates for each sample are shown (with error bars depicting SEM).

Figure 3: BayCysLT2 selectively antagonizes the human CysLT2 receptor calcium responses. Intracellular calcium mobilization in HEK cells expressing either CysLT1 (HEK-CysLT1R) or CysLT2 (HEK-CysLT2R) receptor, expressed as the ratio between fluorescence detected at 340
nm and 380 nm, was elicited via stimulation with 300 nM leukotriene D₄ (LTD₄). Tracings and images depict one cell representative of its experimental group (n = 10-15). Color coding representative of relative intracellular calcium concentrations is indicated on the vertical scale bar, with red being highest and purple being lowest. At the end of each LTD₄ stimulation, 20 μM cyclopiazonic acid (CPA) was added to test for non-selective calcium release. HEK-CysLT₁R cells responded to leukotriene stimulation both in the absence (A) and presence (B) of 100 nM BayCysLT₂ with a sharp increase in intracellular calcium. HEK-CysLT₂R cells responded to leukotriene stimulation in the absence (C), but not in the presence (D) of 100 nM BayCysLT₂.

(E) Antagonist dose response curve for HEK-CysLT₂R (●) cells was constructed by plotting the fluorescence in response to challenge with 300 nM LTD₄ at each antagonist concentration and used to determine the IC₅₀ value. HEK-CysLT₁R (○) cells did not exhibit significant decrease in calcium response to leukotriene stimulation even in the presence of 100 μM BayCysLT₂. Values given as mean ± SEM (n = 3-8).

**Figure 4:** BayCysLT₂ is a potent selective antagonist at the CysLT₂ receptor *in vivo*. Vascular permeability in murine ears in response to intradermal LTD₄ or NMLTC₄ challenge was measured by quantifying Evans blue dye extravasation and normalizing values against vehicle-injected controls. (A) LTD₄ injection caused increased vascular permeability in wildtype mice in the presence of the CysLT₁R antagonist MK-571, but not in mice treated with BayCysLT₂. NMLTC₄ injection in BayCysLT₂-treated mice yielded similar results to LTD₄ injected counterparts. (B) LTD₄ injection in hEC-CysLT₂R transgenic mice resulted in increased vascular permeability compared to vehicle-treated controls, and this phenomenon was attenuated by BayCysLT₂ pre-treatment. * = p < 0.05; n = 4-5, values given as mean ± SEM.
**Figure 5:** BayCysLT₂ attenuates CysLT₂R-mediated exacerbation of myocardial ischemia/reperfusion injury in mice. (A) Representative TTC-stained ventricular sections from wildtype (WT) and hEC-CysLT₂R (TG) mice given no injection (ctrl), vehicle injections (veh), 3 mg/kg BayCysLT₂ 45 min prior to ischemia (drug -45) or 3 mg/kg BayCysLT₂ 45 min post-reperfusion (drug +45) are presented here. Morphometric analysis of (B) LV volume at risk (C) and infarct size in the groups mentioned above showed that infarction volume was significantly greater in TG mice compared to WT, and that administration of BayCysLT₂ either prior to or post-ischemia/reperfusion attenuated this response. * = p < 0.05; ** = p < 0.01; n = 5-7, values given as mean ± SEM.

**Figure 6:** BayCysLT₂ attenuates indirect measures of leukocyte recruitment in ischemia/reperfusion injury-induced diapedesis. Transgenic (TG) mice showed significantly increased (A) VCAM-1 and (B) ICAM-1 mRNA expression post I/R in comparison to non-infarcted controls. Treatment with 3 mg/kg BayCysLT₂ post-I/R resulted in significant attenuation of VCAM mRNA upregulation. ICAM mRNA upregulation was also reduced, albeit at borderline significant levels. (C) Infarcted transgenic (TG) mice also showed significantly increased myeloperoxidase enzyme activity (1 unit is defined as the amount of MPO required to convert 1 μmol of H₂O₂ in 1 min) present in the myocardial area at risk, indicating increased presence of neutrophils, compared to infarcted wildtype (WT) animals. Increase in neutrophil presence in the tissue was significantly attenuated by treatment with 3 mg/kg BayCysLT₂ post-I/R. n = 3-4 for mRNA quantification, n = 5 for MPO assay, * = p < 0.05, values given as mean ± SEM.
**Table 1**: Primers used in qualitative real-time PCR reactions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
<th>AP (bp)</th>
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<td>Cysltr2</td>
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<td>TGGAGATCGAAAGTCCGGAG</td>
<td>110</td>
</tr>
</tbody>
</table>

Gene names, primer sequences, and amplicon (AP) sizes in base pairs (bp) for primers used in this study.
FIGURE 3

A. CysLT₁R

B. CysLT₁R

C. CysLT₂R

D. CysLT₂R

E. % Maximal RFU vs. [BayCysLT₂] (log M)
FIGURE 4

A

B

Fold Increase

Fold Increase

LTD$_4$
NMLTC$_4$
MK-571
BayCysLT$_2$

+  +  -  -
-  -  +  +
+  -  -  +
-  +  +  +
Supplementary Section

A Selective Cysteinyl Leukotriene Receptor 2 Antagonist Blocks Myocardial Ischemia/Reperfusion Injury and Vascular Permeability in Mice

By

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1 These authors contributed equally to this work.
NMR spectra (\(^1\textrm{H} \) Fig. 1, \(^{13}\textrm{C} \) Fig. 2, and HSQC Fig. 3) of the final compound BayCysLT\(_2\) were obtained on an automated Bruker Avance-400 MHz spectometer and referenced to deuterated (d6)-DMSO as solvent. Spectra were analyzed with MestRec (Escondido, CA) software.
Figure S1: $^1$H NMR Spectrum of BayCysLT$_2$ in $d_6$-DMSO.
Figure S2: 13C NMR Spectrum of BayCycl17 in δ-DMSO.
Figure S3: HSQC NMR Spectrum of BayCysLT$_2$ in $d_6$-DMSO.
Figure S4: Calculated (top) and Observed (bottom) High Resolution Mass Spectra (ESI-TOF) of BayCysLT₂ in MeOH.