**Introduction**

Myocardial infarction 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. Although the only remedy to this problem is timely restoration of blood flow (“reperfusion”), this results in the en masse formation of reactive oxygen species, creating oxidative stress (Zweier, 1988). Reactive oxygen species formation, in tandem with chemoattractant release during myocardial infarction, invokes the innate inflammatory response, resulting in adhesion protein up-regulation, leukocyte infiltration, and extracellular matrix degradation (Vinten-Johansen, 2004). It is these postreperfusion events that contribute the majority of myocardial infarction-induced damage (Yellon and Hausenloy, 2007).

The cysteinyl leukotrienes (CysLTS), so termed because of the common cysteine moiety in their structures, are a group of proinflammatory arachidonic acid metabolites consisting of LTC4, LTD4, and LTE4. CysLTS exert their effects primarily by binding two characterized G protein-coupled receptors: CysLT1R (CysLT receptor 1) (Lynch et al., 1999) and CysLT2R (Heise et al., 2000). CysLTs have also likely by binding two characterized G protein-coupled receptors: CysLT1R (CysLT receptor 1) (Lynch et al., 1999) and CysLT2R (Heise et al., 2000). CysLTs have also
been shown to interact with the putative cysteiny1 leukotriene E receptor (Maekawa et al., 2008), the P2Y12 receptor (purinergic receptor P2Y, G protein-coupled, 12) (Nonaka et al., 2005), and G protein-coupled receptor 17 (Ciana et al., 2006; Maekawa et al., 2009). Of these receptors, CysLT1R is the most extensively studied (Moos and Funk, 2008) and has been shown to mediate bronchoconstriction and airway inflammation (Funk, 2005). CysLT1R has not received as much attention as CysLT2R, although there has been a wave of interest concerning this receptor in cardiovascular disease (Funk, 2005). 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 (Lötzer et al., 2003; Uzonyi et al., 2006), 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) and is regulated 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 and leukotriene A4 hydrolase are linked to increased risk of myocardial infarction (Helgadottir et al., 2004, 2006). Some studies have also shown that the 5-lipoxygenase pathway is involved in atherosclerosis (Spanbroek et al., 2003; Funk, 2005; Qiu et al., 2006; Di Gennaro et al., 2010). As well, plasma LTC4 levels (Takase et al., 1996) and both CysLT2R and CysLT3R expression (Jiang et al., 2008) have been shown to increase after 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 after coronary vessel ligation, which was attenuated by the nonselective 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 up-regulation as well as leukocyte infiltration (Jiang et al., 2008). However, because of the absence of a CysLT2R-selective antagonist (Moos and Funk, 2008), the precise pathological contributions of CysLT2R and CysLT3R 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 [3-((3-carboxycyclohexyl)larnino)carbonyl)-4-(3-((4-phenoxybutoxy)phenyl)-propoxy]benzoic acid (HAMI3379) (Wunder et al., 2010). In this article, we characterize the pharmacological properties of another CysLT2R-selective antagonist 3-((3-carboxycyclohexylamino)carbonyl)-4-(3-(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 myocardial infarction injury seen in mice overexpressing CysLT2R and accomplishes this by reducing leukocyte infiltration.

Materials and 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). 1H and 13C) NMR spectra were recorded at 25°C on a Bruker Avance Spectrometer (Bruker Daltonics, Billerica, MA) at 300 (75) or 400 (100) MHz. The desired compound, 3-(((3-carboxycyclohexyl)larnino)carbonyl)-4-(3-(4-phenoxybutoxy)phenyl)-propoxy]benzoic acid (BayCysLT2) is a patented antagonist from Bayer Pharmaceuticals (Montville, NJ) (Haerter et al., 2009) and was synthesized according to the route in Fig. 1 from four commercially available starting materials. 3-(4-Hydroxypyrrol) propionic acid (1), 4-hydroxyisophthalic acid (7), and 3-aminoaclohexane-1-carboxylic acid (10) were purchased from TCI America (Portland, OR), and 4-phenoxy-1-buty1 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 of 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 in vacuo to give a yellow oil in quantitative yield. MS: m/z 150.0 [M]+.

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 of acetonitrile was mixed with K2CO3 (2.5 g, 2 mmol) and heated to reflux for 15 h. Solvent was removed, and the residue was 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 (Lewin et al., 2005).

3-(4-(Phenoxybutoxy)phenyl)-1-propanol (5). LiAlH4 (1 M in 5 ml of THF) was introduced dropwise, while stirring, to a solution of 4 (1.3 g, 4.16 mmol) in minimal (5–8 ml) THF. The solution was stirred at room temperature for 30 min. MeOH (1–2 ml) was cautiously added to the suspension to destroy excess LiAlH4. The mixture was then added to 1 M 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 of THF, to which was added triphenylphosphine (1 g, 4 mmol) and tetrabromomethane (1.2 mg).
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 was 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 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 concentration under reduced pressure. The combined yield of monoester 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, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydroxybenzotriazole, Et3N, CH2Cl2, rt, 15 h, 69%; (i) K2CO3, C5H10N, reflux, 15 h, 17%; (j) NaOH, MeOH, THF, 60°C, 4 h, 100%.

Fig. 1. Chemical synthesis of BayCysLT2. Compounds are indicated by bold numbers and are described under Materials and Methods. Reagents, conditions, and percentage yields are indicated 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%; (f) C5H5N(aq), reflux, 18 h, 80%; (g) (CH3)3SiCl, MeOH, rt, 18 h, 36%; (h) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydroxybenzotriazole, Et3N, CH2Cl2, rt, 15 h, 69%; (i) K2CO3, C3H6O2N, reflux, 15 h, 17%; (j) NaOH, MeOH, THF, 60°C, 4 h, 100%.
concentrated in vacuo. The crude product was then 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).

**Methyl 3-(((3-(Methoxycarbonyl)cyclohexyl)amino)carbonyl)-4-(3-(4-(4-phenoxoxygeno)phenyl)propoxy) Benzoxate (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 was heated to reflux. The alkyl bromide 6 (0.6 g, 1.7 mmol) was added over 15 h until 12 was consumed as judged by TLC. A 5% solution of NaH2PO4 (10 ml) was then added to the reaction mixture, and 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 CysLT1R activation was described recently (Yan et al., 2011). In brief, Bay-u9773 (Cayman Chemical, Ann Arbor, MI) or BayCysLT2 (10 pM to 10 μM final concentration) were preincubated for 15 min with C2C12 myoblast cells expressing modified hCysLT1R and β-arrestin-2 (25,000 cells/well in 96-well plates; 100 μl at 27°C. Cells were stimulated with LTD4 (30 or 300 nM, correlating approximately to the EC50 and 10× EC50 values, respectively) (Cayman Chemical) 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 incubation at 28°C.

In separate assays, C2C12 cells expressing modified hCysLT1R and β-arrestin-2 were preincubated with BayCysLT1R 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 were 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 (Geneticin) (2 mg/ml); Invitrogen) in Dulbecco’s modified Eagle’s medium (Millipore Corporation, Billerica, MA) containing 10% fetal bovine serum, 2 mM l-glutamine, and 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 Hanks’ 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), and fluorescence at the 340- and 380-nm wavelengths was measured using a Nikon Eclipse TS-100 microscope (Nikon, Melville, NY) and analyzed using Northern Eclipse 7.0 software (EMPIX Imaging Inc., Mississauga, Canada). At the end of the assay period, nonspecific 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 into 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). Maximum fluorescence at 516 nm, indicating the changes in intracellular calcium concentrations, was then measured using a SpectraMax Gemini XS Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA).

**Animal Models.** Transgenic human endothelial cell-specific CysLT2R overexpresser (hEC-CysLT2R) mice (Hui et al., 2004) were described previously. The transgenic mice express seven copies of the human CYS1TR2 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 wild-type (WT) controls.

**Vascular Ear Permeability Assay.** A vascular ear permeability assay was conducted as described previously (Hui et al., 2004). In brief, mice received either 1) 3 mg/kg BayCysLT2, diluted in 2.5% DMSO and 97.5% x phosphate-buffered saline (PBS) via intraperitoneal injection or 2) no injection 15 min before anesthesia. Mice were anesthetized intraperitoneally 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 intravenous injection through the tail vein once sedated. After 15 min, the right ear was injected intradermally with 5 ng of NMLTc or 5 ng of LTD4, 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 or LTD4, with vehicle-injected ears.

**Ischemia/Reperfusion Surgical Model.** Ischemia was induced via ligation of the left anterior descending coronary artery (LAD). In brief, mice 14 to 18 weeks of age were administered 20 mg/kg tramadol (Ultran) at least 1 h before surgery. Mice were then anesthetized with 5% isoflurane, intubated, and constantly ventilated (150 breaths/min) with 1 to 5% isoflurane throughout the procedure. An incision was made at the fourth intercostal space, with 50 μl of 50% lidocaine/50% bupivacaine 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. Thirty minutes 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. After ischemia, the ligature was loosened to allow reperfusion. The ribs, muscle, and skin layers were closed, isoflurane delivery was stopped, and animals were extubated as soon as they exhibited signs of consciousness. Exsanguinated animals were given 0.5 to 1.0 ml of warm lactated Ring-er’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 1× PBS (final concentration 2.5% DMSO) and delivered to the mice via intraperitoneal injection either 45 min before ischemia or 45 min after 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 ligation was reighted, and coronary circulation was perfused with 150 to 200 μl of phtha-
locyanine blue ink dye (Liquitex, Cincinnati, OH) via the aorta to mark the nonrisk area. The heart was then rinsed in ice-cold PBS, blotted dry, wrapped in plastic 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 (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, Tokyo, Japan).

The infarct area (pale white), the area at risk (brick red), the nonrisk 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 described previously (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 left ventricle 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). In brief, tissue was manually homogenized while immersed in TRI reagent (Sigma-Aldrich). Chloroform (Thermo Fisher Scientific, 2008). In brief, tissue was manually homogenized while immersed in TRI reagent (Sigma-Aldrich). Chloroform (Thermo Fisher Scientific, Ottawa, ON, Canada) was then added, and the suspension was separated into three phases via centrifugation (10 min, 13,000 g). The clear upper aqueous layer was isolated, and RNA was separated into three phases via centrifugation (10 min, 13,000 g). The clear upper aqueous layer was isolated, and RNA was separated into three phases via centrifugation (10 min, 13,000 g). The clear upper aqueous layer was isolated, and RNA was separated into three phases via centrifugation (10 min, 13,000 g).

**Gene names, primer sequences, and amplicon sizes in base pairs (bp) for primers used in this study are shown.**

### TABLE 1

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**Synthesis and Chemistry.** 3-(((3-Carboxycyclohexyl) amino)carbonyl)-4-(3-(4-(4-phenoxybutoxy)phenyl)-propoxy)-benzoic acid, termed BayCysLT2, was synthesized (as outlined in Fig. 1) using a similar protocol to that used by Haerter et al. (2009). In lieu of purchasing it, we synthesized compound 2 via esterification of 1 as described by Lewin et al. (2005). Furthermore, in lieu of the literature (Haerter et al., 2009), where 8 was purchased, we generated compound 8 by pyridine hydrolysis of the intermediate diester 9 according to Coutts et al. (1981). 1H-NMR, 13C-NMR, and high-resolution mass spectra, as well as melting point characterization of our BayCysLT2 were consistent with the literature report (Haerter et al., 2009) and are provided as Supplemental Material.

**BayCysLT2 Is More Potent than Bay-u9773.** A β-galactosidase/β-agarase assay was used to determine the relative potencies of BayCysLT2 and the dual CysLT1R/CysLT2R antagonist Bay-u9773. Cells were preincubated for 15 min before stimulation with either 30 or 300 nM LTD4. The IC50 value for BayCysLT2 was determined to be 53 nM when cells were stimulated with 30 nM LTD4 (≈EC50 for LTD4 in this assay). Increasing the stimulus concentration 10-fold to 300 nM resulted in an increase in the IC50 to 274 nM. Comparatively, Bay-u9773 was found to have an IC50 of 4.6 μM when stimulated with 300 nM LTD4 (Fig. 2A). Thus, BayCysLT2 is an approximately 20-fold more potent CysLT2R inhibitor, as tested via the β-agarase 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 used were not potent enough to elicit a plateau response in antagonist-treated cells (preventing the generation of a Schild
BayCysLT2 is selective for CysLT2R than CysLT1R in HEK cells stimulated with 300 nM LTD4 (Fig. 3E).

The selectivity of BayCysLT2 was examined in vivo by examination of vascular leakage in the ear after agonist stimulation. Evans blue dye was injected into the circulation of anesthetized 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 CysLT2 challenge, as measured by absorbance at 610 nm. In WT mice injected with the CysLT2R antagonist MK-571 [(E)-3-(((3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)(3-(dimethylamino)-3-oxopropyl)thio) methyl(thio)-propanoic acid], stimulation with LTD4 resulted in significantly greater dye extravasation than vehicle-injected controls (Fig. 4A). Mice injected with BayCysLT2 showed significantly less dye extravasation in both LTD4- and NMLTC4-treated ears compared with mice treated with MK-571 (Fig. 4A). In hEC-CysLT2R mice, LTD4 injection resulted in significantly greater dye extravasation compared with vehicle control, a phenomenon that was almost eradicated when mice were treated with BayCysLT2 (Fig. 4B).

BayCysLT2 Attenuates Exacerbation of Myocardial Damage in hEC-CysLT2R Mice. Given our previous findings (Jiang et al., 2008) using the nonselective CysLT receptor antagonist Bay-u9773 in a myocardial ischemia/reperfusion (I/R) 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 after 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 (Fig. 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 before ischemia attenuated infarct damage. Mice subjected to vehicle injections did not differ in infarction volume compared with sham counterparts. WT mice injected with BayCysLT2 did not differ in infarction volume compared with vehicle or sham groups (Fig. 5C).

Given that selective CysLT2R inhibition in this study and nonselective CysLT2R inhibition in a previous study (Jiang et al., 2008) did not affect infarct volume in wild-type mice and 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 45 min after reperfusion. Risk volumes did not differ significantly between experimental groups (Fig. 5B). However, infarct volume in this group was significantly reduced compared with transgenic mice that did not receive BayCysLT2 (Fig. 5C).

Up-Regulation 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 after I/R compared with basal levels (Jiang et al., 2008). We report here that ICAM-1 mRNA expression is significantly up-regulated in hEC-CysLT2R transgenic mice at 48 h post-I/R relative to basal levels, and application of BayCysLT2 45 min postreperfusion attenuated
this response (Fig. 6A). Concordantly, VCAM-1 mRNA was also significantly up-regulated in transgenic mice at 48 h post-I/R relative to basal levels. Application of BayCysLT₂ 45 min postreperfusion also attenuated this response (Fig. 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/I/R. MPO activity was significantly increased in hEC-CysLT₂R transgenic mice after I/R compared with wild-type counterparts. However, transgenic mice treated with BayCysLT₂ 45 min postreperfusion showed significantly less MPO activity than untreated transgenic mice (Fig. 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 postmyocardial infarction inflammatory response and vascular leakage, respectively.

BayCysLT$_2$ is one of a new family of CysLT$_2$-selective antagonists to be characterized that also includes HAMI3379 (Wunder et al., 2010). BayCysLT$_2$ differs only
in one ring structure from HAMI3379 (benzyl versus cyclohexyl ring). BayCysLT2 is ≈10-fold less potent than HAMI3379 (IC50 50 to 4.3 nM, respectively, in response to 30 nM LTD4 [Haerter et al., 2009]), but is ≈20-fold more potent than the dual antagonist Bay-u9773. In addition, unlike Bay-u9773, BayCysLT2 does not show partial agonist properties, because intracellular calcium mobilization was not observed during antagonist incubation (data not shown). Both BayCysLT2 and HAMI3379 are highly selective for CysLT2R, with HAMI3379 showing more than 10,000-fold affinity for CysLT2R versus CysLT1R (Wunder et al., 2010). Our data indicate that BayCysLT2 is at least 500-fold more selective for CysLT2R and seems 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 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, because BayCysLT2 treatment attenuated Evans blue dye extravasation in both WT and hEC-CysLT2R transgenic animals. The mechanism seems to result from calcium signaling, which in turn regulates transendothelial endocytosis/exocytosis of caveloae-generated vesicles (Moos and Funk, 2008). It was also noted that CysLT2R blockade 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 after myocardial infarction, as well as increased CD45+ cell infiltration, intermyofibril 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 postinfarction (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. Although the mechanism underlying this effect is not fully clear, our data indicate that it probably involves alterations in the postreperfusion 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 proapoptotic 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).

Our previous studies observed no reduction in cardiac damage after infarction in mice lacking CysLT2R or mice lacking 5-lipoxygenase, the enzyme responsible for leukotriene synthesis, compared with wild-type controls (Jiang et al., 2008). In the current study, BayCysLT2 treatment attenuated increased infarction damage when applied either before ischemia or after reperfusion. Finally, treatment with BayCysLT2 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 after 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 CysLT2R 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 CysLT2R activation can be produced via a transcellular mechanism (Zarini et al., 2009). In the myocardium, CysLT production also probably occurs transcellularly with neutrophils (the first inflammatory cells arriving after reperfusion) being the main LTA4 source and other cell types (e.g., endothelial cells) converting neutrophil-
donated LTA₄ into CysLTs. As such, ischemia/reperfusion tissue damage may result in a positive feedback loop resulting in uncontrolled inflammation. Certainly, endothelial or heterogeneous CysLT₂R activation has been shown to result in the activation of transcription factors and chemokine genes (Uzonyi et al., 2006; Thompson et al., 2008). 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 up-regulation of pro-inflammatory genes? What is the exact interplay between endothelial 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 (A.B.-E., N.C.N., and C.D.F., 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 postinfarction in our model and to move beyond mice to humans.

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 have now provided pharmacological tools to fully exploit CysLT₂R biological signaling in health and disease.

**Note Added In Proof.** While this manuscript was under review, a paper appeared (Carnini et al., 2011) describing the use of a compound referred to as CysLT2cpd (Haerter et al., 2009), which is the same as BayCysLT₂ used in our studies.

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**Authorship Contributions**

*Participant in research design:** Ni, Yan, Barajas-Espinosa, Pratt, and Funk.

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

*Contributed new reagents or analytic tools:** Yan and Pratt.

*Performed data analysis:** Ni and Yan.

*Wrote or contributed to the writing of the manuscript:** Ni, Yan, Pratt, and Funk.

**References**


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