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Inhibition of p38 Mitogen-Activated Protein Kinase Improves Nitric Oxide–Mediated Vasodilatation and Reduces Inflammation in Hypercholesterolemia

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Background—Oxidized low-density lipoprotein reduces endothelial nitric oxide production (an important mediator of vasoregulation) and activates p38 mitogen-activated protein kinase (MAPK), a mediator of vascular inflammation. Animal models of vascular stress have previously predicted improvements in vascular function after p38 MAPK inhibition. We hypothesized that a selective p38 α/β MAPK inhibitor (losmapimod; GW856553) would improve compromised nitric oxide–mediated vasoregulation in patients with hypercholesterolemia.

Methods and Results—Untreated hypercholesterolemic patients (low-density lipoprotein cholesterol >4.1 mmol/L) were randomized to receive losmapimod 7.5 mg (n=27) or placebo (n=29) twice daily for 28 days. Patients with known vascular disorders (eg, diabetes mellitus, coronary heart disease) were excluded. Forearm blood flow was measured by venous occlusion plethysmography in response to serial intra-arterial infusion of acetylcholine, sodium nitroprusside, and N^G-monomethyl-L-arginine (L-NMMA). Acetylcholine and L-NMMA responses were significantly impaired ($P=0.01$ and $P=0.03$) compared with responses in control subjects (n=12). In hypercholesterolemic patients treated with losmapimod, responses to acetylcholine were improved by 25% (95% confidence interval, 5 to 48; $P=0.01$), to sodium nitroprusside by 20% (95% confidence interval, 3 to 40; $P=0.02$), and to L-NMMA by 10% (95% confidence interval, –1 to 23; $P=0.07$) compared with placebo. C-reactive protein was reduced by 57% (95% confidence interval, –81 to –6%; $P<0.05$) in patients treated with losmapimod compared with placebo.

Conclusions—Losmapimod improves nitric oxide–mediated vasodilatation in hypercholesterolemic patients, which is consistent with findings in previous translational animal models. These data support the hypothesis that attenuating the inflammatory milieu by inhibiting p38 MAPK activity improves NO activity. This suggests p38 MAPK as a novel target for patients with cardiovascular disease.

Clinical Trial Registration—URL: <http://clinicaltrials.gov>. Unique identifier: NCT00474864. (*Circulation*. 2011;123:515-523.)

Key Words: endothelial function ■ hypercholesterolemia ■ nitric oxide ■ p38 MAPK ■ vasodilation

Atherosclerosis is regarded as a complex condition in which inflammation plays a pivotal role, involving low-density lipoprotein (LDL) deposition and oxidation, recruitment of inflammatory cells, and release of cytokines and endothelial dysfunction.^{1,2} Reduced nitric oxide (NO) bioavailability accompanies all stages of atherosclerosis and is associated with increased cardiovascular risk.³

Clinical Perspective on p 523

The release of NO is a complex process that can be affected by a number of physiological and pathophysiological factors,⁴ including serum levels of LDL, which, when oxidized, have increased atherogenic potential.⁵ Oxidized LDL reduces NO bioavailability, destabilizes endothelial NO synthase mRNA,⁶

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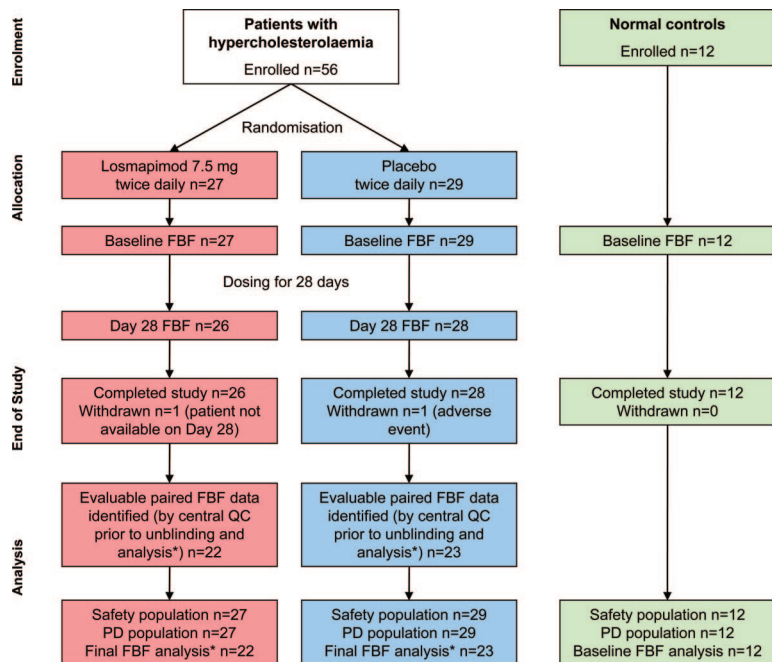
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*Reasons for non-evaluable data and consequent exclusion from final FBF analysis were incomplete data set (ie, FBF not available at both baseline and Day 28), poor quality data, or FBF procedure variation.

Figure 1. Flow diagram of participants through the study. Normal control subjects participated in baseline (day 1) assessments only and did not receive any intervention. The safety population comprised all hypercholesterolemic patients who received at least 1 dose of study drug and normal control subjects. The pharmacodynamic (PD) population comprised all participants who provided pharmacodynamic data on at least 1 occasion. *Reasons for exclusion from final FBF analysis (before unblinding and statistical analysis) included incomplete data sets, nonevaluable data sets, and FBF procedure variation.

and activates p38 mitogen-activated protein kinase (MAPK). As a pivotal intracellular signaling kinase, p38 MAPK activation (phosphorylation) plays a critical role in orchestrating transcriptional and translational aspects of the inflammatory response in endothelial cells and macrophages. The ensuing cascade of inflammatory events, involving the production and release of proinflammatory cytokines such as interleukin 6 (IL-6), results in profound dysfunction of the arterial endothelium⁷ and raises systemic inflammatory markers such as high sensitivity C-reactive protein (hsCRP). A prominent feature of this vascular inflammatory response is the generation of reactive oxygen species (ROS) mediated by the activation of NADPH oxidases.⁸ ROS not only scavenge NO but also oxidize soluble guanylate cyclase and reduce the production of cGMP in the vascular smooth muscle, further limiting NO signaling. Thus, vascular inflammation is often characterized by both reduced NO bioavailability and impaired smooth muscle reactivity. Evidence suggests that local amplification of this inflammatory cascade occurs because p38 MAPK functions in an amplification circuit both upstream and downstream of ROS generation. Specifically, the predominant forms of NADPH oxidases (2 and 4) in the endothelium cause ROS-apoptosis signal-regulating kinase–dependent activation of p38 MAPK (downstream of ROS). In turn, activated p38 MAPK produces ROS further by upregulating NADPH oxidase subunits and cytokine production (upstream of ROS).⁹ Consistent with this evidence, we and others have previously shown that endothelial dysfunction in patients with inflammatory conditions such as vasculitis and rheumatoid arthritis can be reversed by antiinflammatory agents.^{10–12} Endothelial dysfunction is present in patients with risk factors for atherosclerosis such as hypercholesterolemia, hypertension, smoking, and diabetes mellitus.^{13–16} Importantly, endothelial dysfunction independently predicts future cardiovascular events.¹⁷

Several p38 MAPK inhibitors are effective in a variety of in vitro and in vivo animal models that mimic cardiovascular disorders.^{18–21} We have recently shown in an animal model (spontaneously hypertensive stroke-prone rats fed a salt-fat diet) that long-term treatment with a selective p38 α/β MAPK inhibitor (losmapimod; previously known as GW856553 or GSK-AHAB) significantly and dose-dependently improved endothelium-dependent and -independent vascular relaxation in isolated aortas.²² To translate this into humans, we chose subjects with hypercholesterolemia because they exhibit a proinflammatory state thought to be driven by elevated oxidized LDL, and we evaluated their vascular function using the technique of venous occlusion plethysmography²³ before and after p38 MAPK inhibition. We hypothesized that treatment with the novel antiinflammatory drug losmapimod for 28 days would improve endothelial function as evidenced by an increased vasodilatory response to intra-arterial acetylcholine infusion (primary end point). Our prespecified secondary end points included forearm responses to intra-arterial sodium nitroprusside (SNP) as a measure of endothelial-independent function, forearm responses to *N*^G-monomethyl-L-arginine (L-NMMA) as a measure of basal NO synthesis, measurement of total and phosphorylated heat shock protein 27 (HSP27) as a pharmacodynamic marker of p38 MAPK inhibition, and safety and tolerability parameters, including 12-lead ECGs, hematology, biochemistry, urinalysis, and hemodynamic observations. A number of exploratory end points, including hsCRP and IL-6, were also measured to explore postulated mechanisms.

Methods

This was a double-blind, placebo-controlled, parallel-group study in which hypercholesterolemic patients were randomly assigned (1:1) to receive oral losmapimod 7.5 mg or placebo twice daily for 28 days; a separate cohort of healthy volunteers were recruited as control subjects (see Figure 1). The study was conducted at 3 sites

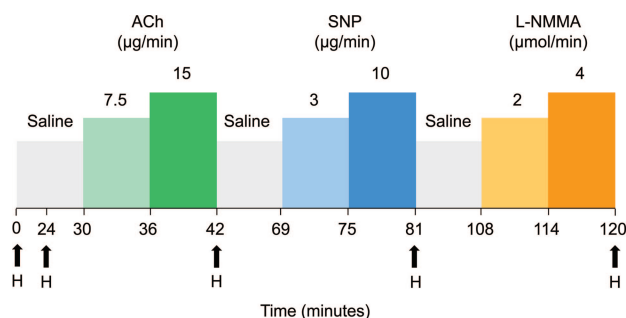


Figure 2. Study schematic for timing of measurements for forearm blood flow. ACh indicates acetylcholine; H, haemodynamic measurements; SNP, sodium nitroprusside; L-NMMA, N^G-monomethyl arginine.

(University of Cambridge, University of Wales College of Medicine, and Barts and The London School of Medicine and Dentistry) in the United Kingdom. Study personnel and participants were blinded to treatment assignment for the duration of the study. Healthy subjects did not receive any intervention.

The protocol was approved by a multicenter ethics committee across all 3 sites (Cambridgeshire 4 NHS Research Ethics Committee) and was registered at clinicaltrials.gov (NCT00474864). The study complied with the Declaration of Helsinki, and full written informed consent was obtained from all participants before the performance of any study-specific procedures.

Study Population

Patients 18 to 75 years of age with untreated hypercholesterolemia (or washed out from lipid-lowering therapies for 28 days), fasting LDL cholesterol >4.1 mmol/L, fasting triglycerides <4.5 mmol/L, and a body mass index of 19 to 32 kg/m² were eligible for the study. Patients with cardiovascular, hepatic, or renal disease; poorly controlled diabetes mellitus; chronic inflammatory conditions; and malignancy were excluded. Twelve age-matched healthy, nonsmoking men 18 to 75 years of age with fasting LDL cholesterol <2.6 mmol/L, fasting triglycerides <1.7 mmol/L, fasting high density lipoprotein cholesterol >1.0 mmol/L, and blood pressure ≤140/90 mm Hg were recruited to a control group.

Interventions

The structure and activity profile of losmapimod (previously known as GW856553²⁴ or GSK-AHAB²²) are described in Figure I in the online-only Data Supplement. Nonselective inhibitors have previously been shown to be toxic; therefore, the development of more selective inhibitors of p38 MAPK (for the α and β isoforms) has been ongoing over the last 20 years²⁵; losmapimod is one such agent that has been optimized to potentially inhibit inflammatory cytokine production while providing safety and efficacy in animal models. The half-life of losmapimod approximates 12 hour; thus, this agent is provided as twice-daily dosing. The drug has been evaluated in >11 clinical studies in healthy volunteers and patients²⁴ and has been shown to be safe and well tolerated. It has been shown to dose-dependently inhibit ex vivo production of lipopolysaccharide-stimulated tumor necrosis factor- α and sorbitol-stimulated phosphorylated HSP27. We have targeted an approximate average of 50% enzyme inhibition for the current dosing level. Losmapimod tablets and matching placebo tablets were manufactured by GlaxoSmithKline (Harlow, UK).

Forearm Blood Flow

Forearm blood flow (FBF) was measured by venous occlusion plethysmography (Hokanson Inc, Bellevue, WA) as previously described²³ using the protocol illustrated in Figure 2. Wrist circulation was excluded by inflating wrist cuffs above the systolic blood pressure. Upper arm cuffs were intermittently inflated (to 40 mm Hg) and deflated at short intervals over 3 minutes to measure FBF with

mercury-in-Silastic gauges. The dominant arm was established as a control arm without cannulation or test infusions. In contrast, acetylcholine (Novartis Pharmaceuticals, Basel, Switzerland), SNP (Nitroprussiat FIDES, Madrid, Spain), and L-NMMA (Bachem Distribution Services GmbH, Weil am Rhein, Germany) were infused in a fixed order into the brachial artery of the nondominant (test) arm via a 27-gauge needle inserted under local anesthesia. All drugs were prepared aseptically and diluted in sterile saline (0.9% Baxter Healthcare, Norfolk, VA). All infusions were performed at a rate of 1 mL/min. Saline was infused to establish a baseline before infusion of each challenge agent of acetylcholine, SNP, and L-NMMA (Figure 2). Each challenge agent was infused at 2 doses, and each dose was infused for 6 minutes. FBF was recorded in both arms over the last 3 minutes of each infusion.

Hemodynamics (blood pressure and heart rate) were measured in the brachial artery of the dominant, noninfused arm at baseline and at the end of the infusion period for each challenge agent with a validated oscillometric machine (Omron HEM-705CP, Omron Corp, Kyoto, Japan).²⁶ The measurements were taken after 24 minutes and then at the end of each challenge period (Figure 2).

Measurements were taken on day 1 for normal control subjects, before dose on day 1 (baseline), and after dose on day 28 for hypercholesterolemic patients. All measurements were conducted in the morning in a quiet, temperature-controlled (22°C to 24°C) clinical laboratory. Participants fasted overnight and abstained from alcohol and caffeine-containing drinks for 24 hours before measurement. At the end of the whole study, all the FBF data sets were sent to the University of Cambridge site for analysis and quality assessment. Any nonevaluable and incomplete data sets were removed from the database before subsequent unblinding and statistical analysis.

Laboratory Assessments

p38 MAPK activity can be estimated by its effect on the 27-kDa HSP27, which is a known downstream substrate of the p38 MAPK signaling pathway.^{27,28} Activation of p38 MAPK by sorbitol results in rapid phosphorylation of MK2, which then phosphorylates HSP27. Therefore, we measured phosphorylation of HSP27 as an in vivo biological assay of p38 MAPK inhibition. Whole-blood samples were collected on days 1 and 28 before dose and 3 and 6 hours after dose. Samples were divided into 2 tubes and incubated for 1 hour at 37°C with sorbitol or with RPMI 1640 medium as a control. Samples were then lysed on ice, and lysates were stored at −80°C. Each sample was analyzed for total and phosphorylated HSP27 with commercially available ELISA-based assays at a central laboratory.

Blood samples were collected before dose on days 1, 14, and 28 for the measurement of other inflammatory biomarkers, including hsCRP and IL-6. All analyses were conducted centrally with standard laboratory methods.

Safety Assessments

A detailed collection of safety data, including adverse events and serious adverse events, was monitored throughout the study as required by regulatory authorities and in accordance with good clinical practice. These data were reviewed during the study period on a weekly basis during study visits. A complete set of safety observations, including heart rate, blood pressure, and 12-lead ECGs, was recorded on days 1, 14, and 28 and at follow-up. Biochemical safety data, including blood and urine samples for hematology, clinical chemistry, and urinalysis, were collected at weekly intervals. Pharmacodynamic responses were measured with the ex vivo method of HSP27 inhibition and by an in vivo method to assess endothelial function using FBF.

Statistical Methods

Sample size calculation was based on the variability of change in FBF after acetylcholine (our primary end point) from the preceding baseline and relative to the noninfused arm. Using an SD of 0.234 on the log scale of change from baseline FBF ratio,²⁹ we estimated that a sample size of 25 patients per treatment arm would provide 90%

Table 1. Characteristics of Hypercholesterolemic Patients and Normal Control Subjects

	Hypercholesterolemic Patients		Healthy Control Subjects
	Placebo	Losmapimod	
Participants, n	29	27	12
Male:female	19:10	20:7	12:0
Age, mean (range), y	55 (35–72)	54 (23–71)	43 (33–64)
BMI, mean (range), kg/m ²	26.6 (22.0–35.2)	26.5 (20.5–31.9)	25.9 (20.9–30.2)
LDL cholesterol, mean (SD), mmol/L			
Day 1 before dose	4.7 (0.52)	4.7 (0.87)	2.1 (0.48)
Day 28 before dose	4.5 (0.58)	4.5 (0.93)	
HDL cholesterol, mean (SD), mmol/L			
Day 1 before dose	1.4 (0.29)	1.2 (0.19)	1.5 (0.36)
Day 28 before dose	1.4 (0.24)	1.3 (0.21)	
Triglycerides, mean (SD), mmol/L			
Day 1 before dose	1.9 (1.05)	1.8 (0.60)	0.9 (0.21)
Day 28 before dose	1.9 (0.89)	1.9 (1.57)	

BMI indicates body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Healthy control subjects were evaluated on day 1 only.

power to detect a 20% difference in change from baseline FBF ratio for the acetylcholine response with an α level of significance of 5%. Statistical analyses were performed with SAS version 8.02 (SAS Institute, Cary, NC).

The FBF ratio was calculated as FBF in the infused arm divided by FBF in the control arm. Ln-transformed FBF ratio data in hypercholesterolemic patients were analyzed by infusion agents using a mixed-effects model with a term for treatment, visit day, infusion dose within day, gender, and interaction of treatment and dose within a day, in which saline was treated as infusion dose zero, and subjects as random effects. To compare hypercholesterolemic patients and normal control subjects at baseline, Ln-transformed FBF data on day 1 were analyzed using a mixed effects model with a term for the cohort (hypercholesterolemic patients or normal control subjects), infusion dose, and interaction of cohort and dose, and subjects as random effects. Finally, to demonstrate any improvement toward normality of our active treatment group, a similar mixed-effects model was used to compare the FBF ratio for hypercholesterolemic patients in the losmapimod group on day 28 and the normal control subjects (on day 1). Ln-transformed total and phosphorylated HSP27 ratio data (concentration after sorbitol stimulation divided by concentration in control medium) were analyzed with repeated measures ANOVA with autoregressive¹ covariance structure. Ln-transformed concentrations of inflammatory biomarkers (including hsCRP) were analyzed with ANCOVA with baseline biomarker at day 1 as a covariate.

Results

Fifty-six hypercholesterolemic patients were randomized to the study, and 12 normal control subjects were enrolled (Figure 1). The demographics and baseline characteristics of the 2 groups of hypercholesterolemic patients were well matched (Table 1). There were no changes in levels of LDL cholesterol, high-density lipoprotein cholesterol, or triglycerides over the 28-day course of the study in these patients (Table 1).

Forearm Blood Flow

Hypercholesterolemic patients had impaired vascular function before dose on day 1 compared with normal control subjects (Figure 3), as evidenced by a significantly attenuated vasodilator response to acetylcholine (24% lower; 95% confidence interval [CI], -40 to -5 ; $P=0.01$), significantly attenuated vasoconstrictor response to L-NMMA (16%

lower; 95% CI, -29 to -1 ; $P=0.03$), and a difference in vasodilator response to SNP that approached statistical significance (20% lower; 95% CI, -37 to 1 ; $P=0.06$). Given the difference in age between the hypercholesterolemic and healthy groups (Table 1), a posthoc analysis was conducted to explore the age effect on FBF, which revealed no significant effects (P for the age effects = 0.23 in acetylcholine, 0.13 in SNP, and 0.55 in LNMMA). Moreover, restricting the analysis to male participants only did not alter the observation.

In hypercholesterolemic patients, after 28 days of treatment with losmapimod, endothelium-dependent vasodilatation to acetylcholine and endothelium-independent vasodilatation to SNP improved from before dose on day 1; the vasoconstrictor response to L-NMMA was not altered significantly, although there was a trend toward more vasoconstriction in the losmapimod group at day 28 (Figure 4 and Table 2). No changes were observed in the placebo group over the 28-day treatment period (Figure 4). When overall comparisons were made between the 2 groups (Table 2), statistically significant differences in acetylcholine-induced vasodilatation (25% higher for losmapimod; 95% CI, 5 to 48; $P=0.01$) and SNP-induced vasodilatation (20% higher for losmapimod; 95% CI, 3 to 40; $P=0.02$) were observed. We also saw an enhanced vasoconstrictor effect with L-NMMA in the losmapimod group (10%; 95% CI, -1 to 23; $P=0.07$), although it did not reach statistical significance.

During the FBF studies, no changes were observed in systemic hemodynamics in either the losmapimod or placebo groups during infusion of acetylcholine, SNP, or L-NMMA.

In a posthoc analysis, FBF ratios for hypercholesterolemic patients treated with losmapimod for 28 days were not significantly different from those of normal control subjects, suggesting normalization of vascular function. The acetylcholine response on day 28 for hypercholesterolemics was 19% lower than for normal control subjects (95% CI, -37.2 to 5.0 ; $P=0.11$), SNP response was 11% lower (-29.0 to 11.6 ; $P=0.30$), and L-NMMA response was 5% lower (-19.8 to 11.7 ; $P=0.50$).

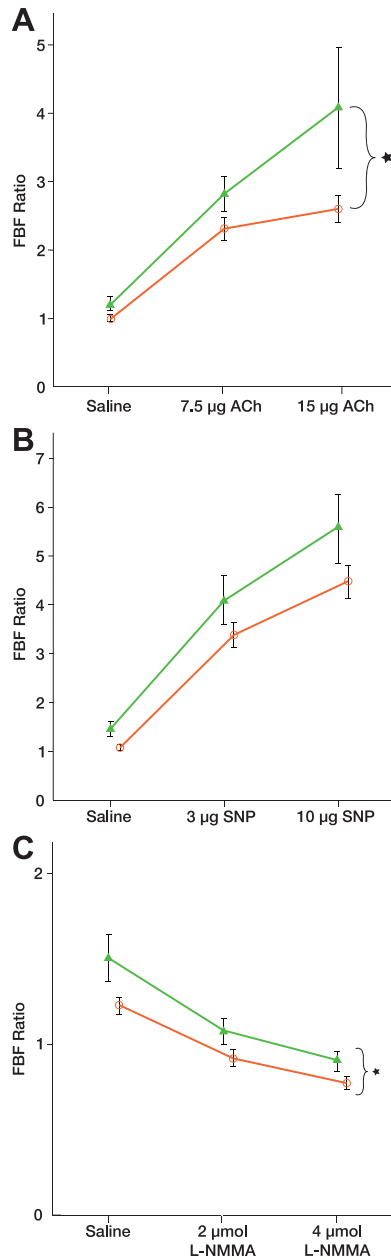


Figure 3. FBF ratio in all hypercholesterolemic patients (orange line; n=45) and normal control subjects (green line; n=12) before dose on day 1 in response to (A) acetylcholine (ACh), (B) SNP, and (C) L-NMMA infusion. Values represent geometric mean and SE. * $P < 0.05$, overall effects of hypercholesterolemic patients vs normal control subjects.

Laboratory Assessments

In hypercholesterolemic patients, HSP27 phosphorylation was inhibited at 3 and 6 hours after dosing with losmapimod on days 1 and 28 (Figure 5). From the repeated measures ANOVA, the phosphorylated HSP27 ratio decreased by 36% (95% CI, -53 to -13; $P = 0.004$) at 3 hours after dose and 33% (95% CI, -47 to -16; $P = 0.005$) at 6 hours after dose on day 1 and by 45% (95% CI, -61 to -23; $P < 0.001$) at 3 hours after dose and 35% (95% CI, -49 to -15; $P = 0.001$) at 6 hours after dose on day 28.

The concentration of hsCRP was reduced from 1.85 to 1.09 mg/L after treatment with losmapimod for 28 days (Figure 6).

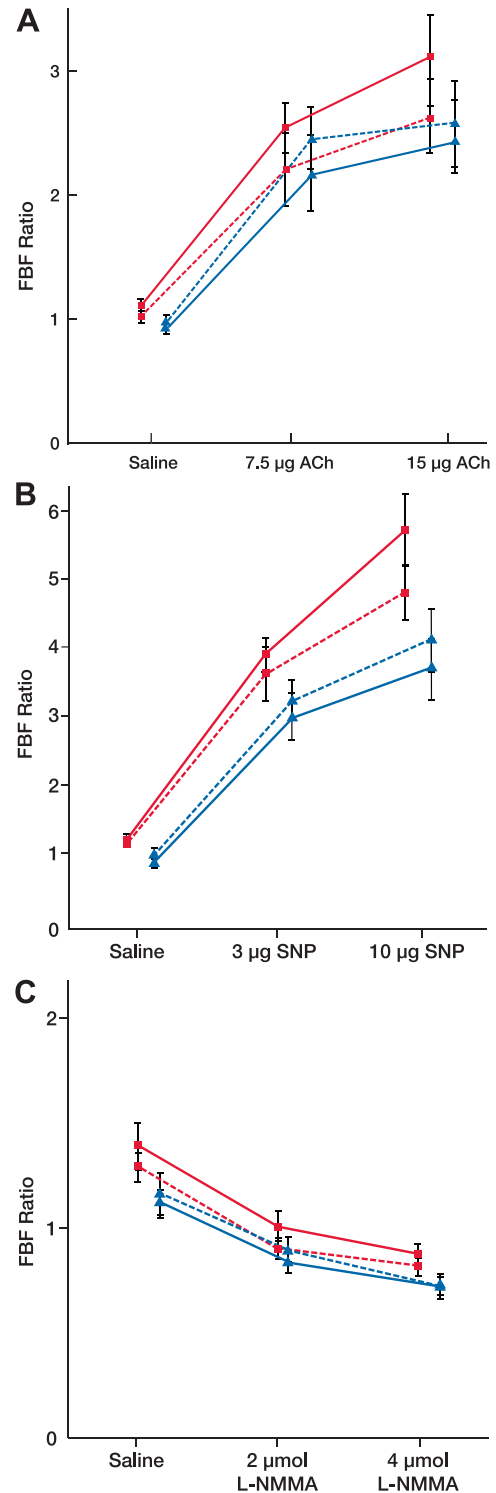


Figure 4. FBF ratio in hypercholesterolemic patients in the losmapimod group (n=22) before dose on day 1 (red dashed line) and after dose on day 28 (red solid line) and in the placebo group (n=23) before dose on day 1 (blue dashed line) and after dose on day 28 (blue solid line) in response to (A) acetylcholine (ACh), (B) SNP, and (C) L-NMMA infusion. Values represent geometric mean and SE. $P = 0.01$ for ACh, $P = 0.02$ for SNP, and $P = 0.07$ for L-NMMA for overall comparison of losmapimod vs placebo from repeated measures mixed-effects model in which saline was treated as infusion dose 0.

Table 2. Geometric Mean (95% CI) FBF Ratio Values in Hypercholesterolemic Patients

	Placebo		Losmapimod	
	Day 1	Day 28	Day 1	Day 28
ACh				
Baseline	0.97 (0.85–1.10)	0.91 (0.80–1.04)	1.02 (0.93–1.11)	1.10 (0.97–1.24)
ACh 7.5 μg	2.45 (1.97–3.04)	2.16 (1.64–2.84)	2.18 (1.79–2.65)	2.54 (2.13–3.03)
ACh 15 μg	2.59 (2.00–3.35)	2.42 (1.86–3.15)	2.62 (2.10–3.28)	3.14 (2.50–3.93)
Overall comparison of losmapimod vs placebo (for day 28 vs 1)*				
Treatment difference (95% CI), %				25 (5–48)
<i>P</i>				0.01
SNP				
Baseline	1.07 (0.93–1.23)	0.96 (0.83–1.11)	1.13 (1.04–1.23)	1.15 (1.02–1.29)
SNP 3 μg	3.22 (2.65–3.92)	2.98 (2.28–3.89)	3.62 (2.87–4.57)	3.88 (3.41–4.41)
SNP 10 μg	4.16 (3.39–5.12)	3.67 (2.85–4.74)	4.84 (3.84–6.10)	5.73 (4.93–6.66)
Overall comparison of losmapimod vs placebo (for day 28 vs 1)*				
Treatment difference (95% CI), %				20 (3–40)
<i>P</i>				0.02
L-NMMA				
Baseline	1.17 (1.03–1.33)	1.13 (0.95–1.34)	1.29 (1.16–1.44)	1.41 (1.25–1.60)
L-NMMA 2 μmol	0.90 (0.77–1.05)	0.84 (0.73–0.98)	0.94 (0.81–1.08)	1.01 (0.90–1.13)
L-NMMA 4 μmol	0.73 (0.65–0.81)	0.73 (0.63–0.84)	0.82 (0.74–0.92)	0.88 (0.77–1.00)
Overall comparison of losmapimod vs placebo (for day 28 vs 1)*				
Treatment difference (95% CI), %				10 (–1–23)
<i>P</i>				0.07

*FBF ratio data for losmapimod and placebo groups were compared by use of a repeated measures mixed-effects model using ln-transformed data, fitting fixed terms for regimen, day, infusion dose within day, gender, and the interaction of regimen and dose within a day, and subject as a random effect. Saline was treated as infusion dose zero in the analyses. Point estimates (expressed as percentage treatment difference) and corresponding 95% CIs for comparison of losmapimod and placebo are shown for both doses of acetylcholine (ACh), SNP, or L-NMMA combined. Percentage treatment difference was calculated as follows: (point estimate–1)×100.

From the ANCOVA, there was a 57% decrease (95% CI, –81 to –6; $P=0.036$) in hsCRP in the losmapimod group compared with placebo on day 28. There was no difference in the concentrations of other protein biomarkers tested.

Safety Assessments

Losmapimod was safe and well-tolerated in hypercholesterolemic patients. The adverse event profile was similar across

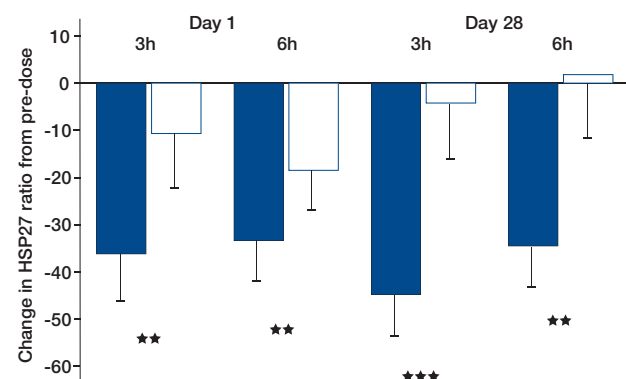


Figure 5. Percentage reduction from before dose in phosphorylated HSP27 ratio in whole blood (concentration after sorbitol stimulation divided by concentration in control medium) and at 3 and 6 hours after dose on days 1 and 28 in the losmapimod group (blue bars) and placebo group (open bars). Values represent geometric mean and SE. ** $P<0.01$; *** $P<0.001$.

groups. There were no deaths or serious adverse events. One patient in the placebo group was withdrawn from the study because of a lung nodule that was diagnosed before treatment. Frequently reported adverse events are shown in Table I in the online-only Data Supplement; the most common adverse event was headache. There were no clinically relevant differences between groups for clinical chemistry (including liver function tests), hematology, heart rate, blood pressure, or ECG monitoring. In the losmapimod group, no patient had an alanine aminotransferase or aspartate aminotransferase value above the normal clinical range, and no other liver function test met predefined criteria for clinical concern (alkaline phosphatase ≥ 2 times the upper limit of normal or total bilirubin ≥ 1.5 times the upper limit of normal).

Discussion

We designed a proof-of-concept study using hypercholesterolemia as a well-accepted model of endothelial dysfunction to determine the role of p38 MAPK on NO-mediated vasoregulation. For the first time in humans, we demonstrate that p38 MAPK inhibition improves NO-mediated vasomotor function in vivo in patients with hypercholesterolemia. Using losmapimod as a specific tool, we demonstrated that after 28 days of therapy, the drug inhibited p38 MAPK activity at the cellular level, as shown by the reduced phosphorylation of HSP27, a well-established downstream bioassay of p38 MAPK activity. This

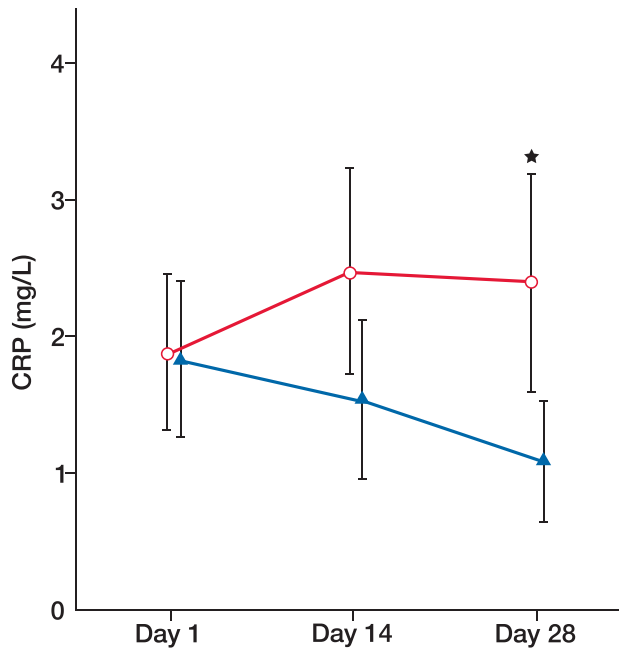


Figure 6. hsCRP values on days 1, 14, and 28 in hypercholesterolemic patients in the losmapimod group (blue line) and placebo group (red line). Values represent geometric mean and SE. * $P < 0.05$ for comparison on day 28.

effect was accompanied by a reduction in systemic inflammation as evidenced by a significant reduction in hsCRP.

We chose hypercholesterolemia as a stable model of endothelial dysfunction to test our hypothesis because hypercholesterolemia is one of many cardiovascular risk factors that have consistently been associated with impairment of endothelial function in the literature.^{14,15,30} As expected,^{30,31} vascular responses were impaired in untreated patients with hypercholesterolemia compared with normal control subjects: significantly decreased endothelium-dependent vasodilatation (acetylcholine induced) and a trend toward impairment of endothelium-independent vasodilatation (SNP induced). There was also a significantly reduced response to the NO synthase inhibitor L-NMMA, indicating reduced basal NO release.

Treatment with losmapimod for 28 days improved both endothelium-dependent vasodilatory responses to acetylcholine and endothelium-independent vasodilatory responses to SNP compared with placebo. We also saw an enhanced vasoconstrictor effect with L-NMMA in the losmapimod group, although this just failed to reach statistical significance, which may be explained by the smaller effect size for L-NMMA compared with that for acetylcholine and SNP.

The improvements in vascular function occurred without a corresponding change in the levels of LDL cholesterol over the 28-day course of the study (see Table 1), suggesting that this was not the cause for improvement in endothelial function. We confirmed that losmapimod was inhibiting p38 MAPK by demonstrating a significant inhibition of HSP27 phosphorylation (a known downstream substrate of the p38 MAPK signaling pathway^{27,28}) on both the first day (after dose) and last day of dosing. These results concur with data from an ex vivo animal model of endothelial dysfunction and

a selective p38 MAPK inhibitor,²² confirming that the model is relevant to human pathophysiology.

The finding that losmapimod enhances both endothelium-dependent and -independent vasodilatation contrasts with responses observed after 4 weeks of treatment with a statin (simvastatin), which lowered LDL cholesterol and affected only endothelium-dependent responses.²⁹ Hypercholesterolemia is known to cause a reduction in both endothelium-dependent^{29,32–34} and endothelium-independent NO responses.¹⁴ Although endothelium-independent effects are not as widely reported, this may be due primarily to the maximum dose of SNP used in previous studies, which is much lower than the highest dose we used in our study, as well as the much smaller sample size in those studies favoring a negative result.

NO-related vascular responses are determined by NO bioavailability (rate of NO production and breakdown) and smooth muscle NO sensitivity. Our observations suggest that the improvement in vascular function after p38 MAPK inhibition is likely to represent increased sensitivity to NO and potentially NO bioavailability. The precise mechanisms underlying these changes cannot be answered by our study. Although losmapimod did not alter LDL levels, like a statin, it may have altered oxidized LDL levels, but this was not measured in this study. Alternatively, we postulate that p38 MAPK inhibition (as evidenced by HSP27 inhibition) may result in reduced ROS generation in hypercholesterolemia,³⁵ which would enhance the half-life of active NO, by limiting its conversion to, for example, peroxynitrite (ONOO[−]) and limiting or reversing oxidation of soluble guanylate cyclase (the NO receptor), thus restoring NO sensitivity.³⁶ We reported similar results in our animal model of stroke-prone spontaneously hypertensive rat.²² A direct antioxidant would also have a similar effect. However, losmapimod has no antioxidant activity at pharmacologically relevant doses in vitro, as shown by a Cu²⁺ antioxidant assay (see Figure II in the online-only Data Supplement).

The novel finding in humans in this study suggests that p38 MAPK inhibition may reverse the effects of chronic inflammation on the sensitivity of vascular smooth muscle to NO as previously described in baseline vascular function in subjects with rheumatoid arthritis.³⁷ Whether this global improvement in vasomotor function is of benefit in terms of event rates and mortality remains to be answered and should be the subject of further investigation.

p38 MAPK has long been postulated as an attractive therapeutic target for atherosclerosis because of its critical role in the generation and signal transduction of proinflammatory cytokines,^{38–40} the latter relevant to chronic inflammatory disease,^{41–45} often in the context of oxidized LDL cholesterol.⁴³ Selective inhibitors of p38 MAPK have been shown to inhibit lipopolysaccharide-stimulated IL-1 and tumor necrosis factor- α production in human monocytes and the production of several other cytokines, including IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor.^{46,47} hsCRP is a stable representation of these activities, as well as an established predictive inflammatory biomarker for cardiovascular risk assessment.^{2,48,49} After 28 days of treatment with losmapimod, we observed a significant reduction in the concentration of hsCRP (from 1.85 to 1.09 mg/L) in hypercholesterolemic patients. We

did not observe differences in IL-6, which could be due to the shorter half-life of IL-6 and its higher variability.^{2,50} Therefore, we have demonstrated that p38 MAPK inhibition (as measured by the specific bioassay HSP27) resulted in a significant reduction in inflammation, as measured by hsCRP. Posthoc analysis, however, did not show any significant correlation between the change in hsCRP and the change in acetylcholine response. This finding likely reflects the small sample size in our study, which means we were not powered to detect a modest correlation.

We have shown an improvement in vascular function despite inhibiting only $\approx 40\%$ of HSP27, suggesting that clinical benefits may occur without maximal blockade. This may ultimately help mitigate against the hepatotoxicity seen at higher doses of p38 MAPK inhibition and other p38 MAPK inhibitors.⁵¹ Indeed, we found no liver function adverse signals, and losmapimod was well tolerated. There were no serious adverse events or significant adverse events compared with placebo, nor were there any clinical safety parameters (hemodynamic, biochemical, or hematologic) of concern throughout the study.

Several limitations of this study merit consideration. This proof-of-concept study in patients with hypercholesterolemia was designed using a surrogate end point (response in the forearm vascular bed after pharmacological challenge) rather than clinical outcomes. Consequently, it needs to be verified in an appropriately designed and powered study to determine whether the observed improvements in vascular function will translate into a clinical benefit (eg, reduction in incidence of cardiovascular events). Moreover, although patients were treated for 28 days, this is still a relatively short period of time with regard to affecting a response, and it is expected that in future studies patients would be treated for longer periods. Only 1 dose level of losmapimod (7.5 mg twice daily) was evaluated in this study. Evaluation of a higher dose of losmapimod or a more potent p38 MAPK inhibitor may result in a greater response.

Conclusions

Our observations indicate that losmapimod improved both endothelium-dependent and -independent vasodilatation in hypercholesterolemic patients, as anticipated on the basis of equivalent results from preclinical models. Results support p38 MAPK inhibition as an approach to improving vascular health and thereby benefiting patients with cardiovascular disease.

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Disclosures

Drs Sarov-Blat, Willette, Lepore, and Sprecher are employees of and own stock in GlaxoSmithKline. Dr Fang is an employee of GlaxoSmithKline. Dr Morgan and M. Elkhawad are former employees of GlaxoSmithKline. Dr Cheriyan is employed by Cambridge University Hospitals NHS Foundation Trust and is obligated to spend 50% of his time on GlaxoSmithKline clinical trial research, representing a significant relationship; however, he receives no other benefits or compensation from GlaxoSmithKline. Dr Wilkinson has received academic sponsorship of posts from GlaxoSmithKline and is a consultant for this and other compounds. Professor Cockcroft received a research grant from GlaxoSmithKline to do this study. Drs Webb and Collier have received academic sponsorship of posts and equipment from GlaxoSmithKline. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

Hypercholesterolemia is associated with impaired vasomotor endothelial function, which is a recognized surrogate marker of outcome. We tested the hypothesis that a novel p38 MAP kinase inhibitor, losmapimod, could improve nitric oxide-mediated responses in such a cohort. We demonstrated for the first time that moderate blockade of this pathway improved endothelial-dependent and -independent nitric oxide-mediated vasodilatation in addition to reducing systemic inflammation, as evidenced by an almost 60% reduction in high-sensitivity C-reactive protein, without alteration in cholesterol levels. Inhibition of p38 may be an attractive target in patients with underlying vascular inflammation.

SUPPLEMENTAL MATERIAL

Supplemental Table 1. Adverse events reported by more than 1 patient in either group

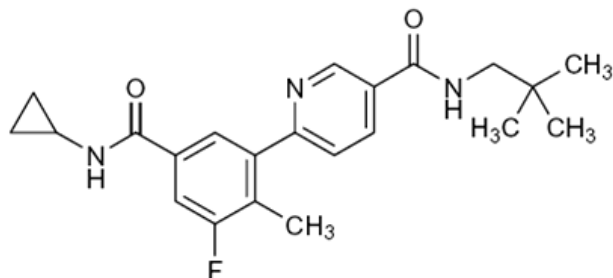
	Number (%) of patients with adverse event	
	Placebo N=29	Losmapimod N=27
Patients with any adverse event	18 (62)	22 (81)
Headache	10 (34)	9 (33)
Influenza like illness	0	3 (11)
Cough	2 (7)	2 (7)
Gamma-glutamyltransferase increased	2 (7)	2 (7)
Bacteria urine identified	1 (3)	2 (7)
Dizziness	1 (3)	2 (7)
Protein urine present	1 (3)	2 (7)
White blood cells urine	1 (3)	2 (7)
Dyspepsia	1 (3)	2 (7)
Nasopharyngitis	1 (3)	2 (7)

Fatigue	0	2 (7)
Muscle spasms	0	2 (7)
Pharyngolaryngeal pain	2 (7)	1 (4)
Alanine aminotransferase increased	2 (7)	0

Adverse events were coded using the Medical Dictionary for Regulatory Activities (MedDRA). Adverse events are sorted by decreasing order of frequency in the losmapimod group.

Supplemental Figure 1

A.



Chemical Name: 6-{5-[(Cyclopropyl-amino) carbonyl]-3-fluoro-2-methylphenyl}-N-(2,2-dimethylpropyl)-3-pyridinecarboxamide

Nonproprietary Name: Losmapimod
assn.org/go/asan)

Previously known as: GW856553 or GSK-AHAB

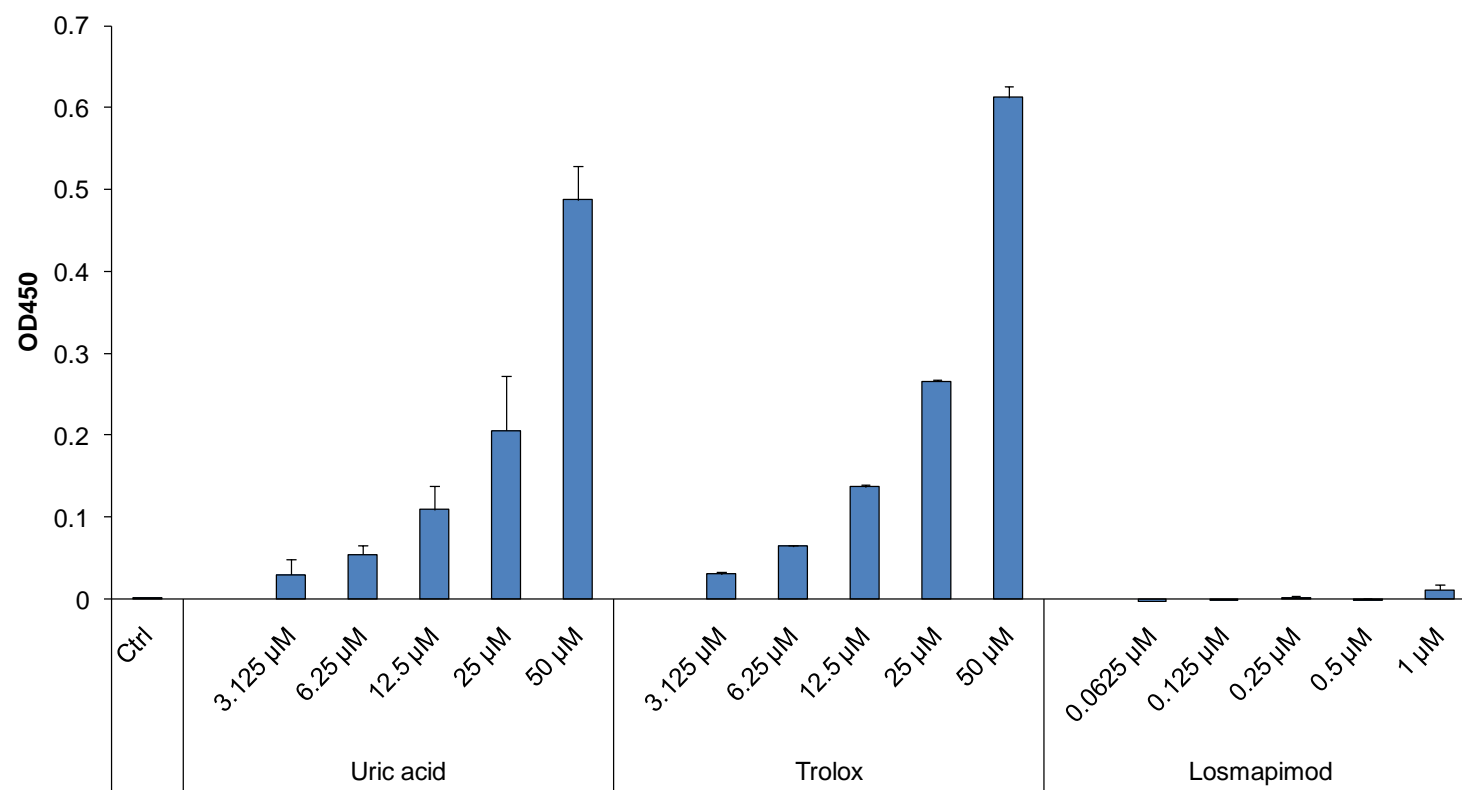
Molecular Formula: C₂₂H₂₆FN₃O₂

Molecular Weight: 383.5

B.

<i>In Vitro</i> Assay	pKi, IC ₅₀ (μM), or inhibition at 10 μM (%)
p38α	8.1
p38β	7.6
p38γ	-20 ± 25%
p38δ	17 ± 5%
Rat PBMC LPS-TNFα	0.60 ± 0.11 μM
Human PBMC LPS-TNFα	0.13 ± 5 μM
COX2 enzyme	>100 μM

Supplemental Figure 2



Supplemental Figure Legends

Supplemental Figure 1: Structural information (A) and compound activity profile (B) of losmapimod (previously known as GW856553 or GSK-AHAB¹). Inhibition of p38 α and p38 β was determined using a ligand displacement fluorescence polarization assay. p38 γ and p38 δ activity was determined by measuring phosphorylation of myelin basic protein using a scintillation proximity assay. LPS-Induced TNF α production was measured in cultured rat and human whole blood. COX2 activity was measured in microsomal preparations from Sf9 cells stably transfected with human COX2 enzyme. Selectivity testing (activity and/or binding) across more than 150 members of the human kinome suggest that losmapimod is a highly selective inhibitor of p38 α and p38 β , i.e. >100-fold more potent (data not shown).

Supplemental Figure 2: Anti-oxidant activities of negative control (Ctrl; dimethyl sulfoxide), uric acid (positive control), Trolox (positive control) and losmapimod using a commercially available antioxidant activity assay (Oxford Biomedical, antioxidant TA02 kit). Samples and positive controls were diluted to proper concentrations, mixed with buffer and aliquoted to 96 well plates. 50 μ L of Cu²⁺ reagent was added to each well for 3 minutes, followed by optical density reading at 450 nM (OD450). Dimethyl sulfoxide had no antioxidant activity (0.5% and 1%). Uric acid and Trolox were potent antioxidants at the dose range tested (3 to 50 μ M). Losmapimod had no antioxidant activity at pharmacologically relevant doses (0.0625 to 1 μ M).

Supplemental References

- (1) Willette RN, Eybye ME, Olzinski AR, Behm DJ, Aiyar N, Maniscalco K, Bentley RG, Coatney RW, Zhao S, Westfall TD, Doe CP. Differential effects of p38 mitogen-activated protein kinase and cyclooxygenase 2 inhibitors in a model of cardiovascular disease. *J Pharmacol Exp Ther* 2009;330:964-70.