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Title Page:

Differential Effects of p38 MAPK and COX2 Inhibitors in a Model of Cardiovascular Disease

Robert N Willette, Marianne E. Eybye, Alan R. Olzinski, David J. Behm, Nambi Aiyar, Kristeen Maniscalco, Ross G. Bentley, Robert W. Coatney, Shufang Zhao, Timothy Westfall, and Chris P Doe

Heart Failure Discovery Performance Unit and Laboratory Animal Sciences (RWC, SZ)

Metabolic Pathways Center of Excellence in Drug Discovery (RNW, MEE, ARO, DJB, NA, KM, RGB, TW, CPD)

GlaxoSmithKline Pharmaceuticals, King of Prussia, PA

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Running Title Page:

Running Title: Differentiation of Anti-inflammatory Agents in CV Disease

Corresponding author:

Robert N. Willette, Ph.D.

GlaxoSmithKline Pharmaceutics

709 Swedeland Rd.

King of Prussia, Pennsylvania

USA PA 19406

robert.n.willette@gsk.com

tele. 610-270-6052

fax. 610-270-6720

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Introduction = 520

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Abbreviations: SHR-SP, spontaneously hypertensive-stroke prone rat; SFD, salt-fat diet, PRA, plasma renin activity; GSK-AHAB, aryl heteroaryl bis-carboxyamide.

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Abstract:

Aim: There is compelling evidence for a role of inflammation in cardiovascular diseases, however, the chronic use of anti-inflammatory drugs for these indications have been disappointing. The present study compares the effects of two anti-inflammatory agents (COX2 and p38 inhibitors) in a model of cardiovascular disease. *Methods:* Vascular, renal and cardiac effects of rofecoxib (COX2 inhibitor) and GSK-AHAB (a selective p38 MAPK inhibitor), were examined in the spontaneously hypertensive stroke prone rat (SHR-SP). *Results:* In SHR-SP receiving a salt/fat diet (SFD), chronic treatment with GSK-AHAB significantly and dose-dependently improved survival, endothelial-dependent and -independent vascular relaxation, and indices of renal function, and attenuated dyslipidemia, hypertension, cardiac remodeling, plasma renin activity (PRA), aldosterone and interleukin -1β (IL-1β). In contrast, chronic treatment with a COX2 selective dose of rofecoxib exaggerated the harmful effects of the SFD, i.e. increasing vascular and renal dysfunction, dyslipidemia, hypertension, cardiac hypertrophy, PRA, aldosterone and IL-1β. *Conclusion:* The protective effects of a p38 MAPK inhibitor are clearly distinct from the deleterious effects of a selective COX2 inhibitor in the SHR-SP and suggest that anti-inflammatory agents can have differential effects in cardiovascular disease. The results also suggest a method for evaluating long term cardiovascular efficacy and safety.

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Introduction:

A substantial body of evidence suggests that chronic low-grade inflammatory processes contribute to the pathogenesis of cardiovascular disease e.g. coronary artery disease, heart failure, hypertension and other components of the metabolic syndrome (Forrester and Libby, 2007;Berg and Scherer, 2005;Savoia and Schiffrin, 2007;De Tena, et al., 2005;Grundy, 2003). Manifestations of vascular inflammation are believed to include early pathogenic changes such as endothelial dysfunction and plaque formation as well as later events related to plaque rupture, thrombosis and end-organ disease. At a cellular level, vascular inflammation is associated with activation of a variety of intracellular signaling pathways (e.g. p38 MAPK, NFk-B and eicosanoid) in endothelial, inflammatory and synthetic smooth muscle cells. These often intersecting and overlapping pathways promote cellular adhesion, infiltration, apoptosis, proliferation and remodeling of the extracellular matrix (Touyz, 2003). Specific mediators that have been associated with cardiovascular disease include: pro-inflammatory cytokines (IL-6, IL-1β, TNFα, MCP-1), lipid mediators (oxLDL, Lp-PLA2, TXA2) and acute phase proteins, e.g. C-RP (Willerson and Ridker, 2004). Evidence also suggests that some drugs prescribed to reduce the risk of cardiovascular disease (e.g. statins and ACEi) reduce biomarkers of inflammation associated with cardiovascular disease (Forrester and Libby, 2007;Schieffer, et al., 2004).

Despite the compelling case for a role of inflammation in cardiovascular disease, drugs that specifically target inflammatory pathways have not been approved for the treatment of cardiovascular disease and the use of current anti-inflammatory drugs in the setting of cardiovascular disease has been disappointing. In fact, high dose

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glucocorticoids, targeted TNFα inhibition and some COX2 inhibitors have been associated with increased risk of cardiovascular disease (Mann, et al., 2004; Zarraga and Schwarz, 2007).

Recent preclinical studies suggest that p38 MAPK inhibitors, novel anti-inflammatory agents, demonstrate efficacy in a variety of atherosclerotic and non-atherosclerotic preclinical models of cardiovascular disease (Behr, et al., 2001; Ju, et al., 2002; Behr, et al., 2003; Olzinski, et al., 2005; Bao, et al., 2007; Park, et al., 2007; Morris, et al., 2008). However, preclinical models used to assess chronic cardiovascular safety and efficacy don't always predict clinical outcome. In the case of COX2 inhibitors, preclinical studies failed to predict the elevated risk of thrombotic events, heart failure and hypertension (not stroke) observed in the clinic use of rofecoxib, a selective COX2 inhibitor, and other NSAIDs (for review see Zarraga and Schwarz, 2007). Both p38 MAPK and COX2 inhibitors act by inhibiting complex intracellular inflammatory pathways activated by cellular stress, i.e. hypertonicity, toll-receptor activation, pro-inflammatory cytokines, neurohormones, reactive oxygen species (for review Schieven, 2005).

In the present study we have conducted a side-by-side evaluation of a novel selective p38 MAPK inhibitor and a prototypic selective COX2 inhibitor, rofecoxib (Chan, et al., 1999), in a non-atherosclerotic model of cardiovascular disease. This comparison is important for the following reasons: 1) p38 MAPK and COX2 inhibitors regulate COX2 expression and activity, respectively (Schieven, 2005); 2) direct COX2 inhibitors (i.e. rofecoxib) have cardiovascular liabilities; and; 3) p38 MAPK inhibitors are in advanced stages of clinical development. The present results clearly distinguish

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the efficacious effects of a p38 MAPK inhibitor from the deleterious effects of a COX2 inhibitor and suggest benefits of broad-spectrum cytokine suppression in the treatment of cardiovascular disease.

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Methods:

SHR-SP Preparation:

Male SHR-SP rats (n = 70) were obtained from Charles River Laboratories, Inc. (Wilmington, MA**)** and randomized according to body weight into 5 groups (n=14 per group): normal diet controls (ND), high salt/fat diet controls (SFD), SFD + GSK-AHAB (1.2 mg/kg/day) and), SFD + GSK-AHAB (12 mg/kg/day) and SFD + rofecoxib (18 mg/kg/day). For clarity, normal diet control data are not always presented graphically but are described in the text. All drugs were administered in the diet by mixing with the SFD. A subgroup of animals from each group (n=6 per group) were anesthetized with isoflourane and surgically instrumented with radiotelemetry units (DSI, St. Paul, MN) for the conscious measurement of mean arterial blood pressure (MBP) and heart rate (HR). These animals were allowed to recover for at least 7 days prior to the start of the study. All experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals (NIH Publication 85-23), and experimental protocols were reviewed and approved by the GlaxoSmithKline Animal Care and Use Committee.

Experimental Protocol:

At the age of 7-8 weeks, rats were placed into metabolism cages for 24 hours for the collection of baseline urine and blood samples. Following baseline measures, the animals were randomized to the 5 groups. The ND group received a standard chow and tap water; the SFD diet groups received a fat enriched diet 24.5% fat and 1% NaCl in the drinking water ad lib (Behr, et al., 2001). Urine collections to determine electrolyte and

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protein excretion rates were repeated on weeks 2, 4, 6 and 8 and blood samples were taken to determine plasma creatinine and plasma sodium levels after each urine collection period to determine creatinine clearance (CrCl) and fractional sodium excretion (*FE*Na). Blood samples were also analyzed for HDL cholesterol (HDL), LDL cholesterol (LDL), and triglycerides (TRIGs). All urine and plasma samples were analyzed using an Olympus AU640 Chemistry Analyzer (Olympus America Inc. Diagnostic Systems Group, Melville NY) during week 3. Drug plasma levels were determined in a subgroup of animals (n=4) from samples obtained at 7 am, noon and 4 pm (this represents the peak to trough profile following dietary dosing).

Animals were considered moribund, and promptly euthanized with pentobarbital (65 mg/kg, ip), when they exhibited decreased locomotion and seizure and/or persistent loss of body weight.

Transthoracic echocardiograms (GE/Vingmed SystemV, Milwaukee WI) were performed, as described previously (Behr, et al., 2001), on randomly chosen animals (n=8/group) at baseline prior to treatment (week 0) and at week 4 in high dose and control groups. Inhalation anesthesia was induced with 4–5% isoflurane, and maintained at 1.5–2.0% during the procedure. The leading edge method was used to determine left ventricular (LV) thickness and volumes. Relative wall thickness (RWT) was calculated as RWT=(AWd+PWd)/LVDd, where AWd is diastolic anterior wall thickness, PWd is diastolic posterior wall thickness, and LVDd is the left ventricular diameter in diastole. The t/b ratio is the average LV wall thickness (t) divided by the average LV radius (b) in diastole. Left ventricular systolic diameter (LVDs), Stroke volume (SV) and cardiac output (CO) were also determined by a modified Simpson's method.

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At the end of the study, following the 8-9 week treatment period, surviving rats were anesthetized with 5% isoflourane in O_2 and euthanized by exsanguination. Blood samples were obtained for subsequent determination of plasma renin activity (PRA) and aldosterone (Aldo). Radio-immunoassays were used for determination of PRA (GammaCoat Plasma Renin Activity, DiaSorin, Minnesota), Interleulin-1β (R&D Systems, Minneapolis, MN) and Aldo (Diagnostic Products, Los Angeles, CA). The wet weight of the left and right kidneys and the whole heart were taken and expressed as tissue to body weight ratios.

Vascular reactivity:

Following exsanguination, the proximal descending thoracic aortae were removed and prepared for *in vitro* vascular relaxation studies as described previously (Behm, et al., 2002). Vascular ring segments (2–3 mm) were suspended in 10-ml tissue baths containing oxygenated (95% O_2: 5% CO_2) Krebs-Henseleit buffer (pH 7.4, 37°C) of the following composition (mM): NaCl 112.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_2 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and dextrose 11.0. The isolated aortae were suspended on two 0.1 mm diameter tungsten wire hooks in the tissue bath. Changes in force were measured isometrically under 1.0 g resting tension using force-displacement transducers (MLT0201/D; Letica Scientific Instruments) and recorded using Chart 5.0 software (ADInstruments). After a 60 min equilibration period, each tissue was contracted to equilibrium with 60 mM KCl, washed with 37°C Krebs solution and allowed to relax to the resting tension. The 60 mM KCl contraction was repeated. Each tissue was then contracted to equilibration with 1 μM phenylephrine and washed with Krebs solution and allowed to relax to the resting

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tension. Following washout, cumulative concentration-response curves to phenylephrine were obtained by adding 0.5 log unit increments (1 nM – 10 μM) and the EC_{80} for each tissue was determined. Following several washes, each vessel was contracted to equilibrium with an EC_{80} concentration of phenylephrine and tone was relaxed by adding cumulative concentration of carbachol at 0.5 log unit intervals (10 nM – 100 μM). Following washout, the tissues were again contracted to equilibrium with an EC_{80} concentration of phenylephrine and tone was relaxed by adding cumulative concentrations of sodium nitroprusside (SNP) at 0.5 log unit intervals (0.01 nM – 100 nM).

Whole blood COX1 actvity:

Heparinized whole blood (100 ul) was incubated at 37^{o}C for 1 hour in a cell culture incubator. A volume of 0.5 μl of Ca ionophore (A23187) was added to each well **(**final concentration of 50 uM) and the plate was incubated for an additional 30 minutes in the incubator. The incubation was stopped by centrifuging the plate at 4^{o}C for 5 minutes. Plasma was removed and stored at –80^{o}C until assayed for TXB_2 by ELISA according to the manufacturer's instructions (Cayman, Ann Arbor, MI). Rat plasma was usually diluted 1:300 with ELISA buffer.

Whole blood COX2 activity:

Heparinized whole blood was incubated for 1 hour at 37^{o}C with aspirin (100 uM final) to inactivate COX1. LPS (100ug/ml, final concentration) was added to the heparinized whole blood (100 μl) and incubated at 37^{o}C for 24 hours in a cell culture incubator. The incubation was stopped by centrifuging the plate at 4^{o}C for 5 minutes.

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Plasma was removed and stored at –80^{o}C until assayed for PGE2 by ELISA according the manufacturers instructions. Rat plasma was diluted 1:100 with ELISA buffer (modified from Patrignani et al., 1994)

Drugs and Materials:

Rofecoxib,,a well-characterized, potent and selective COX2 inhibitor, was obtained from a commercial source (Hanna's Pharmaceutical Supply, Wilmington, DE) and GSK-AHAB was synthesized at GlaxoSmithKline Pharmaceuticals (PA). Both compounds were administered by the dietary route and were formulated by blending with the high fat powdered diet (Ziegler Brothers Inc., PA). The rofecoxib dose (18 mg/kg/day) was extrapolated from the literature to achieve selective COX2 inhibition (Chan, et al., 1999). An eight week low (4 mg/kg/day) and high (54 mg/kg/day) dose pilot study was performed with rofecoxib. The low dose had inconsistent effects on COX2 activity and the high dose achieved plasma levels exceeding previously published selectivity limits. All rofecoxib doses had similar insignificant effects on survival. Only the data for the selective 18 mg/kg/day dose is described in this report. High and low doses of GSK-AHAB (1.2 and 12 mg/kg/day) were calculated as a free base and selected to bracket rat whole blood IC_{50} values (see Fig. 1). The doses of GSK-AHAB used in this study had no significant toxicological or cardiovascular effects in normal rats. Normal diet controls were given standard powdered rodent chow (Purina 5001) and maintained on tap water. All rats receiving the high fat diet or high fat diet containing drug were maintained on 1% NaCl in the drinking water.

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Data Analysis:

Summary data values are reported as mean±standard error of the mean (SEM) and $p<0.05$ was considered significant. Time and treatment related effects were compared using a 2-way ANOVA for repeated measures followed by post test comparisons to SFD and/or rofecoxib values using the Bonferroni test for multiple comparisons, unless stated in the text (GraphPad Software Inc., CA). Morbidity results were assesses by Kaplan Meier survival analysis followed by the Holm-Sidak test for comparisons between treatment groups (GraphPad Software Inc., CA).

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Results:

Activity profile of GSK-AHAB (6-{5-[(Cyclopropylamino)carbonyl]-3-fluoro-2-methylphenyl}-N-(2,2-dimethylpropyl)-3-pyridinecarboxamide):

GSK-AHAB, is a selective and potent, orally active p38 MAPK inhibitor that acts by competing for the kinase ATP binding site. GSK-AHAB has single digit nM potency (pKi) in isolated p38α and p38β enzyme assays, sub-micromolar potency in both rodent and human LPS-stimulated peripheral blood mononuclear cell (PBMC) assays and no detectable direct effect on COX2 activity (Fig. 1).

Chronic dietary administration of high (12 mg/kg/day) and low (1.2 mg/kg/day) doses of GSK-AHAB provided dose-linear plasma concentrations above and below the rat PBMC IC_{50} (Fig. 2A). A COX2 selective dose of rofecoxib (18 mg/kg/day), based on literature precedence, was confirmed in the present study (approximately 2 μM plasma concentration) and had no effect on rat whole blood COX1 activity (Fig. 2A&B). A low dose rofecoxib group (3.6 mg/kg/day) had only marginal effects of COX2 activity (Supplemental Figure 1).

Effects of GSK-AHAB and rofecoxib on survival and BP:

Introduction of a high salt/fat diet (SFD) induces a progressive and malignant hypertension in the SHR-SP. Untreated animals receiving the SFD had a 50% survival rate at 8 weeks and a continual increase in mean BP throughout the observation period (Fig. 3A&B). In the rofecoxib groups, the effect of the SFD on mean BP was

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significantly exaggerated and/or accelerated ($p<0.05$) and there was a tendency toward greater mortality (Fig. 3A&B). Blood pressure results were similar in a low dose rofecoxib group (3.6 mg/kg/day, see Supplemental Figure 2), but did not reach statistical significance. In contrast, treatment with low and high doses of GSK-AHAB significantly improved survival (Fig. 3A). The high dose group of GSK-AHAB also ameliorated the progressive increase in mean BP induced by the SFD (Fig. 3B). Survival was 100% and mean BP was only marginally increased (136 $\pm$ 2.3 to 140 $\pm$ 2.2 mmHg) in SHRSP receiving a normal diet (see Supplemental Figure. 2).

Indices of Renal Function and Lipid profile:

Introduction of the SFD rapidly and progressively increased urine flow, the fractional excretion of sodium (*FE*Na), albumin excretion and kidney weight (Fig. 4A, Table 1). Treatment with rofecoxib tended to exacerbate the effects of the SFD and reduced creatinine clearance at 6 weeks (Fig. 4B). Results tended to be similar in the low dose rofecoxib group (Supplemental Figure 3) and in preliminary studies of naproxen and celecoxib (Supplemental Figure 4). In contrast, treatment with GSK-AHAB dose-dependently delayed and attenuated SFD-induced changes in urine flow, *FE*Na, albumin excretion, creatinine clearance and kidney weight (Fig. 4A&B, Table 1).

Cardiac remodeling:

Based on echocardiographic analysis, the SFD produced a concentric left ventricular hypertrophy with increased LV mass, LV wall thickness and preserved ejection; consistent with a hypertensive cardiomyopathy (Table 1&2). Treatment with

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GSK-AHAB reduced LV mass and wall thickness, improved stroke volume and cardiac output and normalized chamber dimensions. In contrast, rofecoxib had no significant effect on the cardiac remodelling induced by the SFD (Table 2).

The SFD also produce a progressive dyslipidemia characterized by a delayed but progressive increase in plasma concentrations of LDL, HDL and the LDL/HDL ratio (Supplemental Fig 1) and a more abrupt increase in triglycerides (Fig 5A-C). At 6 and 8 weeks the increases in HDL, LDL and triglycerides were greater in the rofecoxib group than in untreated SHR-SP on the SFD. In contrast, GSK-AHAB treatment dose–dependently reduced HDL, LDL and Triglycerides as well as the LDL/HDL ratio (Fig. 5A-C, Supplemental Fig 5).

Endothelial function and vascular reactivity:

Both endothelial-dependent and nitrate-dependent (sodium nitroprusside) vasorelaxation were attenuated by the SFD (Fig. 6A&B). Treatment with GSK-AHAB dose-dependently improved both endothelial-dependent (Fig 6A) and endothelium-independent (Fig. 6B) vasorelaxation. In contrast, treatment with rofecoxib markedly attenuated endothelial-dependent vasorelaxation induced by carbachol (Fig. 6A) and had no significant effect on sodium nitroprusside (SNP)-dependent vasorelaxation (Fig. 6B). Vasorelaxation induced by carbachol and SNP in SHRSP receiving a normal diet did differ significantly from GSK-AHAB high dose group (data not shown).

Effects on plasma renin activity, aldosterone, and interleukin-1β:

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Somewhat paradoxically, the chronic SFD did not reduce plasma renin activity (PRA) and aldosterone at 4 weeks (data not shown) and 8 weeks (Fig. 7), see normal diet below. Treatment with GSK-AHAB reduced PRA, aldosterone and IL-1β (Fig. 7A-C) at both time points. In contrast, rofecoxib tended to increased PRA, aldosterone and IL-1β (Fig. 7A-C). PRA, aldosterone and IL-1β in the normal diet group did not differ significantly from the SFD group (10, 222 $\pm$ 3271 pg ang-1/ml/hr, 68.0 $\pm$ 7.6 pg/ml, 29.1 $\pm$ 5.0 pg/ml, respectively).

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Discussion:

In the present study, chronic treatment with two distinct classes of anti-inflammatory agents had markedly different effects in a rodent model of cardiovascular disease. Exemplars of orally active selective inhibitors of COX2 and p38 MAPK (rofecoxib and GSK-AHA, respectively) were compared in the SHR-SP fed a SFD. In most cases chronic treatment with a COX2 selective dose of rofecoxib exaggerated the harmful effects of a SFD in the SHR-SP, i.e. increasing vascular and renal dysfunction, hypertension, dyslipidemia, cardiac hypertrophy, PRA, aldosterone and IL-1β. In contrast, chronic treatment with GSK-AHAB dose-dependently improved survival, endothelial-dependent and independent vascular relaxation, lipid profiles and indices of renal function, and attenuated hypertension, cardiac remodeling, plasma renin activity (PRA), aldosterone and interleukin-1β (IL-1β). This differentiation is important when considering the advanced phase of clinical development of p38 MAPK inhibitors, the role of p38 MAPK in regulating COX2 and the known cardiovascular liabilities of COX2 inhibitors.

The opposing effects on vasoregulation were among the most striking, and perhaps revealing, observations. Chronic treatment with GSK-AHAB significantly improved both endothelial-dependent (carbachol) and nitrate-induced vasorelaxation in SFD-SHR-SP. In contrast, rofecoxib treatment markedly attenuated endothelial-dependent vasorelaxation. These results are consistent with previous reports of endothelial protection following chronic treatment with p38 MAPK inhibitors and is also consistent with reports of impaired endothelial function following rofecoxib treatment

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(Bao, et al., 2007;Widder, et al., 2004;Ju, et al., 2003;Li, et al., 2004;Li, et al., 2008). The mechanism underlying these differences appears to involve opposing effects on the generation of reactive oxygen species (ROS) that may be related to the regulation of NADPH oxidase (NOX). In the case of p38 MAPK inhibitors, several reports have shown that p38 MAPK inhibitors reduce NOX/subunit expression and ROS generation (Bao, et al., 2007;Li et al., 2008). These effects in the vasculature would be expected to increase the bioavailability of nitric oxide and reduce the nitrate insensitive form of oxidized soluble guanylate cyclase – thereby improving endothelial-dependent and nitrate-dependent vasorelaxation. On the other hand, COX2 inhibitors and NSAIDs have recently been shown to upregulate NOX(s) and increase ROS in blood vessel and heart (Li et al., 2008). Despite this explanation of the results, it should be noted that the precise mechanism(s) for regulation of NOX by either agent is not well understood and that the clinical effect of COX2 inhibitors on endothelial function is controversial and may depend upon the study population (Chenevard, et al., 2003;Widlansky, et al., 2003;Wong, et al., 2007).

The role of blood pressure in the protective effects of p38 MAPK inhibitors was investigated by assessing the effects of a blood pressure neutral low dose and a higher dose that abolished the hypertension induced by the SFD. Although the antihypertensive effects may contribute to the end-organ protection observed at the high doses, it was clear that significant improvements in renal function, lipid profiles, and survival were also observed at a blood pressure neutral dose of GSK-AHAB. However, the antihypertensive effects of the high dose of GSK-AHAB were associated with improved endothelial-dependent and nitrate-dependent vasorelaxation and were not observed in the low dose

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treatment group. It is tempting to suggest that improved vasoregulation underlies the antihypertensive effects of GASK-AHAB however it is likely that the reduced plasma renin activity and aldosterone concentrations observed at this dose also play an important role. It is also likely that augmented hypertension observed in the rofecoxib group contributes to the exaggerated end organ damage but the single dose evaluation precludes any further speculation. Modest elevations in blood pressure associated with rofecoxib treatment have also been reported in clinical studies (Aw et al., 2005).

Given the elevated risk of cardiovascular events associated with the use of rofecoxib in the clinic and in the SHR-SP it is tempting to suggest that the SHR-SP is a translation model for predicting cardiovascular liabilities. Although the model possesses vascular, coagulation, metabolic, cardiac and renal impairments commonly associated with cardiovascular disease it is important to note that it does not exhibit coronary atheroma or myocardial infarction – also important features of human cardiovascular disease.

The present study suggests that the effects of modulating of COX2 in the context of the pleiotropic actions of p38 MAPK inhibition are very different than the effects of selective enzyme inhibitors specifically targeting COX2. However, the study has several limitations that could be addressed in future studies. First, further examination of the rofecoxib dose-response may help to define the correlation of deleterious effects with COX2 inhibition. Secondly, rofecoxib comparisons with other anti-inflammatory agents, i.e. selective COX2 inhibitors and non-selective COX inhibitors, are needed to determine the validity of using: 1) rofecoxib as a prototype COX2 inhibitor and, 2) the SHR-SP as a

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translational tool to assess cardiovascular risk. Finally, additional mechanistic studies directed at determining the cellular and molecular events underlying damage and protection in the various end organs, e.g. generation and inhibition of ROS, would enhance our understanding of beneficial and harmful anti-inflammatory drug targets.

In conclusion, the protective effects of a p38 MAPK inhibitor are clearly distinct from the deleterious effects of a selective COX2 inhibitor in SHR-SP on SFD and suggest that anti-inflammatory agents can be differentiated in cardiovascular disease. The results also suggest a model for evaluating long term cardiovascular efficacy and safety.

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Legends for Figures:

Figure 1: Structure (A) and activity profile (B) of GSK-AHAB an aryl heteroaryl bis-carboxyamide series p38 MAPK inhibitor (6-{5-[(Cyclopropylamino)carbonyl]-3-fluoro-2-methylphenyl}-N-(2,2-dimethylpropyl)-3-pyridinecarboxamide). Inhibition of p38a and p38b were determined using a ligand displacement fluoresence polarization assay. p38g and p38d activity was determined by measuring phosphorylation of myelin basic protein using a scintillation proximity assay. LPS-Induced TNFa 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.

Figure 2: Plasma concentration of GSK-AHAB and rofecoxib after 4 weeks of dietary dosing (panel A; n=4 per group). Pilot studies indicate that concentrations obtained at 0800 and 1600 hours represent peak and trough levels, respectively. COX1 and COX2 activity was determined in rofecoxib samples obtained at 0800 hours (B). Groups compared by t-test, *$p<0.05$ (n=4-6).

Figure 3: Effects of treatment on survival (A) and mean arterial blood pressure (B) in stroke prone spontaneously hypertensive rats (SHR-SP) placed on a salt-fat diet (SFD). Following a 3 day run in period, SFD with or without treatments was begun at time 0 when animals were 12 weeks of age. In A,SFD and rofecoxib groups differed signifcantly from the GSK-AHAB groups from day 40 to the end of the study on day 56 (Kaplan-Meier Survival Analaysis with 95% confidence intervals, log rank $p<0.002$).

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Blood pressure values were significantly greater (#) or less than(*) the SFD group ($p<0.05$) by two-ANOVA for repeated measures and Bonferroni test for multiple comparisons.

Figure 4: Urinary albumin excretion and creatinine clearance was determined at baseline prior to introduction of the salt fat diet (SFD) and at 2, 4 and 6 weeks of the study in all groups. All groups (n=6-12 per group) were compared by a two-way ANOVA and Bonferroni's test for multiple comparisons. All groups were compared to the SFD ($^{*}p<0.05$, $^{**}p<0.01$ and $^{****}p<0.001$) and rofecoxib ($^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$).

Figure 5: Plasma lipoprotein concentrations, HDL (A), LDL (B) and Triglycerides (C), were determined at 2, 4, 6 and 8 weeks of the study in all groups. All groups (n=6-12 per group) were compared by a two-way ANOVA and Bonferroni's test for multiple comparisons. All groups were compared to the SFD ($*p<0.05$) and rofecoxib ($\#p<0.05$). Abbreviation: HDL, high density lipoprotein; LDL, low density lipoprotein; ND, normal diet; SFD, salt/fat diet. Drug treatments were added to the salt fat diet.

Figure 6: Vascular relaxation studies were performed in isolated thoracic aorta ring segments obtained from stroke prone hypertensive rats maintained on a salt/fat diet (SFD) for 8 weeks. Chronic treatments with GSK AHAB (1.2 and 12 mg/kg/day) and rofecoxib (18 mg/kg/day) were concomitant with the SFD. All vessels were pre-contracted with phenylephrine and endothelial-dependent (A) and -independent (B) relaxations were determined by addition of carbachol (A) and sodium nitroprusside (SNP, B), respectively.

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Statistical comparisons of R_{max} values were made using one way ANOVA or two way ANOVA for repeated measures with a Bonferroni's post-test where $^{*}P<0.05$, $^{**}P<0.01$ and $^{****}P<0.001$ versus SFD and $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ for rofecoxib vs 12 mg/kg/d GSK-AHAB.

Figure 7: Plasma renin activity (PRA), plasma concentrations of aldosterone and IL-1β were measured from blood samples obtained at 4 and 8 weeks of the study and in all groups. All groups (n=6-12 per group) were compared by ANOVA and Bonferroni's test for multiple comparisons. All groups were compared to the salt-fat diet control (*SFD) or rofecoxib (#). Results were similar at 4 and 8 weeks (8 week results shown).

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Table 1

Physiological Parameters		Normal Diet			Salt/Fat Diet			AHAB (1.2 mg/kg/d)			AHAB (12 mg/kg/d)			rofecoxib (18 mg/kg/d)		
	Weeks	*MEAN*	*SEM*	*N*	*MEAN*	*SEM*	*N*	*MEAN*	*SEM*	*N*	*MEAN*	*SEM*	*N*	*MEAN*	*SEM*	*N*
Body Weight	**0**	177.5	11.5	12	181.6	8.5	14	185.4	11.4	13	166.8	9.5	13	185.3	10.8	12
(g)	**4**	254.4	6.8	12	256.5	8.8	14	254.8	7.6	13	250.8	6.5	13	254.3	6.7	12
	8	286.7##	7.9	12	278.6	9.6	7	282.2	8.6	12	276.8	6.1	13	219.2**	13.3	6
Urine Flow	**0**	11.83	0.83	12	10.71	0.74	14	13.85	1.57	13	14.92	4.65	13	10.92	0.98	12
(ml/d)	**4**	13.50***,###	1.14	12	43.79	5.27	14	37.08	3.88	13	25.69	2.63	13	51.50	6.79	12
	8	22.33***,###	5.15	12	89.00	15.08	7	52.08***	6.46	12	33.77***	3.87	13	83.40	19.98	5
***FE*Na**	**0**	0.45	0.02	11	0.50	0.03	14	0.50	0.01	12	0.48	0.04	13	0.49	0.02	11
(%)	**4**	0.48***,###	0.04	12	3.16	0.49	14	2.50	0.53	13	1.67*	0.10	13	3.60	0.40	12
	8	0.81***,###	0.20	12	5.39	1.21	7	3.26**	0.38	12	2.51***	0.19	13	8.17**	0.67	5
Hematocrit	**8**	41.3	2.7	7	40.7	0.8	11	44.0	0.8	11	41.1	1.2	13	36.0	1.6	5
(%)																
Platelet Count	**8**	436.6	74.7	10	285.1	57.1	8	475.1	29.5	11	461.3	33.1	13	209.5	71.5	4
(x1000/μl)																
RBC Count	**8**	8.12##	0.18	11	7.84	0.64	7	8.52#	0.23	11	8.35#	0.24	13	5.98**	0.36	5
(x10E6/μl)																
HW/BW	**8**	4.2#	0.1	12	5	0.25	8	4.7#	0.1	12	4.5*,#	0.08	13	5.6	0.14	6
(mg/g)																
Kidney/BW	**8**	4.4###	0.05	12	5.5	0.48	8	4.74##	0.15	12	4.5**,##	0.05	13	8.1***	0.31	6
(mg/g)																

*p<0.05, **p<0.01, ***p<0.001 *vs*. Salt/Fat Diet
#p<0.05, ##p<0.01, ###p<0.001 *vs*. rofecoxib

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Table 2

Echocardiographic Analysis		Normal Diet			Salt/Fat Diet			AHAB (12 mg/kg/d)			rofecoxib (18 mg/kg/d)		
	Weeks	***MEAN***	***SEM***	***N***	***MEAN***	***SEM***	***N***	***MEAN***	***SEM***	***N***	***MEAN***	***SEM***	***N***
CO	**0**	79.4	4.0	9	74.5	4.3	11	77.5	2.9	10	78.9	4.0	7
(ml/min)	**4**	103.2	11.1	6	87.3	9.9	10	109.57*,##	9.0	10	63.0	8.1	5
SV	**0**	243.6	8.1	9	242.8	10.7	11	251.0	8.9	10	256.4	9.6	9
(µl)	**4**	300.5	20.8	6	282.3	23.1	10	341.7*,#	15.3	10	272.5	32.4	7
EF	**0**	60.3	1.3	9	62.7	1.5	11	60.2	1.0	10	61.1	0.7	9
(%)	**4**	56.6	1.9	7	56.6	1.2	10	57.5	1.6	10	56.1	2.7	7
LVDd	**0**	7.06	0.12	9	6.84	0.14	11	7.01	0.13	10	7.16	0.21	9
(mm)	**4**	8.23*,#	0.22	6	7.41	0.21	10	7.92	0.15	10	7.33	0.36	7
LVDs	**0**	3.81	0.13	9	3.75	0.17	11	3.78	0.11	10	3.73	0.23	9
(mm)	**4**	4.60**,##	0.30	6	3.62	0.18	10	4.249*,#	0.15	10	3.69	0.29	7
LVM/BW	**0**	2.55	0.08	9	2.42	0.08	11	2.48	0.06	10	2.52	0.07	9
(mg/g)	**4**	2.41**,##	0.08	6	3.01	0.20	10	2.69	0.07	10	3.30	0.23	7
T/B	**0**	0.369	0.010	9	0.365	0.013	11	0.358	0.007	10	0.354	0.008	9
	4	0.390*,##	0.017	6	0.474	0.029	10	0.39***,#	0.011	10	0.501	0.042	7

*p<0.05, **p<0.01, ***p<0.001 *vs*. Salt/Fat Diet
#p<0.05, ##p<0.01, ###p<0.001 *vs*. rofecoxib

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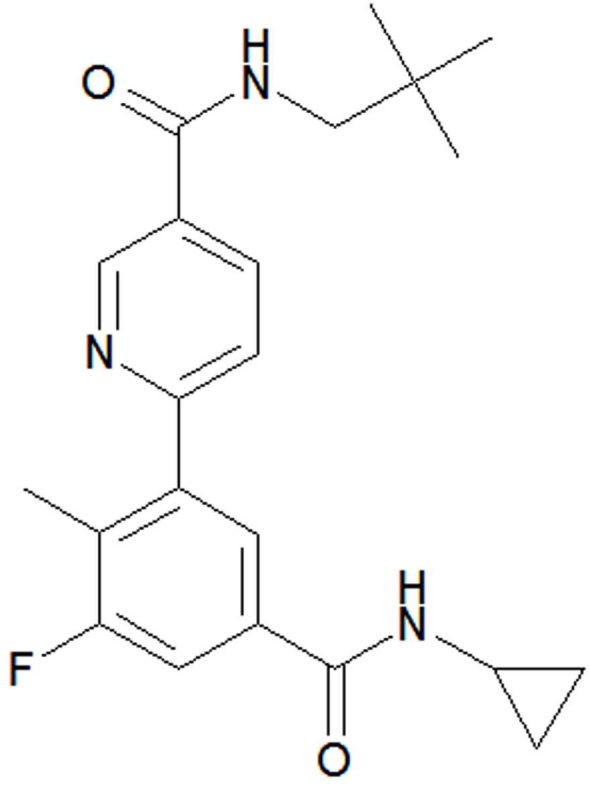
A.



GSK-AHAB

B.

In Vitro **Assay**	**pKi, IC_{50} (μM), or inhibition @ 10μM (%)**
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

Figure 1

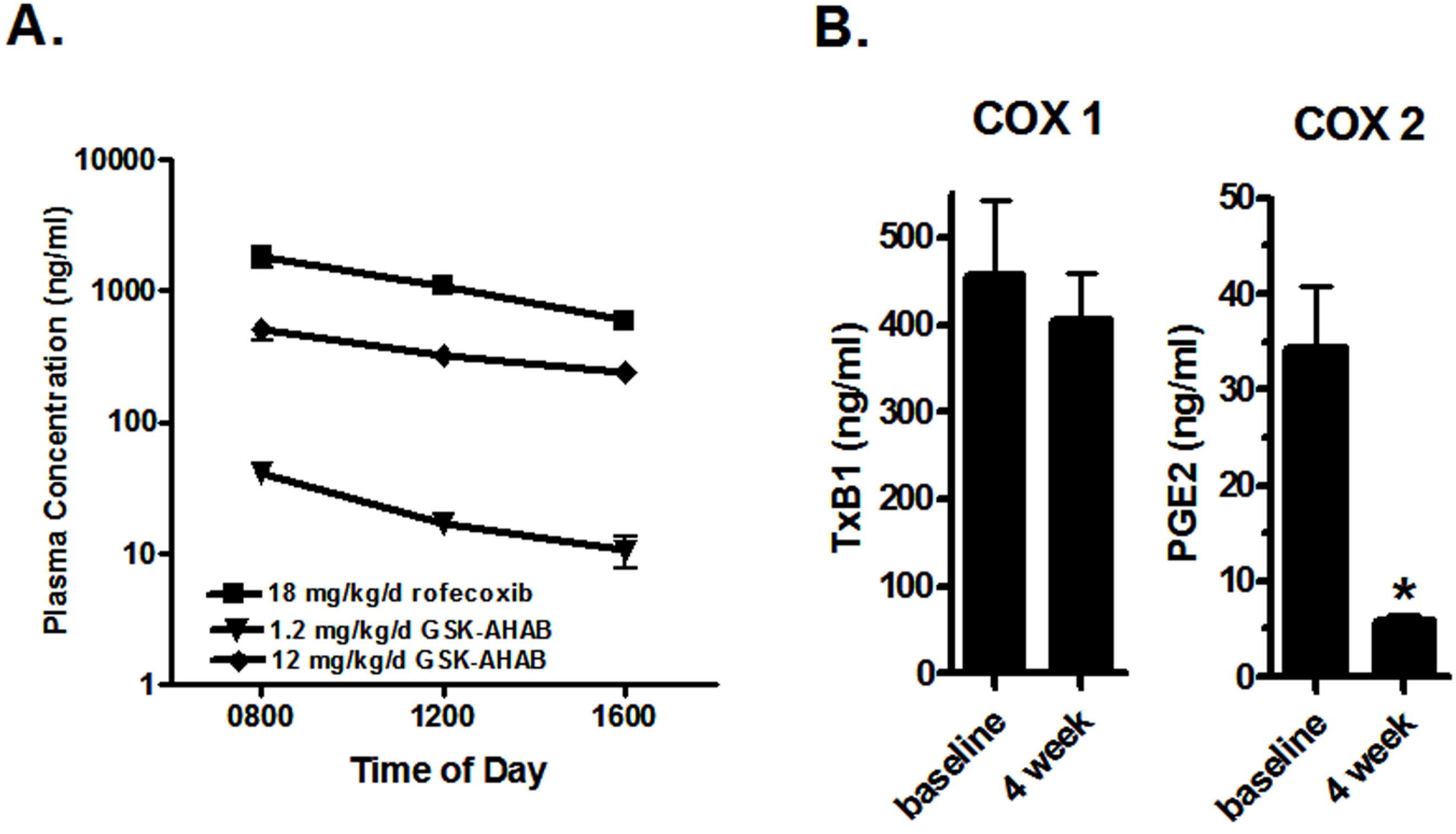
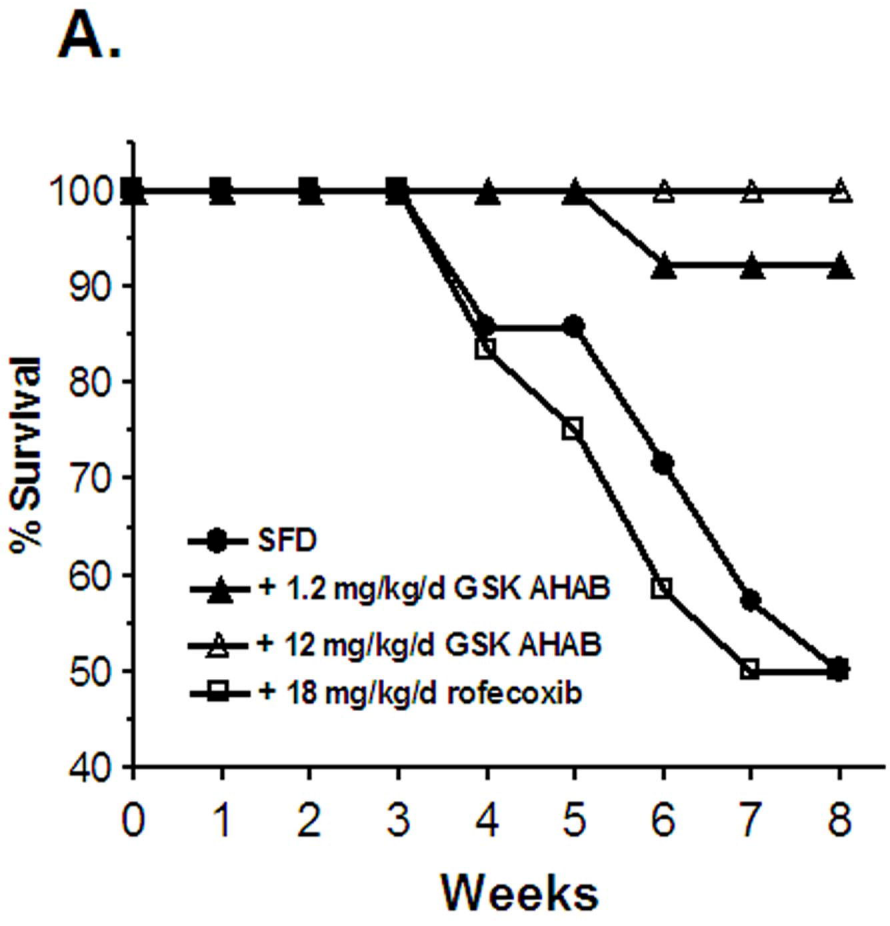

A.
B.
Plasma Concentration (ng/ml)
10000
1000
100
10
1
0800
1200
1600
Time of Day
18 mg/kg/d rofecoxib
1.2 mg/kg/d GSK-AHAB
12 mg/kg/d GSK-AHAB
COX 1
COX 2
TxB1 (ng/ml)
500
400
300
200
100
0
PGE2 (ng/ml)
50
40
30
20
10
0
*
baseline
4 week
baseline
4 week

Figure 2


A.
100
90
80
70
60
50
40
% Survival
SFD
+ 1.2 mg/kg/d GSK AHAB
+ 12 mg/kg/d GSK AHAB
+ 18 mg/kg/d rofecoxib
0
1
2
3
4
5
6
7
8
Weeks

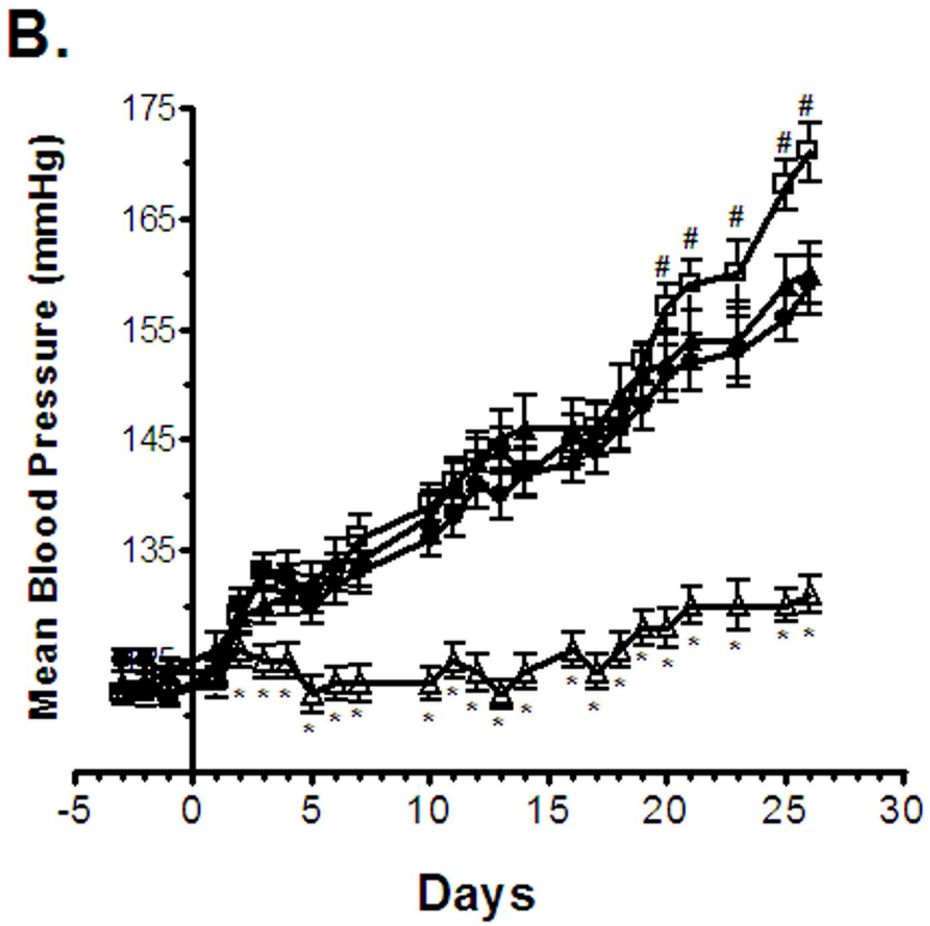

B.
175
165
155
145
135
Mean Blood Pressure (mmHg)
-5
0
5
10
15
20
25
30
Days

Figure 3

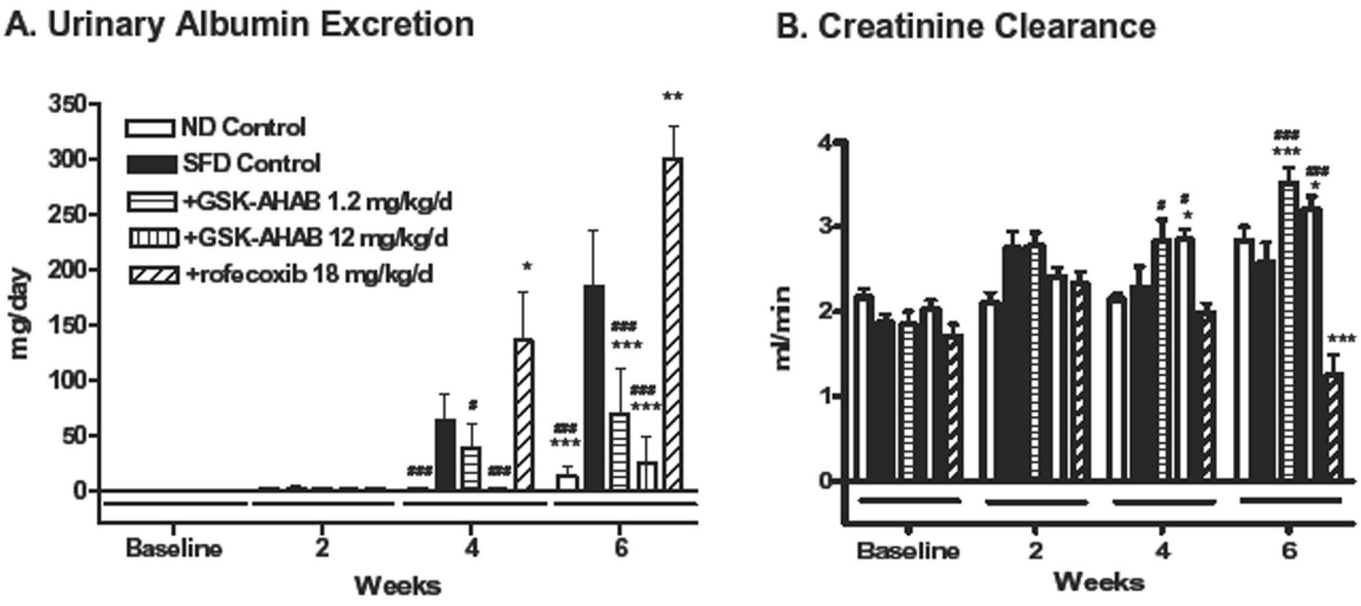

A. Urinary Albumin Excretion
B. Creatinine Clearance
ND Control
SFD Control
+GSK-AHAB 1.2 mg/kg/d
+GSK-AHAB 12 mg/kg/d
+rofecoxib 18 mg/kg/d
mg/day
ml/min
Baseline
2
4
6
Weeks

Figure 4

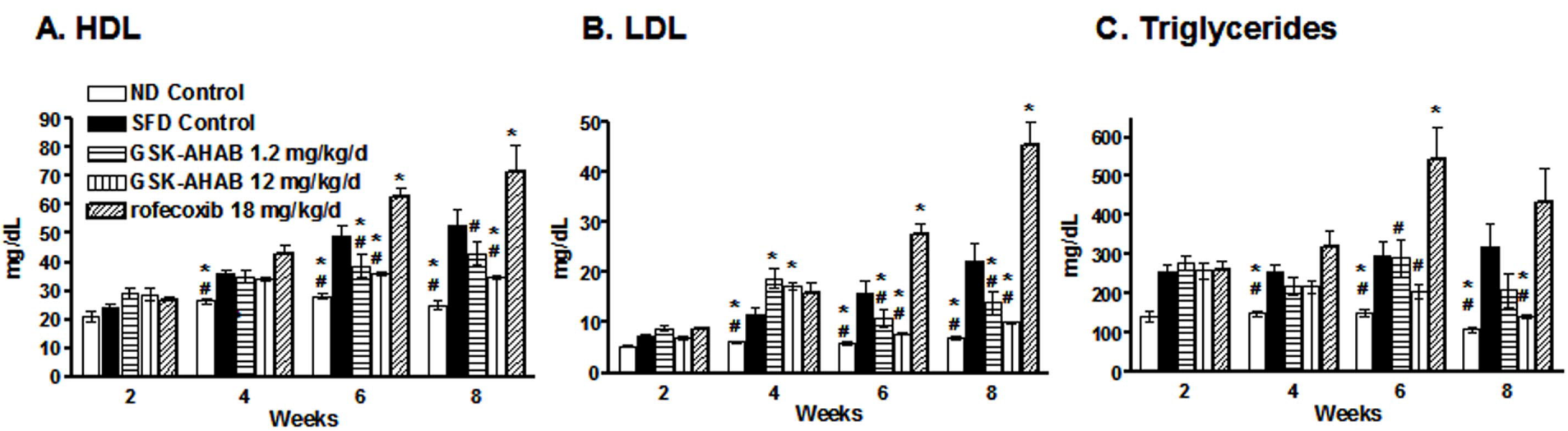

A. HDL
B. LDL
C. Triglycerides
ND Control
SFD Control
GSK-AHAB 1.2 mg/kg/d
GSK-AHAB 12 mg/kg/d
rofecoxib 18 mg/kg/d
mg/dL
Weeks
2
4
6
8

Figure 5

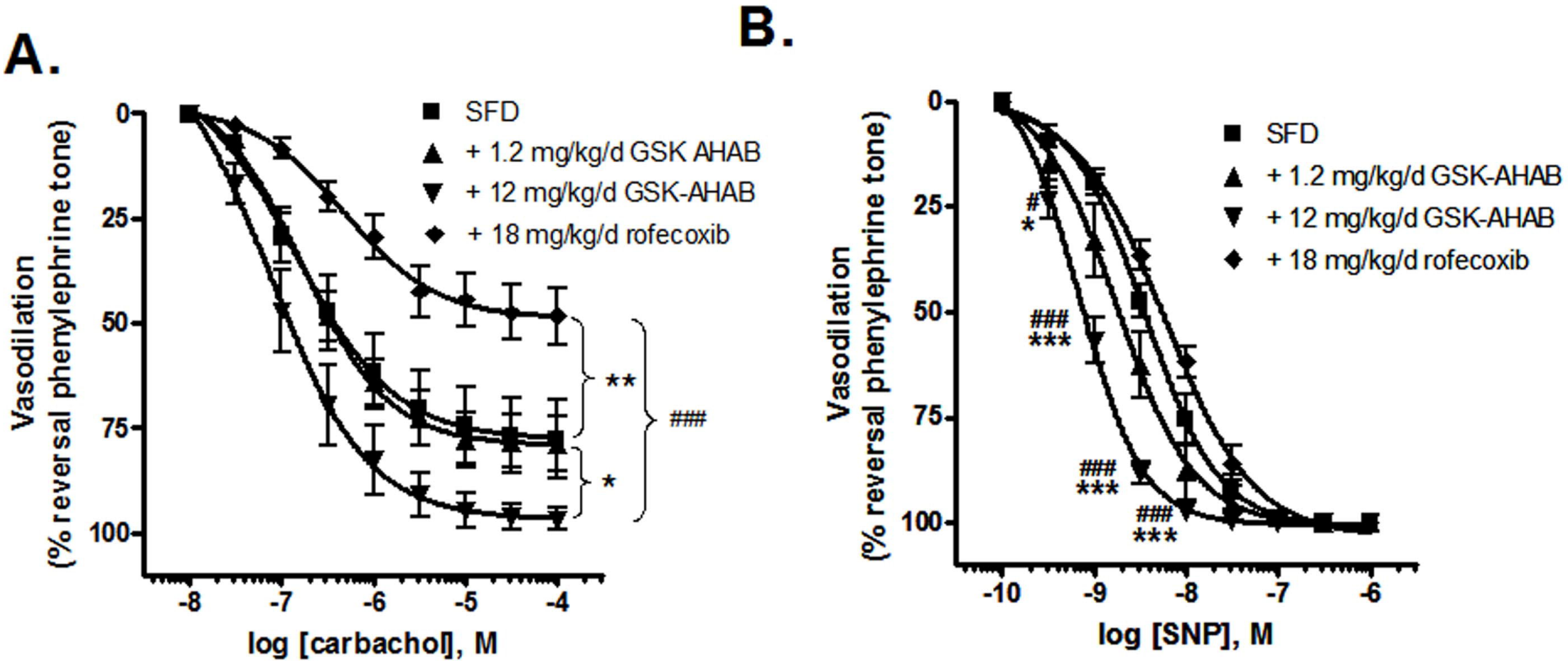

A.
B.
SFD
+ 1.2 mg/kg/d GSK AHAB
+ 12 mg/kg/d GSK-AHAB
+ 18 mg/kg/d rofecoxib
Vasodilation
(% reversal phenylephrine tone)
log [carbachol], M
log [SNP], M
**
*
###
#
###
***

Figure 6

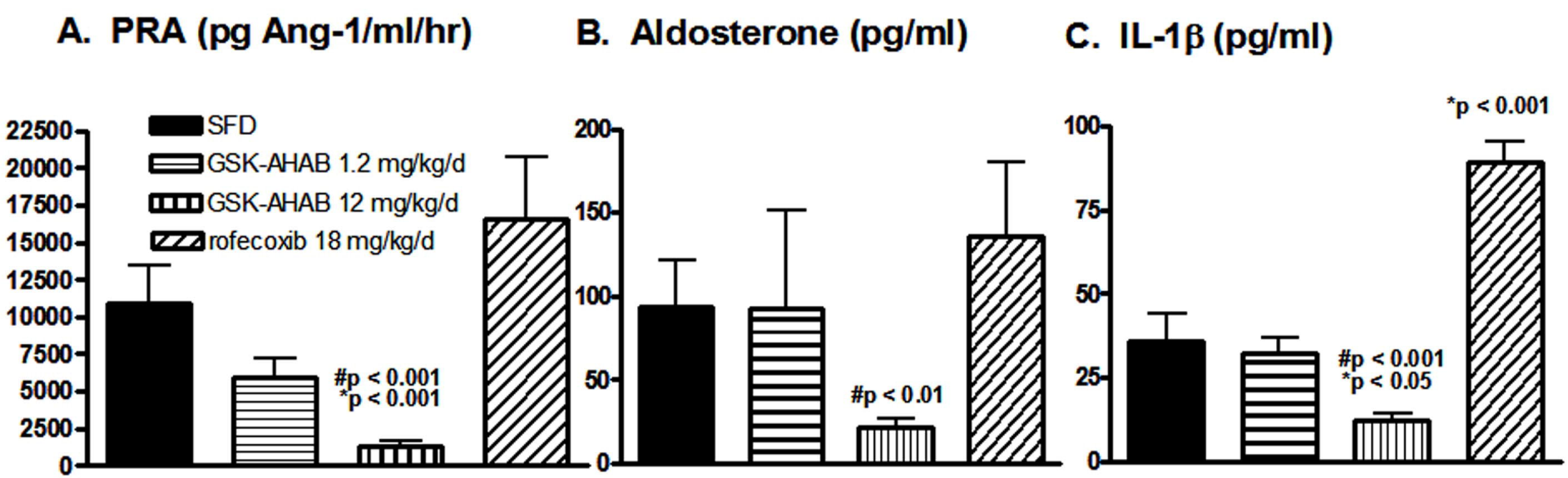

A. PRA (pg Ang-1/ml/hr)
B. Aldosterone (pg/ml)
C. IL-1β (pg/ml)
SFD
GSK-AHAB 1.2 mg/kg/d
GSK-AHAB 12 mg/kg/d
rofecoxib 18 mg/kg/d
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
#p < 0.001
*p < 0.001
200
150
100
50
0
#p < 0.01
100
75
50
25
0
*p < 0.001
#p < 0.001
*p < 0.05

Figure 7