Novel Rho Kinase Inhibitors with Anti-inflammatory and Vasodilatory Activities


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Abbreviations:

ROCK: Rho Kinase
MLC: Myosin light chain
MLCK: Myosin light chain kinase
MYPT: Myosin light chain phosphatase
MBS: Myosin binding subunit of MYPT
DOCA: Deoxycorticosterone acetate
SHR: Spontaneously hypertensive rat
WKY: Wista-Kyoto rat
L-NAME: N(omega)-nitro-L-arginine-methyl-ester
AngII: Angiotensin II
BP: Blood pressure
GEF: GTP exchange factor
LPS: lipopolysaccharide

Generic Names:

SB-772077-B: 4-(7-{{(3S)-3-amino-1-pyrrolidinyl}carbonyl}-1-ethyl-1H-imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-amine

GSK269962A: N-(3-{{2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-1H-imidazo[4,5-c]pyridin-6-yl}oxy}phenyl)-4-{{2-(4-morpholinyl)ethyl}oxy}benzamide
Abstract

Increased ROCK activity contributes to smooth muscle contraction and regulates blood pressure homeostasis. We hypothesized that potent and selective ROCK inhibitors with novel structural motifs would help elucidate the functional role of ROCK and further explore the therapeutic potential of ROCK inhibition for hypertension. In this paper, we characterized two aminofurazan-based inhibitors, GSK269962A and SB-772077-B, as members of a novel class of compounds that potently inhibit ROCK enzymatic activity. GSK269962A and SB-772077-B have IC$_{50}$s of 1.6 and 5.6 nM towards recombinant human ROCK1 respectively. GSK269962A also exhibited more than 30 fold selectivity against a panel of serine/threonine kinases. In LPS-stimulated monocytes, these inhibitors blocked the generation of inflammatory cytokines such as IL-6 and TNF-α. Furthermore, both SB-772077-B and GSK269962A induced vasorelaxation in pre-constricted rat aorta with an IC$_{50}$ of 39 and 35 nM respectively. Oral administration of either GSK269962A or SB-772077-B produced a profound dose-dependent reduction of systemic blood pressure in spontaneously hypertensive rats (SHR). At doses of 1, 3 and 30 mg/kg, both compounds induced a reduction in blood pressure of approximately 10, 20 and 50 mmHg. In addition, administration of SB-772077-B also dramatically lowered blood pressure in DOCA salt-induced hypertensive rats. SB-772077-B and GSK269962A represent a novel class of ROCK inhibitors that have profound effects in the vasculature, and may enable us to further evaluate the potential beneficial effects of ROCK inhibition in animal models of cardiovascular as well as other chronic diseases.
Introduction

Rho kinase (ROCK) belongs to a family of Ser/Thr protein kinases that is primarily activated via interaction with the small GTP binding protein RhoA. Growing evidence suggests that RhoA and ROCK participate in a variety of important physiological functions in vasculature including smooth muscle contraction, cell proliferation, cell adhesion, migration, and many aspects of inflammatory responses (Riento and Ridley, 2003). Two isoforms, ROCK1 and ROCK2, have been identified (Ishizaki et al., 1996; Leung et al., 1996; Matsui et al., 1996). They share significant sequence homology in the kinase domain (>90%) while the regulatory domains at the C-terminus show significant divergence (Riento and Ridley, 2003; Rikitake and Liao, 2005). Both are ubiquitously expressed in various human and rodent tissues. Mouse gene knockout (KO) studies suggest that ROCK1 and ROCK2 have non-overlapping functions. The ROCK1 KO results in developmental abnormalities in the eye lids whereas the ROCK2 KO was associated with placenta defects and failed to develop to term (Thumkeo et al., 2003; Shimizu et al., 2005).

ROCK activities play pivotal roles in modulating tonic smooth muscle contraction in various tissues including blood vessels, bronchial trachea, intestine, carvenosum and bladder (Leung et al., 1996; Wang et al., 2002; Wibberley et al., 2003). It is a major player in enhancing the calcium sensitivity of smooth muscle by phosphorylating the myosin binding subunit (MBS) of myosin light chain phosphatase (MYPT), leading to increased phosphorylation of myosin light chain (MLC) at lower intracellular calcium concentrations, thus maintaining tonic smooth muscle contraction (Amano et al., 2000; Somlyo and Somlyo, 2003). In addition to its role in smooth muscle contraction, ROCK
may also participate in diverse biological functions by phosphorylating numerous substrates such as adducin, ezrin-radixin-moesin (ERM), collapsin response mediator protein 2 (CRMP2) and sodium hydrogen exchanger1 (NHE1), thereby affecting a wide range of cellular activities (Shimokawa, 2002; Riento and Ridley, 2003; Hu and Lee, 2005). The mechanisms associated with these ROCK-mediated biological functions are just beginning to be elucidated.

Increased ROCK activity plays an important role in the blood pressure regulation and the development of hypertension. For example, enhanced ROCK activity as evidenced by increased ROCK substrate (MYPT) phosphorylation was observed in spontaneously hypertensive rat (SHR), DOCA-salt induced hypertensive rat and other rodent hypertension models (Seko et al., 2003). SHR and DOCA-salt induced hypertensive rats represent two subclass of hypertension: high renin (SHR) and low renin (DOCA) form (Pinto et al., 1998). The enhanced ROCK activity in both of these models suggests that this is a universal feature of the vasoconstrictive state observed in hypertension. Two widely employed ROCK inhibitors, fasudil and Y-27632, have provided preliminary but compelling evidence supporting the potential benefits of ROCK inhibition in animal disease models and in clinical trials. For example, administration of Y-27632 reduced blood pressure in SHR and other models of hypertension (Uehata et al., 1997), improved cardiac function in heart failure (Satoh et al., 2003) and suppressed lesion formation in a model of atherosclerosis (Mallat et al., 2003). However, given the limited potency and kinase specificity for these two compounds (Davies et al., 2000), additional ROCK inhibitors with novel structures and improved potency and selectivity may provide better tools to further evaluate the role of ROCK in various biological
functions. Here we describe aminofurazan-based compounds that are novel and potent ROCK inhibitors. Our results demonstrate a good correlation between the potency of ROCK enzyme inhibition with the efficacy of smooth muscle relaxation. These aminofurazan-based ROCK inhibitors also inhibited cytokine production in macrophages. In addition, we further demonstrated that these inhibitors had vasodilatory activity and lowered blood pressure in spontaneously hypertensive rat (SHR) and DOCA salt-treated hypertensive rats.
Methods

Reagents. Y-27632 and fasudil were purchased from Calbiochem Inc. (San Diego, CA) and Bio Mol (Plymouth Meeting, PA), respectively. LPS was also obtained from Calbiochem Inc. (San Diego, CA). TNF-α and IL-6 ELISA kits were from Roche Molecular Biochemicals, Inc (Indianapolis, IN). Human primary aortic smooth muscle cells were purchased from Camberex Bioscience (Walkersville, MD). Phenylephrine and other chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Rhodamine phalloidin was obtained from Molecular Probe (Invitrogen Inc.) (Carlsbad, CA). Nitrocellulose membrane for northern blot was purchased from Schleicher & Schuell Inc. (Keene, NH).

Expression of recombinant ROCK1 kinase domain. Human ROCK1 (amino acid 3-543) was expressed in baculovirus in Sf9 cells. A histidine tag was incorporated at the N-terminus. Affinity and conventional chromatography procedures were used to purify the protein to homogeneity. Purified protein was used for subsequent kinase assay. A separate manuscript describing the details of the recombinant expression, purification and characterization of human ROCK1 has been published elsewhere (Khandekar et al., 2005).

ROCK Enzyme assays. The enzyme activity and kinetics of the purified ROCK1 (3-543) were determined using scintillation proximity assay (SPA). In this assay, purified ROCK1 was incubated with peptide substrate (Biotin-Ahx-AKRLSSLRA-CONH2, purchased from Synpep,) and $^{33}$ATP (Redivue, Amersham Biosciences) and the
subsequent incorporation of $^{33}$P into the peptide was quantified by streptavidin bead capture.

For IC$_{50}$ determination, test compounds were dissolved at 10 mM in 100% DMSO, with subsequent serial dilution in 100% DMSO. Compounds were typically assayed over an eleven point dilution range with a concentration in the assay of 10 µM to 0.2 nM, in 3-fold dilutions. For dose response curves, data were normalized and expressed as % inhibition using the formula $100\times((U-C1)/(C2-C1))$ where $U$ is the unknown value, $C1$ is the average of the high signal (0%) control wells, and $C2$ is the average of the low signal (100%) control wells. Curve fitting was performed with the following equation: $y = A+((B-A)/(1+(10^x/10^C)^D))$, where $A$ is the minimum response, $B$ is the maximum response, $C$ is the log$_{10}$(IC$_{50}$), and $D$ is the Hill slope. The results for each compound were recorded as pIC$_{50}$ values (-C in the above equation).

Assays for determining inhibitory activity were performed in opaque, white walled, 384 well plates, in a total assay volume of 10 µl. The assays contained: 1 nM ROCK1; 1 µM biotinylated peptide (biotin-Ahx-AKRRRLSSLRA-CONH$_2$); 1 µM ATP; 1.85 kBq per well ATP (gamma-$^{33}$P); 25 mM HEPES, pH 7.4; 15 mM MgCl$_2$; 0.015% BSA. The reactions were incubated at 22°C for 90 minutes and terminated by the addition of a 10 µl solution containing 150 mM EDTA and streptavidin coated polystyrene SPA beads (Amersham Biosciences). The beads were added to a concentration of 0.08 mg per well. The plates were allowed to incubate at 22°C for 10 minutes before centrifugation at 1500 rpm for 1 minute. $^{33}$P incorporation was quantified by liquid scintillation counting using a TopCount (Packerd).
Cell culture and cytokine mRNA and protein expression in human smooth muscle cells and macrophages. Human aortic smooth muscle cells were purchased from Camberex Biosciences (Walkersville, MD) and grown in SmGM medium according to manufacturer’s instructions. For stress fiber formation assay, cells were grown on cover slips and at approximately 50% confluent, they were serum starved overnight. Cells were subsequently stimulated with AngII (100 nM) for 2 hours before fixing and stained with Rhodamine phalloidin (see section below).

Human primary macrophages were prepared as follows: leukopacks enriched for monocytes were obtained from Biological Specialty Corporation (Colmar, PA). Cells were washed twice in HBSS w/o Ca++, w/1 mM EGTA, then layered onto 10mls Histopaque gradient (Sigma, St. Louis, MO); after centrifugation, the harvested interface layer contains both monocytes and lymphocytes. To obtain a pure monocyte population, non-monocytic cells (i.e., T cells, granulocytes, NK cells, B cells, dendritic cells, and basophils) are labelled using a cocktail of hapten-modified CD3, CD7, CD19, CD45RA, CD56, and anti-IgE antibodies (Miltenyi Biotec, Inc., Auburn, CA). The non-monocytes are then magnetically labelled using MACS MicroBeads coupled to an anti-hapten antibody, and the entire cell population is passed through a magnetized column. Unlabelled monocytes pass through the column, and are collected, washed, and plated into Costar 24 well plates. Growth medium is RPMI-1640 with 2 mM L-glutamine, 5% human AB serum (Sigma), penicillin-streptomycin (100 U/ml, 100 µg/ml final concentration), and granulocyte-macrophage colony stimulating factor (G-MCSF) at 1.0 nanogram/ml final concentration (Life Technologies, Rockville, MD). Cells are allowed
to differentiate in culture for 6 to 8 days prior to experiments, and reach nearly 100% confluence and purity.

Similar to smooth muscle cells, these macrophages were serum starved overnight and stimulated with LPS (100 ng/ml). Expression of IL-6 mRNA and protein were measured using northern blot techniques and IL-6 ELISA. Supernatants were also analyzed for TNF-α expression using ELISA. ROCK inhibitors were added 30 minutes prior to stimulation. Cellular RNA was isolated for Northern analysis while supernatants were used for cytokine measurement. RNA isolation and Taqman and ELISA measurements were performed as instructed in manufacturer’s protocol and published procedures (Sambrook et al., 1989). All cell culture experiments were repeated at least three times, and representative blots are presented.

**Immunofluorescence for actin stress fiber formation and confocal microscopy.**
Detection of actin stress fiber was monitored using Rhodamine phalloidin as described by Ueda et al (Ueda et al., 2000). Upon the completion of staining, slides were examined using argon laser in a confocal microscope (Olympus, Melville, NY). The images were taken at 40x with a PMT voltage of 664, gain of 1.6 and a 4% offset, with a procedure similar to an earlier study (Grygielko et al., 2005).

**Aorta relaxation assay.** Rat aorta contraction was performed as described (Behm et al., 2002). Male Sprague-Dawley rats (350-400g) were anesthetized with 5% isoflurane in O2 and killed by exsanguination. Aortic rings, approximately 2-3 mm in length, were suspended by two 0.1mm diameter tungsten wire hooks in 10 ml tissue baths containing
Krebs of the following composition (mM): NaCl 112.0, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.0, dextrose 11.0, indomethacin 0.01, and L-NAME 0.01. Krebs was maintained at 37°C and aerated with 95% O₂, 5% CO₂ (pH 7.4). Changes in isometric force were measured under optimal resting tension (1 g) using FT03 force-displacement transducers (Grass Instruments, Quincy, MA) coupled to Model 7D polygraphs. Following a 60 min equilibration period, the vessels were treated with standard concentrations of KCl (60 mM) and phenylephrine (1 µM). Cumulative concentration-response curves to phenylephrine were obtained for each tissue by dosing at 0.5 log unit intervals (1nM to 10 µM). Following several washes, each vessel was contracted to equilibrium with an EC₈₀ concentration of phenylephrine and tone was reversed by adding cumulative amounts of either GSK269962A or SB-772077-B at 0.5 log unit intervals (0.1 nM to 1 µM). Responses were expressed as percent reversal of the tone established with phenylephrine.

**SHR blood pressure (BP) studies.** SHR, obtained from the National Institutes of Health (Bethesda, MD), were bred in the Department of Laboratory Animal Science at GlaxoSmithKline (King of Prussia, PA). Age-matched normotensive rats [Wistar-Kyoto (WKY) and Sprague-Dawley (SD)] were purchased from Charles River Laboratories, Inc. (Wilmington, MA). 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.

Blood pressure (BP) measurements were performed using a telemetry system as described (Ju et al., 2003). Briefly, male SHR (8-10 weeks of age) maintained on a
normal powdered diet (Purina Diet 5001) were anesthetized with 2% isoflurane anesthesia and a telemetry transmitter (TA11PA-C40; Data Sciences International, St. Paul, MN) was implanted. The transmitter catheter was inserted into the femoral artery and advanced into the lower abdominal aorta. Baseline measurements of systolic and diastolic blood pressure, heart rate, and activity were obtained 1 week before experiments. Recordings were obtained each week thereafter for a continuous period of 24 h with data acquisition of 10-s averages every 5 min. ROCK inhibitors (SB-772077-B and GSK269962A) were administered via oral gavage and BP responses were monitored immediately following drug administration. 4-6 animals were examined for each dose in treated and vehicle group.

**Statistical Analysis.** Maximal blood pressure changes were analyzed for statistical significance. A two-way ANOVA analysis was performed. Significance if indicated was compared to the vehicle response. P value was described in figure legends. All statistical analyses were performed using Prizm (GraphPad Software Inc., San Diego, CA) and $P \leq 0.05$ was considered significant.
Results

Identification of selective aminofurazan ROCK inhibitors. In an effort to identify potent and selective Rho kinase inhibitors, we expressed and purified the kinase domain of human ROCK1 (amino acid 3-543) (Khandekar et al., 2005) and used the purified recombinant protein to screen the GSK compound collection. Several aminofurazans were identified as putative ROCK inhibitors. Subsequent optimization led to improved potency, selectivity and pharmacokinetics and resulted in the identification of SB-772077-B and GSK269962A. The structures of SB-772077-B and GSK269962A are depicted in Figure 1. Both are highly potent towards human ROCK1 with IC\textsubscript{50}s of 5.6 nM for SB-772077-B and 1.6 nM for GSK269962A, respectively (Figure 2). These compounds also potently inhibited human ROCK2 with similar potency (Table 1). In comparison, widely used Rho kinase inhibitors Y-27632 and Fasudil inhibited ROCK1 with much lower potency, with an approximate IC\textsubscript{50} of 150 nM for Y-27632 and 300 nM for Fasudil. Both Y-27632 and Fasudil also inhibited human ROCK2 with similar IC\textsubscript{50}. These data are consistent with previously published results for these two compounds (Fasudil and Y-27632) (Uehata et al., 1997). In addition to ROCK1 and 2, SB-772077-B potently inhibits several other kinases (e.g. MSK1 and RSK1). On the other hand, GSK269962A has a significantly improved kinase selectivity profile with at least >30 fold selectivity against the panel of protein kinase tested. The IC\textsubscript{50} value of SB-772077-B and GSK269962A for ROCK1, ROCK2 and profiled kinases are shown in Table 1. These data suggest that SB-772077-B and GSK269962A represent a novel class of ROCK inhibitors with high potency and good protein kinase selectivity.
Aminofurazan ROCK inhibitors disrupt actin stress fiber formation in smooth muscle cells and suppress inflammatory cytokine production from macrophages. In order to evaluate the cellular effect of novel ROCK inhibitors, we employed actin stress fiber formation as a functional assay. ROCK regulates actin stress fiber formation via the activation of LIM kinase and ROCK inhibition has been shown to decrease actin fiber content in cells (Amano et al., 1997). SB-772077-B and GSK269962A completely abolished the actin stress fiber formation induced by angiotensin II in human smooth muscles (Figure 3). Such suppressive effect on actin fiber formation was observed beginning at around 1 µM for both SB-772077-B and GSK269962A. Similar effects were also observed with 30µM of Y-27632 treatment.

ROCK inhibitors such as Y-27632 and fasudil have been demonstrated to suppress cytokine expression from several types of cells in the vasculature including monocytes, endothelial cells and lymphocytes (Hippenstiel et al., 2002; Aihara et al., 2003; Segain et al., 2003). We evaluated the effects of the aminofurazan ROCK inhibitors in primary human macrophages. When THP-1 macrophages were pre-treated with increasing concentrations of SB-772077-B, LPS-induced cytokine production including TNFα and IL-6 were dose-dependently reduced (Figure 4). Similar results were obtained with GSK269962A and other structurally related aminofurazans with inhibitory activities towards ROCK. The effect of ROCK inhibitors on cytokine production is likely to act through modulating the transcriptional activation or mRNA stability since the LPS-induced increase in IL-6 mRNA is abolished when macrophages were pre-treated with Y-27632 and two structurally-related aminofurazan ROCK inhibitors (SB-
729743 and SB-742548), but not by an aminofurazan with no activity towards ROCK1 (SB-739412-B) (Figure 5A and 5B).

**Aminofurazan ROCK inhibitors relax contracted blood vessels in vitro.** Inhibition of ROCK has been associated with relaxation of constricted smooth muscles. We examined the effect of SB-772077-B and GSK269962A on contracted rat aortic rings in tissue baths. Both SB-772077-B and GSK269962A profoundly reversed the tonic tension generated by phenylephrine (Figure 6). The relaxations were highly dose-dependent with IC₅₀s of 39 nM and 35 nM for SB-772077-B and GSK269962A respectively. In comparison, Y-27632 is significantly less potent in this assay with a IC₅₀ of approximately 1.4 µM (Figure 6). In addition, the relaxation induced by SB-772077-B and GSK269962A are reversible. Washing the treated aorta will restore the contractility to KCl as well as phenylephrine (data not shown). This is consistent with the reversible nature of ROCK inhibition by these compounds.

We also examined whether ROCK inhibition potency correlates with the potency to relax aortic rings. As shown in Figure 7, eighteen aminofurazans were examined with a wide range of potency (from < 1 nM to ~1000 nM) toward ROCK1 and the results revealed a correlation coefficient R²=0.65 in linear regression analysis. Since the effects on aortic ring contraction require cell/tissue permeability, some of the variability observed may be due to the differences in cell membrane permeability.

**Aminofurazan ROCK inhibitors are potent antihypertensive agents in vivo.** The effects of SB-772077-B and GSK269962A on blood pressure regulation were examined
in vivo in Spontaneously Hypertensive Rats (SHR) as well as in normotensive and DOCA salt-induced hypertensive rats (DOCA-rat). As shown in Figure 8A and 8B, oral administration of SB-772077-B and GSK269965A (0.3, 1 and 3 mg/kg) induced a dose-dependent reduction in blood pressure in SHR. The reduction of blood pressure was acute and substantial. The maximal effect on blood pressure was observed approximately 2 hours after oral gavages for both compounds. Under a similar setting, oral administration of Y-27632 (10 and 30 mg/kg) also induced a dose-dependent decrease of blood pressure (Figure 8C). For all three Rho kinase inhibitors, the reduction of blood pressure was accompanied by an acute, dose-dependent increase in heart rate, presumably due to the activation of baroreflex mechanism. These results are consistent with the hypothesis that ROCK inhibition induces vasodilation in vivo by relaxing vessel and reducing total peripheral vascular resistance.

In addition, we also evaluated the effect SB-772077-B on blood pressure in normotensive SD rat and DOCA-salt induced hypertensive rats. As shown in Figure 9, oral administration of SB-772077-B at 1 mg/kg profoundly reduced blood pressure in DOCA rats. The reduction in blood pressure was more acute and dramatic in DOCA rats than in SHR, with a maximal decrease of approximately 60 mm Hg reached at approximately 2 hours post dosing. The maximal decrease in blood pressure in DOCA-treated rats is more than the maximal decrease (~ 40 mmHg) in SHR at the same dose (Figure 9A). An increase in heart rate was observed following oral dosing in DOCA rats similar to that of SHR (Figure 8). In comparison, oral administration of SB-7720770B in normotensive rat (SD rat) induced only modest decrease in blood pressure (~ 12 mHg) (Figure 9B).
Discussion

Accumulating evidence suggests that ROCK plays an important role in blood pressure regulation by controlling smooth muscle contraction (Somlyo and Somlyo, 2003; Lee et al., 2004). ROCK regulates the calcium sensitization by enhancing the phosphorylation of myosin binding subunit (MBS) of myosin light chain phosphatase (MYPT) and inhibiting phosphatase activity, consequently increases the phosphorylation of MLC, leading to sustained smooth muscle contraction without significantly elevated intracellular calcium. This mechanism seems to play a pivotal role in blood pressure homeostasis. Furthermore, there is evidence suggesting that ROCK and ROCK pathway components (RhoA, GEFs, ROCK) are increased in SHR (Mukai et al., 2001; Seko et al., 2003; Moriki et al., 2004; Ying et al., 2004) and human hypertensive patients (Masumoto et al., 2001). Elevated levels of these elements lead to enhanced ROCK activity and further results in smooth muscle hyperconstriction which is one of the hallmarks of hypertension and other cardiovascular diseases. Small molecule ROCK inhibitors may offer an attractive strategy to attenuate the enhanced ROCK activity, leading to reduced hypercontraction, and thereby providing potential therapeutic benefits.

We describe here the identification of a novel class of aminofurazan-based small molecules that are highly potent and selective ROCK inhibitors. Aminofurazan ring has not been used as one of the motifs for ROCK inhibitors, and our results demonstrate aminofurazan is a key feature of contact between this small molecule and ROCK ATP binding pocket. In this respect, the identification of this structure motif is a novel finding. Two compounds from this chemical series, SB-772077-B and GSK269962A, demonstrated low nM potency towards human ROCK enzyme and relaxed pre-
constricted blood vessels in vitro. These novel inhibitors showed significantly enhanced potency in vitro and in vivo as compared to the widely studied ROCK inhibitors Y-27632 and fasudil (Uehata et al., 1997). A strong correlation between ROCK inhibition and aorta relaxation was observed for this novel class of inhibitors. We also demonstrated that aminofurazan-based ROCK inhibitors suppress the production of IL-6 and TNF-α from LPS-stimulated human macrophages. The anti-inflammatory effects of ROCK inhibitors such as Y-27632 or fasudil were previously described in several laboratories (Funakoshi et al., 2001; Eto et al., 2002; Segain et al., 2003; Aihara et al., 2004). Our results showed that in macrophages, SB-772077-B and GSK269962A, as members of a distinct class of ROCK inhibitors, suppress IL-6 mRNA transcription and reduce LPS-induced IL-6 and TNF-α protein production. This, combined with our earlier findings demonstrating that Y-27632 administration reduced cytokine production in rat models of ischemia reperfusion-induced myocardial infarction (Bao et al., 2004), strongly suggest ROCK inhibition per se is sufficient to reduce the inflammatory cytokine expression.

The mechanism responsible for ROCK inhibitor-mediated suppression of cytokine expression is not fully understood. Several investigators (Hippenstiel et al., 2002; Segain et al., 2003) have postulated that NFkB may be involved in this process. Indeed, it was demonstrated that p65Rel phosphorylation in vivo was reduced upon treatment of Y-27632 in murine peritoneal macrophages (Mallat et al., 2003). We have failed to observe significant reduction in p65Rel phosphorylation on Ser-276, Ser536 or Ser-468 residues upon pre-treatment of either Y-27632 or aminofurazan-based ROCK inhibitors in TNF-α treated primary human macrophages in vitro (data not shown).
Whether ROCK-mediated p65Rel phosphorylation is involved in suppression of cytokine production in human primary macrophages warrants further careful analysis.

Aminofurazan ROCK inhibitors exhibited good oral activities in rat models of hypertension. SB-772077-B and GSK269962A potently reduced blood pressure in both SHR and DOCA-treated hypertensive rats, while having a much smaller effect on blood pressure in normotensive animals. These results are consistent with the hypothesis that enhanced ROCK activity contributes to vasoconstriction and elevated blood pressure in these animal models. Concomitant with blood pressure decrease, acute increases in heart rates were observed for both SB-772077-B and GSK269962A in a dose-dependent manner. Interestingly, as compared with these two compounds, Y-27632 induced a more dramatic increase in heart rate with doses that had comparable effect on blood pressure (Figure 8). It is well known that acute decrease in blood pressure induces baroreflex activation and leads to increased heart rate and cardiac output. The observed heart rate is likely due to such baroreflex activation. ROCK inhibitors have been shown influence baroreflex activation by inhibiting brain-specific ROCK2 and reduce neuronal activation (Ito et al., 2003). The differences in heart rate induced by aminofurazan-based inhibitor versus Y-27632 may result from their ability to penetrate blood brain barrier and affect the baroreflex activation. Further analysis is needed to examine this possibility.

In summary, we have identified a novel class of potent and selective ROCK inhibitors that possess vasodilatory and anti-inflammatory activity in tissue baths and cultured cells. When administered in vivo, these compounds are potent anti-hypertensive agents in two models of rat hypertension. Such inhibitors represent additional tools for further exploration of the biological functions of ROCK in smooth muscle biology and
for evaluating the usefulness of ROCK inhibitors in various cardiovascular diseases including hypertension.
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protein betagamma subunits induce stress fiber formation and focal adhesion

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of smooth muscle mediated by a Rho-associated protein kinase in hypertension.


Legends for Figures

Figure 1. Structures of SB-772077-B and GSK269962A

Figure 2. Dose-dependent inhibition of recombinant human ROCK1 kinase domain with SB-772077-B and GSK269962A. Human recombinant ROCK1 (amino acid 3-543) and a biotinylated peptide substrate were used to monitor kinase activity as described in Methods. Signal output was converted to % inhibition and IC50 values were determined as described in Methods.

Figure 3. Inhibition of actin stress fiber formation by novel ROCK inhibitor in human primary aortic smooth muscle cells. Human primary smooth muscle cells were serum starved and treated with Ang II as described in Methods. ROCK inhibitors (3µM for SB-772077-B or GSK269962A) were added 30 min prior to Ang II stimulation and cells were fixed and stained with rhodamine phalloidin. Confocal images of actin stain were obtained as described. Experiments were triplicates and representative micrographs were shown.

Figure 4. Suppression of cytokine production by SB-772077-B and other novel ROCK inhibitors. Primary human macrophage cells were serum starved and stimulated with LPS (100 ng/ml) for 18 hours and supernatants were harvested and analyzed for IL-6 and TNF-α expression (pg/ml) using ELISA as described in Methods. SB-772077-B
was added 30 min prior to LPS. Experiments were repeated at least three times and representative data from one experiment (triplicate samples) was shown.

**Figure 5. Northern blots for IL-6 mRNA expression in human macrophages.** Cell culture and northern blot were performed as described in Methods. Panel A: expression of IL-6 mRNA in control (lane 1), LPS-stimulated (Lane 2) and pre-treated with various ROCK inhibitor and control inhibitors (Lane 3-6). Lane 3: Y-27632 (30 µM); Lane 4 and 5: SB-729743 (10 µM) and SB-742548 (10 µM). These two compounds are aminofurazan-based ROCK inhibitor (IC$_{50}$ < 10 nM for both) with varying degree of selectivity towards ROCK. Lane 6: SB-739412-B (10 µM). This compound has aminofuran core motif, but does not inhibit ROCK (IC$_{50}$ >1 µM). Panel B: dose-dependent suppression of IL-6 expression by SB-742548 (lane 3-7).

**Figure 6. Effects of SB-772077-B, GSK269962A and Y-27632 on contracted rat aorta.** Rat aorta was contracted with phenylephrine and then increasing concentrations of ROCK inhibitors were added to the tissue bath as described in Methods. Aminofurazan-based ROCK inhibitors demonstrated a concentration-dependent relaxing effect on these vessels. Vasodilation (% reversal) was defined as % reduction in tension from phenylephrine-treated pre-constricted aorta based on tension tracing graphs. An average of 4 aorta were tested for each compound at various concentrations.

**Figure 7. Correlation between ROCK inhibition *in vitro* and relaxation of rat aorta.** IC$_{50}$ values of ROCK inhibition *in vitro* and relaxation of rat aorta for 18 aminofurazan-
based ROCK inhibitors was plotted using Prizm (Graph Pad) software and linear regression analysis was performed to obtain correlation coefficient (R²) value.

Figure 8. Effect of SB-772077-B, GSK269962A and Y-27632 on blood pressure and heart rate in SHR. Blood pressure of SHR were monitored via telemetry as described in Methods. Blood pressure telemetry tracings were presented. Data for SB-772077-B (Panel A), GSK269962A (Panel B) and Y-27632 (Panel C) are presented. Drug doses are indicated on the graph and compounds were administered through oral gavage. Mean blood pressure (MBP) decrease with time (Δ mmHg) was defined as decrease from baseline for each group of treated animals. Heart rate (HR) were also monitored. 5 rats were used for each dosing group. The maximal decrease in blood pressure for each dosing group were statistically significant (p<0.05) as compared to vehicle group.

Figure 9: Effect of SB-772077-B on blood pressure and heart rate in normotensive (Sprague-Dawley, SD) and DOCA salt-treated hypertensive rats. SB-772077-B (1 mg/kg) induced a dramatic reduction in blood pressure (~40 mmHg decrease) in DOCA salt-treated hypertensive rats (Panel A), while the same dose of drug induced a small decrease (~12 mmHg) in blood pressure in normotensive SD rats (Panel B). The BP telemetry tracing (Δ mmHg) was presented up to 12 hours after dosing.
Table 1: Protein kinase selectivity profile of SB-772077-B and GSK269962A.

<table>
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<tr>
<th>Protein</th>
<th>SB-772077-B</th>
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Figure 1

SB-772077-B

GSK269962A
Figure 2

![Graphs showing inhibition (%) vs concentration (nM) for SB-772077-B and GSK269962A.](image-url)
Figure 3

(control) A

Ang II

Ang II + SB-772077-B

Ang II + GSK269962A
Figure 4

A bar graph showing cytokine levels (IL-6 and TNF-α) in response to different concentrations of LPS and LPS + SB-772077-B. The x-axis represents the different concentrations: C, LPS, 0.1 μM, 0.3 μM, 1 μM, 3 μM, and 10 μM. The y-axis represents cytokine levels in pg/ml, ranging from 0 to 6000 pg/ml.
Figure 5.
Figure 6

Graphs showing the vasodilation (as a percentage of reversal) in response to different concentrations of three substances: [Y-27632] nM, [SB-772077-B] nM, and [GSK269962A] nM.
Figure 7

[Graph showing the relationship between Rat aorta IC$_{50}$ (nM) and Human ROCK1 kinase IC$_{50}$ (nM). The graph includes data points for SB-772077-B and GSK269962A. The correlation coefficient R$^2 = 0.65$.]

Rat aorta IC$_{50}$ (nM)

Human ROCK1 kinase IC$_{50}$ (nM)
Figure 8B:

- **MBP**
  - Vehicle (n=5)
  - GSK269962A 0.3mg/kg po (n=5)
  - GSK269962A 1mg/kg po (n=5)
  - GSK269962A 3mg/kg po (n=5)

- **HR**
  - Vehicle (n=4)
  - GSK269962A 0.3mg/kg po (n=5)
  - GSK269962A 1mg/kg po (n=5)
  - GSK269962A 3mg/kg po (n=5)
Figure 8C

**MBP**

- **Vehicle (n=5)**
- **Y-27632 10mg/kg po (n=5)**
- **Y-27632 30mg/kg po (n=5)**

**HR**

- **Vehicle (n=5)**
- **Y-27632 10mg/kg po (n=5)**
- **Y-27632 30mg/kg po (n=5)**

Y-axis: Change from baseline (mmHg) or (bpm)
X-axis: Time (hrs)
Figure 9A

MBP

- Vehicle (n=6)
- SB-772077-B 1mg/kg (n=6)

HR

- Vehicle (n=6)
- SB772077B 1mg/kg (n=6)
Figure 9B

MBP

- Vehicle (n=4)
- SB-772077-B 1mg/kg po (n=5)

Change from baseline (mmHg)

Time (hrs)

HR

- Vehicle (n=4)
- SB-772077-B 1mg/kg po (n=5)

Change from baseline (bpm)

Time (hrs)