Linking Phenotype to Kinase: Identification of a Novel Benzoxaborole Hinge-Binding Motif for Kinase Inhibition and Development of High-Potency Rho Kinase Inhibitors

Tsutomu Akama, Chen Dong, Charlotte Virtucio, David Sullivan, Yasheen Zhou, Yong-Kang Zhang, Fernando Rock, Yvonne Freund, Liang Liu, Wei Bu, Anne Wu, Xiao-Qing Fan, and Kurt Jarnagin

Anacor Pharmaceuticals, Inc., Palo Alto, California

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ABSTRACT

Benzoxaboroles are a novel class of drug-like compounds that have been rich sources of novel inhibitors for various enzymes and of new drugs. While examining benzoxaborole activity in phenotypic screens, our attention was attracted by the (aminomethylphenoxyl)benzoxaborole family, which potently inhibited Toll-like receptor–stimulated cytokine secretion from leukocytes. After considering their structure-activity relationships and the central role of kinases in leukocyte biology, we performed a kinome-wide screen to investigate the members of the (aminomethylphenoxyl)benzoxaborole family. This technique identified Rho-activated kinase (ROCK) as a target. We showed competitive behavior, with respect to ATP, and then determined the ROCK2–drug cocrystal structure. The drug occupies the ATP site in which the oxaborole moiety provides hydrogen bond donors and acceptors to the hinge, and the aminomethyl group interacts with the magnesium/ATP-interacting aspartic acid common to protein kinases. The series exhibits excellent selectivity against most of the kinome, with greater than 15-fold selectivity against the next best member of the AGC protein kinase subfamily. Medicinal chemistry efforts with structure-based design resulted in a compound with a Kᵢ of 170 nM. Cellular studies revealed strong enzyme inhibition rank correlation with suppression of intracellular phosphorylation of a ROCK substrate. The biochemical potencies of these compounds also translated to functional activity, causing smooth muscle relaxation in rat aorta and guinea pig trachea. The series exhibited oral availability and one member reduced rat blood pressure, consistent with ROCK’s role in smooth muscle contraction. Thus, the benzoxaborole moiety represents a novel hinge-binding kinase scaffold that may have potential for therapeutic use.

Introduction

During the course of investigating the activities of benzo- xaborole compounds, we identified a subset with shared structural features, a common spectrum of activity, and a similar mechanism of action. These compounds and their improved analogs were discovered to be Rho-activated kinase (ROCK) inhibitors.

ROCK was first characterized by Matsui et al. (1996) as a Rho-regulated kinase from the AGC subfamily of Ser/Thr kinases (Olsen, 2008; Pearce et al., 2010). Two genes encode two highly related enzymes, ROCK1 and ROCK2, which are both expressed in many types of tissues (Mueller et al., 2005). Both enzymes contain an N-terminal kinase domain, a coiled-coil domain associated with dimerization, a mid-protein Rho-binding domain at the C terminus, a membrane-association Pleckstrin homology domain, and a further C-terminal cysteine-rich domain that binds lipid substrates (Mueller et al., 2005; Pearce et al., 2010). Binding of Rho-GTP to the Rho-binding domain deactivates autoinhibition, allowing the kinase to phosphorylate its substrate. At least 11 different substrates have been demonstrated for ROCK kinases, many of which are involved in the regulation of the myosin–actin skeleton and cell adhesion and motility (Matsui et al., 1998; Ivetic and Ridley, 2004; Haas et al., 2007; Pearce et al., 2010). The most well characterized substrate is myosin phosphatase-1 (MYPT1), which activates myosin and increases myosin fiber contraction; these actions lead to tonic smooth muscle contraction in the vasculature and lungs (Uehata et al., 1997).

The first synthetic kinase inhibitor to be approved for therapeutic use was a ROCK inhibitor, fasudil [HA-1077 (5-(1,4-diazepane-1-sulfonyl)isoquinoline], which was approved...
in 1995 for cerebral venospasms secondary to aneurysm (Shibuya et al., 1992; Ono-Saito et al., 1999). This compound was discovered while examining calmodulin inhibitors of the naphthalene-sulfonamide family, a group that was found to inhibit AGC family kinases. ROCK was subsequently identified as the highest affinity target of these compounds (Takayasu et al., 1986; Uehata et al., 1997; Ono-Saito et al., 1999). Other clinical studies have shown that fasudil induces beneficial outcomes in angina (Shimokawa et al., 2002) and pulmonary hypertension (Fukumoto et al., 2005, 2007). Trials for the use of ROCK inhibitors in glaucoma have been initiated with five different compounds (Chen et al., 2011); however, early studies have shown that inadequate pressure lowering and hyperemia (redness) might limit the application of fasudil (Tanihara et al., 2008; Williams et al., 2011).

Human tissue and animal studies have shown that ROCK inhibitors promote wound healing (Bond et al., 2011), improve spinal cord injury recovery (Impellizzeri et al., 2012), and reduce neuropathic pain (Mueller et al., 2005). Human genome-wide association studies connected the ROCK pathway with memory defects and Alzheimer’s disease, which then led to the demonstration that ROCK inhibitors improve memory in aged rats (Huentelman et al., 2009). Cardiac hypertrophy and fibrosis in mice and rats are reduced by ROCK inhibitors through a mechanism that may involve ROCK-mediated phosphorylation of PTEN (phosphatase and tensin homolog)-phosphatase and subsequent activation of the Akt pathway (reviewed by McKinsey and Kass, 2007). Together, these findings indicate that ROCK inhibitors may be beneficial for several significant therapeutic applications.

Inflammation is strongly affected by ROCK inhibitors, because cytoskeletal reorganization is significant for secretion of inflammatory products from the cell, formation of the immune synapse, and chemotaxis (Hogg et al., 2003; Takesono et al., 2010). Indeed, the beneficial effects of fasudil on cerebral venospasm are associated with reduced migration and a reduced number of neutrophils at lesion sites (Sato et al., 1999). Inflammation suppression is part of the mechanism that generates the beneficial effects of ROCK inhibitors on spinal cord injuries (Impellizzeri et al., 2012) and arthritis (He et al., 2008). Given the effects of ROCK inhibitors on smooth muscle contraction and inflammation, which are a consequence and cause of asthma, respectively, asthma is an obvious application for ROCK inhibitors. In mice, guinea pigs, and human tissues, inhaled and systemic administration of ROCK inhibitors blocks eosinophilia, airway contraction, and hyper-responsiveness, which are endpoints associated with asthma suppression (Yoshii et al., 1999; Iizuka, et al., 2000; Hashimoto et al., 2002; Henry et al., 2005; Kume, 2008; Schaafsm et al., 2008a,b).

In this article, we describe the utilization of modern medicinal chemistry and chemogenomic approaches to elucidate the mechanism of certain benzoaxaborole compounds and identify the kinase target as Rho kinase. We demonstrate the activity of the compounds in biochemical, cellular, tissue, and in vivo assays. We also provide a crystal structure for one of the compounds in the active site of ROCK2.

### Materials and Methods

The syntheses of compounds 1–10 were previously described (Xia et al., 2011; Akama et al., 2013). Compounds 11–17 were synthesized in a manner similar to that for compound 5. Compounds 16 and 17 were separated via chiral preparative high-pressure liquid chromatography (HPLC); the absolute configuration for each enantiomer was not determined. Y-39983 [(R)-4-(1-aminooethyl)-N-(4H-pyrrolo[2,3-b]pyridin-4-yl)benzamide] was synthesized following a published procedure (Arita, et al., 1995). Fasudil and hydroxyfasudil were obtained from EMD Merck KGaA (Darmstadt, Germany). All lots of compounds had a purity >95%, as assessed by HPLC and 1H nuclear magnetic resonance.

### Animals

Sprague-Dawley rats were purchased from Charles River Laboratories (Hollister, CA). Spontaneously hypertensive rats were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). Tissue isolation from Wistar rats and Dunkin-Hartley guinea pigs was from animals obtained from MDS Pharma Services Taiwan Ltd. (Taipei, Taiwan). All animal studies were performed with approval from the institutional animal care and use committees in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

#### Cytokine Secretion Inhibition

Human peripheral blood mononuclear cells (PBMCs) were stimulated and treated with different compounds to measure the inhibitory effects on cytokine secretion, as described by Akama et al. (2013) and Dong et al. (2013). In brief, PBMCs were incubated with the following drugs and stimulators: lipopolysaccharide (LPS) for 24 hours to release tumor necrosis factor-α (TNF-α) and interleukin (IL)-6; phyoehemagglutinin (PHA) for 24 hours to release IL-2 and interferon-γ (IFN-γ); and PFA for 48 hours to release IL-6 and IL-13. The cell culture supernatants were collected for cytokine determination using homogeneous time resolved fluorescence cytokine determination kits (Casia Biosays, Codolet, France). IC50 values were determined from dose-response curves defined with an eight-point 1:10 dilution series starting at 10 or 100 µM and were fit to the four-parameter sigmoidal equation.

#### Jurkat Cell Growth

Log-phase Jurkat cells (Clone E6-1, TIB-152; American Type Culture Collection, Manassas, VA), 2 × 104 cells/well, were cultured for 3 days at 37°C in the presence and absence of drugs. Cell growth was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) mitochondrial reduction assay. Under these conditions, untreated Jurkat cells completed at least two division cycles in 3 days. The percent inhibition or dose-response IC50 values were calculated in the same manner as the cytokine assays listed previously.

#### Kinase Binding Panel

Kinase inhibition potency was measured using KINOMEscan technology across 402 human kinases (DiscoverX, Inc., Fremont, CA). This competitive binding assay quantitatively measures the ability of a compound to compete with an immobilized, active site-directed ligand for binding to a DNA tagged kinase molecule (Fabian et al., 2005). Kinase ligand affinity beads labeled with an immobilized active site-directed ligand, DNA-labeled kinases, and test compounds were combined in binding buffer [20% SeaBlock, 0.17 × phosphate-buffered saline, 0.05% Tween-20, 6 mM dithiothreitol (DTT)] for 1 hour at room temperature. The beads were washed and then resuspended in elution buffer (phosphate-buffered saline, 0.05% Tween 20, 0.5 µM nonbiotinylated affinity ligand) for 30 minutes. The kinase concentrations in the eluates were measured using quantitative polymerase chain reaction.

#### Kinase Phosphorylation Assays Using the Select Panel

Kinase phosphorylation assays were performed in collaboration with Reaction Biology (Malvern, PA) (Ma et al., 2008). The ROCK1 enzyme tested was gluthathione S-transferase GST[1-535] (GenBank accession number NP_005397), and the tested ROCK2 enzyme was GST [5-554] (GenBank accession number NP_032705). The buffers contained 20 mM HEPES, 10 mM MgCl2, 2 mM DTT, 0.02 mg/ml bovine serum albumin, 0.1% dimethylsulfoxide (DMSO), 1 mM EGTA, 0.02% Brij-35, 10 µM ATP, and [γ-32P]ATP. Appropriate substrates and activators were provided in each assay; for ROCK, 20 µM S6-peptide (32 amino acids in length) was used. Each enzyme was preincubated with a test compound for 15 minutes, and the reaction was initiated by the addition of ATP. The samples were incubated at room temperature for 120 minutes and were then applied to P81 ion exchange paper and washed.
used the procedures of Garton et al. (2008) with minor modifications. PANC-1 cells (CRL-1469; American Type Culture Collection), 18 × 10^6 cells/well, were grown overnight at 37°C in Dulbecco’s modified Eagle’s media supplemented with 4.5 g/l glucose, 1-glutamine, sodium pyruvate, and 10% fetal bovine serum. The test compounds were applied to the cells for 1 hour in growth media (final DMSO concentration of 1%), and the cells were then lysed. Phosphorylated-MYPT1 was detected using plate-immobilized antibody for MYPT1 (BD Biosciences, Franklin Lakes, NJ), secondary antibody directed to phospho-MYPT1 (T853) (M9925-08; US Biological, Swampscott, MA), and tertiary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (111-035-003; The Jackson Laboratory, Bar Harbor, ME). Phospho-MYPT1 levels were quantified based on the color formed by tetramethylbenzidine–horseradish peroxidase substrate.

**Rat Aorta Smooth Muscle Relaxation.** To link our cellular findings to tissue-level effects, rat aorta smooth muscle relaxation was assessed using the robust model in rat thoracic aortas, as outlined by Eltze and Boer (1992). Thoracic aorta tissues were isolated from adult Wistar rats in oxygenated Krebs-Ringer buffer at 37°C. The assays were initiated from a resting tension of 1 g. Agonist mode, contraction testing, and antagonist mode relaxation testing were performed for all compounds, which were diluted from DMSO stocks to a final concentration of 0.5% DMSO. Relaxation testing was performed in tissues precontracted with 1 μM norepinephrine. This dose is approximately 8-fold the norepinephrine contraction EC50. The test compounds were applied for 5 minutes before the tension values were measured. Two separate dose-response curves were constructed, and the curve-fit asymptotic standard error is reported for the IC50 measurement.

**Trachea Smooth Muscle Relaxation.** Trachea smooth muscle relaxation was assessed using guinea pig tracheas, as detailed by Wasserman and Griffin (1977). Guinea pig trachea tissues were isolated from adult Dunkin-Hartley guinea pigs. The samples were assayed in Krebs solution with 2.8 mM HCO3−, 10% DMSO (pH 5.7) or by oral gavage using a 10 mg/kg dose in 84:16 water/cyclodextrin (pH 4.6). Blood samples were collected and analyzed for drug content using HPLC coupled to tandem mass spectrometry (Dong et al., 2013). In Vivo Blood Pressure Testing in Spontaneously Hypertensive Rats. Male adult, spontaneously hypertensive rats (300–400 g; n = 3 rats per group) were anesthetized with ketamine prior to implanting radio frequency transmission and heart rate (HR) monitors (model No. C50-PXT; Data Sciences International, St. Paul, MN) into their abdomens. The pressure catheters were inserted into the descending aorta below the renal artery and secured to their abdominal musculature. Two ECG leads per rat were placed intracutaneously, one at the right shoulder and the other at the lower left chest. The animals were individually housed and allowed to recover for at least 3 days. Compound 5 (600 mg/kg) and vehicle, losartan [10 mg/kg, a dose widely reported to cause robust blood pressure (BP) changes in rats] and vehicle, or vehicle [15% cyclodextrin in water] was administered to each rat by oral gavage. BP and HR data were recorded continuously for 48 hours (Data Sciences International). The animals were fully conscious for all dosing and recording procedures. All of the animal dosing occurred at approximately 0900 (based on a 7:00AM to 7:00 PM light cycle). BP and HR were analyzed at 10-minute intervals and the hourly means were plotted. Independent t tests compared the BP and HR values after treatment with AN3485 and losartan with the vehicle control. This study and a similar investigation in normal rats were conducted by WuXi AppTec ( Suzhou, China), in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care International as well as the People’s Republic of China Ministry of Science and Technology.

**ROCK2–Compound 4 Cocrystallization and Structure Determination.** ROCK2–compound 4 complexes were crystallized and the structure was determined by Proteos Biostructures (Martinsried, Germany). ROCK2 residues 27–417 (GenBank accession number 3327051) were expressed in Sf9 insect cells, and ROCK2 protein was purified by affinity and gel filtration, yielding homogenous protein with >95% purity, as assayed via Coomassie Brilliant Blue staining and SDS-PAGE analyses. The enzyme was cocrystallized with compound 4 from solutions that comprised 9 mg/ml ROCK2 and 2 mM compound 4 (AN3484, 6-(4-amino phenoxyl)-2-fluorophenoxylbenzoic[1,2][oxaborol-1(3H)-ol]. The cocrystallization buffer contained 1.2 M sodium citrate and 2 mM DTT at pH 6.0. The X-ray diffraction (XRD) spectroscopy data were collected from the ROCK2-compound 4 co-crystals at the Swiss Light Source synchrotron facility (Villigen, Switzerland) using cryogenic conditions. The crystals belong to space group C2 and diffracted to 2.79 Å. The data were processed using XRD spectroscopy and Scala programs. The crystal existed in a space group with a unit cell unique reflections with a multiplicity of 25, completeness of 95.7%, an Rsym of 6.5%, an Rmerge of 9.2%, an R(I) of 6.5, and a mean (I/S.D. of 7.9. Residues 391–393 were not defined in the structure and are omitted from the structure deposited in the Protein Data Bank as 4L6Q. Results

**Initial Identification of ROCK Inhibitors.** While studying the activities of a novel class of drugs, benzoxaboroles, various screening assays indicated that the positional isomers of (aminomethylphenoxy)benzoxaboroles, including compounds 1–3, had moderate potency as inhibitors of cytokine secretion. In human leukocyte cells, cytokine secretion stimulated by LPS or by surface receptor cross-linking was inhibited with potencies in the range of 0.4–13 μM for TNF-α, IL-6, IL-2, IFN-γ, IL-5, and IL-13 (Fig. 1A). These compounds do not halt the growth of human Jurkat cell lines, for which the IC50 values were greater than 25 μM (Fig. 1A). Additional exploratory structure–activity relationship (SAR) studies implied that the primary amine was critical, because the desaminophase lacking activity in all performed assays. Furthermore, the boron atom was essential, because the corresponding carbon analog of compound 3 [6-(3-(aminomethylphenox)-)2,3-dihydro-1H-inden-1-ol] was completely inactive in the cytokine secretion assays. These phenotypic results stimulated further mechanistic investigations.

Consideration of the chemical structure requirements to preserve both the primary amine and the benzoxaborole moiety and the importance of kinase signaling in immune cell activity and cell division regulation led us to speculate that these compounds might be interacting with a kinase. Therefore, we tested compounds 2, 3, and 4 using an Ambit 402 human kinase binding panel (Ambit Biosciences Corporation, San Diego, CA). This platform measures the ability of test compounds (10 μM) to inhibit the binding of an ATP active
site-directed ligand to 402 recombinant human kinases (Fabian et al., 2005). To guard against false positives, we selected three structurally related molecules from the set of initial interesting compounds and investigated the inhibition effects on kinases in the top 3% of all 402 kinase activities measured (12 of 402 kinases in the panel). Figure 1B indicates that ROCK1, ROCK2, cyclin-dependent kinase-like 5, phosphatidylinositol 4-kinase, and p38-γ were early kinase candidates with specific interaction sites for our (aminomethylphenoxy)benzoxaborole analogs. The activities of compounds 2, 3, and 4 against ROCK1 and ROCK2 were reconfirmed using kinase binding and phosphorylation assays at 10 μM; however, in these assays, no significant inhibition of cyclin-dependent kinase-like 5, p38-γ, and phosphatidylinositol 4-kinase β was observed with these three molecules. Thus, the latter three kinases were considered false positives. Further screening via phosphorylation assays using ROCK1 and ROCK2 indicated that compound 4 was consistently the most active, compound 2 was less active, and compound 3 was significantly less active in the series, with IC₅₀ values ranging from 0.5 to 30 μM (Table 1). In addition, we measured the inhibitory activity of compound 5, which showed comparable

![Table A](image)

![Table B](image)

![Figure 1](image)
potency to compound 4 against ROCK1 and ROCK2 enzymatic activity. These studies showed that inhibition was competitive with ATP for both enzymes, exhibiting $K_i$ values of 1.78 ± 0.05 μM and 1.18 ± 0.13 μM for ROCK1 and ROCK2, respectively (Fig. 1C).

**ROCK2–Compound 4 Cocrystal Structure Determination.** We determined the cocrystal structure of ROCK2 with compound 4 bound. The amino acid residues forming the active site and the ligand were well defined in the electron density map. The interpreted XRD data exhibited a clear binding mode and unambiguous ligand orientation and conformation (Fig. 2). The benzoxaborole ring system lies sandwiched between the hydrophobic residues Leu221 and Met172 (below) and Val106 and Ala119 (above). The phenoxy ring rests against the backbone of Ala231 and Asp232, with the fluorine jutting up into a pocket created by Val106 and the d and e carbons of Lys121. The aminomethyl group of compound 4 occupies the magnesium cofactor site, satisfying an ionic interaction with Asp232. Ligand compound 4 forms four hydrogen bonds: two to the main chain atoms of Met172 and Glu170 and two to the side chain atoms of Asn219 and Asp232. Several residues are within 3.9 Å of compound 4 and form a direct contact shell with the ligand (Ile98, Val106, Ala119, Lys121, Val153, Met169, Glu170, Tyr171, Met172, Asp218, Asn219, Leu221, Asp232, and Phe384) (Fig. 2). The boron atom and the three atoms bound to it were constrained to planarity. A space for a fourth boron ligand was not observed either above or below the ring system. Therefore, the boron atom is sp2 hybridized for its interaction with the ROCK2 active site.

**In Vitro SAR Studies against ROCK1/2.** A small-scale medicinal chemistry campaign was initiated with the objective of improving the activity against ROCK1/2. The inhibitory activities of the benzoxaborole analogs are summarized in Table 1. Para-aminomethyl analog compound 2 showed IC$_{50}$ values of 9.7 and 6.5 μM against ROCK1 and ROCK2, respectively. This compound was 4- to 6-fold more potent than meta-aminomethyl analog compound 3. Compound 1 has a 4- (aminomethylphenoxy) group attached to the 5-position of the benzoxaborole core and exhibited comparable activity to that of compound 2. Because the 6-position analog (compound 2) showed a better PK profile than the 5-position analog (compound 1) (data not shown), we explored more 6-position analogs. Conversion of the primary amine of compound 2 to a tertiary amine (compound 6) abolished its activity. When halogen atoms, such as fluorine (compound 4) or chlorine (compound 5), were installed into the phenoxy group of compound 2, the IC$_{50}$ values against both ROCK1 and ROCK2 improved 12- to 19-fold to the submicromolar range. The carboxylate (compound 7),

**TABLE 1**

<table>
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<th>Compound</th>
<th>R$^4$</th>
<th>R$^3$</th>
<th>R$^2$</th>
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<th>5/6</th>
<th>R$^7$</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
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</tr>
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<td>O</td>
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<td>H</td>
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</tr>
<tr>
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<td>O</td>
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<td>F</td>
<td>O</td>
<td>6</td>
<td>Me</td>
<td>0.54</td>
</tr>
<tr>
<td>17 (*enant. 2)</td>
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<td>O</td>
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</table>

enat., enantiomer.
analog (compounds 8 and 9), and thioether (compound 10) analogs did not demonstrate any activity. Fasudil and its active metabolite, hydroxyfasudil, had IC\textsubscript{50} values between 0.12 and 0.19 \(\mu\)M, respectively.

The crystal structure analysis guided modifications to the 7-position of the benzoxaborole (Table 1). The oxaborole serves as a hydrogen bond donor-acceptor pair, interacting with the ROCK2 active site hinge region with a trigonal planar conformation. Benzoxaboroles can adopt a tetrahedral conformation when interacting with biologic molecules, such as leucyl tRNA synthetase (Rock et al., 2007) and phosphodiesterase (Freund et al., 2012). Thus, increasing the pKa of the boron atom should stabilize a trigonal planar conformation, strengthening the interaction with the hinge. In addition, a vacant hydrophobic pocket around the 7-position allows space that might benefit by filling. As expected, electron-donating groups, such as methyl (compound 11) and ethyl (compound 12) moieties, at the 7-position enhanced the potency, reducing the ROCK1/2 IC\textsubscript{50} values to between 0.25 \(\mu\)M and 0.34 \(\mu\)M. In contrast, the 7-chloro analog (compound 13) reduced the potency relative to the unsubstituted compound 5, suggesting that the electron withdrawing chloro group lowers the pKa of boron, making the trigonal planar conformation less favored.

We next installed a methyl group on the benzylic position of the aminomethyl groups of compounds 5 and 11, generating compounds 14 and 15, respectively. These compounds showed similar activity to that of compound 11. The two enantiomers of compound 15 were separated and tested. Enantiomer-2 (compound 17) was 3-fold more potent than enantiomer-1 (compound 16).

**Selectivity.** As shown with the 402 human binding panel assay results, the selectivity of the base (aminomethylphenoxo)benzoxaborole chemical structure family is excellent and comprises approximately 75% of the human kinome, including all subfamilies (Fig. 1B). The selectivity was further tested for several of the highest-affinity compounds against members of the ROCK AGC family using a selection that covers all of the major AGC subfamilies (Pearce et al., 2010) (Table 2). These studies showed that one of the most active compounds (compound 17) is selective against the family, with the next best activity on PKA and with a ratio of 15-fold for ROCK1 and ROCK2.

**Intracellular Activity.** ROCK enzymes have been characterized to phosphorylate at least 11 different substrates (Pearce et al., 2010). MYPT1 is one of the better characterized substrates and is partially responsible for the well described regulation of actomyosin skeleton contraction by ROCK. PAN-1 cells exhibit a high basal level of ROCK activity and endogenous MYPT1 phosphorylation without the requirement of cell stimulation (Garton et al., 2008). In preliminary studies, receptor agonists that would generally be required to stimulate ROCK activity did not further increase ROCK activity in PAN-1 cells (data not shown). We examined the inhibitory effects of a number of compounds on intracellular MYPT1 phosphorylation and compared their activities to the biochemical inhibition of ROCK1 in PAN-1 cells. As shown in Fig. 3, a good correlation (\(R^2 = 0.52\)) was observed between these activities across the 11 measured compounds, including both benzoxaboroles and several literature standards. Although the activities were well correlated across the SAR series, a 90-fold shift in intracellular versus biochemical activity was observed.

**Correlation between ROCK Activity and Cytokine Secretion Inhibition.** Initially, we were attracted to the (aminomethylphenoxo)benzoxaborole compounds by their simple scaffold (e.g., compounds 2 and 3) and their suppression of cytokine release activity from stimulated PBMCs (Fig. 1A). Because our medicinal chemistry efforts afforded higher-potency ROCK inhibitors, cytokine suppression failed to increase in proportion (Fig. 4). The distinct grouping of the less potent ROCK inhibitors (Fig. 4, lower oval) indicates that these compounds are both ROCK inhibitors and cytokine suppressors by a mechanism that is distinct from selective ROCK inhibition. In contrast, potent and selective ROCK inhibitors, including compound 17, hydroxyfasudil, Y-27632 [(1R,4R)-4-(1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide], and Y-39983 [(R)-4-(1-aminoethyl)-N-(1H-pyrrolo[2,3-b]pyridin-4-yl)benzamide], are weaker cytokine suppressors relative to their ROCK activity and possess distinctly different SARs (Fig. 4, upper oval).

**Tissue Activity.** To better characterize the activity of ROCK on the smooth muscle beds associated with cardiovascular disease and asthma, we measured the ability of compound 4 to contract and relax aorta and trachea tissues, as listed in Tables 3 and 4. Rat aortas and guinea pig tracheas

### Table 2

Selectivity of compounds for ROCK1 and ROCK2 and versus several representative members of the AGC family of Ser/Thr kinases.

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Compound 4</th>
<th>Compound 14</th>
<th>Compound 11</th>
<th>Compound 15</th>
<th>Compound 16</th>
<th>Compound 17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} nM</td>
<td>IC\textsubscript{50} nM</td>
<td>IC\textsubscript{50} nM</td>
<td>IC\textsubscript{50} nM</td>
<td>IC\textsubscript{50} nM</td>
<td>IC\textsubscript{50} nM</td>
</tr>
<tr>
<td>ROCK1</td>
<td>730</td>
<td>5</td>
<td>510</td>
<td>3</td>
<td>320</td>
<td>3</td>
</tr>
<tr>
<td>ROCK2</td>
<td>340</td>
<td>4</td>
<td>430</td>
<td>3</td>
<td>240</td>
<td>3</td>
</tr>
<tr>
<td>Selectivity Ratio</td>
<td>20</td>
<td>&gt;39</td>
<td>11</td>
<td>18</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>(Next Best/Rock1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKT1</td>
<td>24,063</td>
<td>&gt;50,000</td>
<td>1</td>
<td>6584</td>
<td>1</td>
<td>24,160</td>
</tr>
<tr>
<td>GRK2</td>
<td>&gt;50,000</td>
<td>&gt;20,000</td>
<td>1</td>
<td>&gt;20,000</td>
<td>1</td>
<td>&gt;20,000</td>
</tr>
<tr>
<td>PKA</td>
<td>14,725</td>
<td>&gt;50,000</td>
<td>1</td>
<td>2658</td>
<td>1</td>
<td>7040</td>
</tr>
<tr>
<td>PKC\textsub{a}</td>
<td>&gt;30,000</td>
<td>&gt;20,000</td>
<td>1</td>
<td>&gt;20,000</td>
<td>1</td>
<td>&gt;20,000</td>
</tr>
<tr>
<td>RSK1</td>
<td>15,458</td>
<td>&gt;50,000</td>
<td>1</td>
<td>3621</td>
<td>1</td>
<td>8836</td>
</tr>
</tbody>
</table>

GRK2, G protein-coupled receptor kinase 2; PKC\textsub{a}, protein kinase Ca; RSK1, ribosomal S6 kinase 1.
were not contracted by compound 4 at concentrations ≤500 μM. For aorta that were precontracted with 1 μM norepinephrine, compound 4 relaxed the aorta tissue, with an IC\text{50} value of 154 μM (Table 3). This potency was similar to its MYPT1 phosphorylation blockade IC\text{50} value of 68.4 μM in PANC-1 cells (Fig. 3). Guinea pig tracheas that were precontracted with carbachol were completely relaxed with 500 μM compound 4 and partially relaxed with 100 μM compound 4 (Table 4).

**PK in Rats.** In preparation for in vivo studies, we examined the PK of compounds 4 and 5 in rat plasma. Studies using 10 mg/kg PO or s.c. doses and 2 mg/kg i.v. doses were conducted. Compound 4 exhibited very rapid and unmeasurable clearance after an intravenous dose was administered. In contrast, compound 5 exhibited a lower clearance of 10,600 ± 2800 ml/kg per hour, with a V\text{ss} of 3150 ± 890 ml/kg per hour after intravenous administration. Consistent with this high clearance, compound 4 exhibited very low exposures with both oral and subcutaneous administration from a saline/PEG400/DMSO solution (44:46:10), demonstrating area under the curve (AUC\text{0–\infty}) values of 0.194 and 0.324 μg·h/ml, respectively, and an unmeasurably low bioavailability. Compound 5 achieved oral and subcutaneous exposures of AUC\text{0–\infty} values of 0.456 μg·h/ml and 1.19 μg/ml per hour from a saline/PEG400/DMSO solution (55:35:10). The bioavailabilities were 45% via the oral route and 120% via the subcutaneous route. Bioavailability and exposure via the oral route could be increased using a water-cyclodextrin solution (84:16) at pH 4.9. This vehicle allowed an AUC\text{0–\infty} of 1.09 ± 0.28 μg·h/ml, a terminal half-life of 1.32 ± 0.34 hours and a bioavailability of 107%. The cyclodextrin oral formulation was selected for further studies.

**In Vivo Activity.** The control of smooth muscle contraction is a feature of the ROCK pathway, and in vivo ROCK inhibition should induce smooth muscle relaxation. To demonstrate that smooth muscle relaxation can be mediated by compound 5 in vivo, we measured BP in spontaneously hypertensive rats and in normotensive rats. A dose of 600 mg/kg was selected based on calculations of the likely plasma concentration at this dose, scaling linearly from the 10 mg/kg PK study. By use of the 145 μM C\text{max}, 8.3 μM C\text{avg}, and the 1–2 μM affinity of compound 5 from the biochemical tests, we estimated that the ROCK enzymes could be inhibited by 85 to 99% during the first few hours of compound exposure. In addition, we noted large shifts between the biochemical affinities and those observed in the cell and tissue assays, and thus chose a higher dose for the in vivo testing. Spontaneously hypertensive rats exhibited substantial BP decreases of 14% at 8 hours and 23% at 18 hours (Fig. 5). Lowered BP can lead to a reflex increase in HR, which we observed with...
compound 5, increasing the heart rate by 18% at 8 hours and 12% at 18 hours. Angiotensin receptor antagonist losartan, a well characterized BP-lowering agent, reduced spontaneously hypertensive rat BP by a similar magnitude, but without the reflex HR increase. The vehicle control animals did not exhibit changes in either BP or HR.

Discussion

Biologic activity studies of benzoxaboroles have been ongoing. Compounds from this class have led to several clinical candidates as inhibitors of leucyl aminoacyl-tRNA synthetase for treating fungi (Rock et al., 2007) and Gram-negative bacteria (Hernandez et al., 2013). Other compounds are phosphodiesterase inhibitors used for treating atopic dermatitis (Freund et al., 2012) or trypanosome growth inhibitors (Nare et al., 2010) used for treating African sleeping sickness. Our initial interest in the (aminomethylphenoxy)benzoxaborole family was stimulated by the inhibition of cytokine secretion in Toll-like receptor–stimulated PBMCs (Fig. 1A). Our mechanistic investigations used the SAR information in Fig. 1A and knowledge of kinases in cell signaling to hypothesize that a kinase might be the target. To investigate this possibility, we used a kinase screening panel developed by (Fabian et al., 2005). To reduce the probability of pursuing false positives, we restricted further tests to hits that were common to three structurally related analogs and to the top 3% of all kinase hits. These studies identified ROCK1/2 as a high-affinity kinase target from the 402 enzymes assayed, which represents approximately 75% of the human kinome (Fig. 1B).

SAR and Crystal Structure Data. On the basis of ROCK target identification, compound 5 was determined to be a competitive inhibitor with respect to ATP for both ROCK1 and ROCK2, with $K_i$ values in the 1–2 μM range (Fig. 1C). The binding mode was investigated after preparing a cocrystal of compound 4 bound to ROCK2 and determining its structure. The overall arrangement of compound 4 adopted a hinge-binding conformation, which has been observed for several other ROCK inhibitors and other protein kinase inhibitors (Fig. 2) (Engl et al., 1996; Mohammadi et al., 1997; Jacobs et al., 2006; Ikuta et al., 2007; Wagner et al., 2009; Tesmer et al., 2010; Lin et al., 2012). The structure revealed that the boron atom is in the trigonal planar conformation as opposed to the tetrahedral configuration. This is the first example of an oxaborole analog that acts as a hydrogen bond donor/acceptor pair to interact with a kinase hinge region. The region around Asp232, which in the magnesium/ATP-bound form would contact the β-γ bridging magnesium ion, is the interaction site of the amino group. This region is large enough to allow the addition of a methyl group to the aminomethyl group of compound 17. Thus, the benzoxaborole moiety is a novel type of hinge-binding motif and a new kinase-interacting scaffold.

Selectivity. Comparing the cocrystal structures of ROCK2–compound 4 and the other AGC family kinase members enables the understanding of the selectivity shown in Table 2 (Jacobs et al., 2006; Ikuta et al., 2007; Wagner et al., 2009; Tesmer et al., 2010; Lin et al., 2012). For example, the PKA active site amino acid difference of Ala231 in ROCK2 and Thr183 in PKA may explain the reduced PKA affinity of several benzoxaborole ROCK inhibitors and other protein kinase inhibitors (Ikuta et al., 2007; Wagner et al., 2009; Tesmer et al., 2010; Lin et al., 2012). The structure revealed that the threonine residue in PKA clashes with the 6-position carbon of compound 4, reducing the affinity. The slightly smaller alanine residue in ROCK does not clash with the 6-position carbon of compound 4 allowing better affinity. Jacobs et al. (2006) proposed a similar role for this residue using a ROCK1–Y-27632 complex. AN3484 shows selectivity against other kinases (Table 2). Bulkier polar residues residing in the other kinases with either a threonine (AKT1, PKA, ribosomal S6 kinase 1) or serine (G protein-coupled receptor kinase 2) are consistent with their lower affinity for AN3484.

Cellular Activity. Cellular activity was assessed after measuring MYPT1 phosphorylation in PANC-1 cells. Comparisons of several benzoxaborole ROCK inhibitors and other types of inhibitors showed that the rank order and correlation between cellular potency and biochemical potency was good ($R^2 = 0.52$) (Fig. 3), which is consistent with the intracellular target being ROCK. Although the activities rank compounds similarly...
across the various scaffold series, there is a 90-fold shift in intracellular activity compared with biochemical activity; this shift is common to the benzoxaboroles and other inhibitor scaffolds, such as pyridine scaffold members Y-27632 or fasudil (Fig. 3). PANC-1 cells have a high basal activation of Rho, and the resultant continuous activation of ROCK may require a very large fractional inhibition of ROCK to achieve a substantial inhibition of endogenous MYPT1 phosphorylation (Garton et al., 2008). This effect may reduce the PANC-1 potency of ROCK inhibitors compared with the biochemical potency.

**ROCK Activity versus Cytokine Secretion.** Our data and that of other researchers have noted that ROCK inhibitors cause the cytokine secretion suppression of TNF-α, IL-6, and IL-1β from human macrophages, PBMCs, and synoviocytes (Doe et al., 2007; Rodriguez et al., 2007; He et al., 2008). The suppression of cytokine secretion by ROCK inhibitors in endothelial cells, leukocytes, and synoviocytes is attributed to the suppression of ROCK-mediated inactivation of IKKβ and inhibition of phosphorylation of the RelA/p65 domain and by induction of IκBα phosphorylation, which leads to its degradation (Anwar et al., 2004; Rodriguez et al., 2007; He et al., 2008). As we improved the activity of ROCK, the suppressive activity against cytokine secretion of TNF-α, IL-1β, IL-6, IFN-γ, IL-4, and IL-13 did not proportionally increase (Fig. 4). We can separate the benzoxaborole compounds into two groups separated by their cytokine suppression activity. Compounds 3, 4, and 5 (Fig. 4, lower oval) are more potent cytokine suppressors than ROCK inhibitors and are approximately 200- to 400-fold more potent cytokine suppression inhibitors than the compounds shown in the upper oval (compounds, 11-17 and Y-39983, Y-27632, hydroxy-fasudil, and fasudil), which are all more potent ROCK inhibitors. These data suggest that these three compounds possess additional activities that are distinct from ROCK, which lead to the suppression of cytokine secretion; further studies may lead to the understanding of the distinct activities of these three benzoxaboroles. In contrast, their optimized analogs (e.g., compound 17), as well as other classes of ROCK inhibitors, including pyridine scaffold members Y-27632 or Y-39983 (Fig. 4, upper oval), are more potent ROCK inhibitors and are less effective at cytokine suppression.

**Tissue Activity.** The ability of benzoxaborole to cause smooth muscle relaxation in rat aortas and guinea pig tracheas was determined. Rat aortas were relaxed after compound 4 (AN3484) administration, with a large potency shift of approximately 200-fold (Table 3). Because of the potential application of ROCK inhibitors in asthma, we investigated the effects on tracheal smooth muscle contraction. Guinea pig tracheas that were precontracted with carbachol were also relaxed after compound 4 (AN3484) administration, with an IC50 value of approximately 200 μM and a potency shift between the biochemical and tissue assays of approximately 250-fold (Table 4). Doe et al. (2007) observed a large (approximately 20-fold) potency shift when examining the relationship between rat aorta relaxation and biochemical inhibition across 18 aminofurazan ROCK inhibitors. Thus, tissues, similar to cells, show large differences in apparent potency between biochemical and other functional endpoints.

**Biochemical, Intracellular, and Tissue Assay Correlation.** The large concentrations of drugs required to cause a MYPT1 phosphorylation decrease in PANC1 cells or in relaxation in the tissues might be attributable to the fact that these inhibitors are ATP active site inhibitors with Ki values in the 1–2 μM range. Cellular ATP generally ranges from 1 to 10 mM (Gupta and Yushok, 1980). Based on the competitive nature of ATP, the magnitude of the ratio between cellular or tissue IC50 and ROCK biochemical IC50 is estimated to be approximately 50- or 500-fold at ATP concentrations of 1 or 10 mM, respectively. Thus, large quantities of drug are required to compete with ATP, and the observed changes of 90- to 250-fold between the cells and tissues are similar to what was expected based on intracellular ATP concentrations.

**In Vivo Effects on BP.** Our investigations of the activities of the oxaboroles were continued by examining the effects of compound 5 (AN3485) on BP in spontaneously hypertensive rats. Consistent with the effect on smooth muscle contraction, we observed decreases in average BP and a reflex increase in HR with compound 5 administration. This system has been used to measure the effects of aminofurazan inhibitors, azaindole, triazines, and the pyridine derivative, Y-27632, with broadly similar findings that include both the effect duration and reflex HR increase (Doe et al., 2007; Kast et al., 2007; Ho et al., 2009). Both the benzoxaborole (compound 5) and losartan positive control caused a prolonged depression in pressure, which was still evident 33 hours after drug administration (25 half-lives for compound 5). The terminal half-life of compound 5 was 1.3 hours; thus, the biologic effect persisted after the drug was cleared. Other ROCK inhibitors and losartan have demonstrated prolonged effects (Morton et al., 1992; Doe et al., 2007; Kast et al., 2007; Ho et al., 2009). Kast et al. (2007) reported that azaindole suppressed the mean arterial pressure at 12 half-lives postdose. Compound 5 and azaindole ROCK inhibitors and losartan cause persistent pharmacodynamic effects due to a long-lived inhibitor-induced change in the basal contraction state of arterial smooth muscle cells. Further studies are required to understand this phenomenon.

While examining the benzoxaborole family, we used phenotypic testing, medicinal chemistry, SAR information, and knowledge of leukocyte biology to hypothesize that (aminomethylphenoxy)benzoxaboroles are kinase inhibitors. A kinome-wide screen identified ROCK as the target for these derivatives. Enzymology studies and the crystallographic determination of a drug–ROCK complex elucidate a mechanism by which benzoxaboroles bind to the kinase hinge region, providing a novel hinge-binding scaffold. The cocrystal structure of the early lead compound with ROCK2 revealed its unique biding mode to the kinase through the oxaborole moiety and the primary amino group. Guided by this structural information, more potent ROCK inhibitors were identified. This class also exhibited cellular and tissue activities consistent with the effects of ROCK on smooth muscle contraction and inflammation. Furthermore, the observed tissue activities translate to BP reduction, as facilitated by oral bioavailability. There are several possible therapeutic applications for ROCK inhibitors, including angina and glaucoma, in which clinical trials have been conducted. In addition, based on animal data, conditions such as pulmonary hypertension, cardiac hypertrophy, asthma, and wound healing may also be areas ripe for application of ROCK inhibitors. The benzoxaborole scaffold provides a highly novel type of kinase inhibitor, which may present new approaches to inhibiting kinase activity. Because ROCK is involved in many disease
processes that include smooth muscle contraction, fibrosis, and inflammation, these compounds may be excellent starting points for the development of novel medicines.

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Authorship Contributions

**Participated in research design:** Akama, Dong, Freund, Rock, Liu, Jarnagin.

**Conducted experiments:** Dong, Virtucio, Sullivan, Bu, Wu, Fan.

**Contributed new reagents or analytic tools:** Akama, Zhang.

**Performed data analysis:** Sullivan, Zhou, Rock, Akam, Jarnagin.

Wrote or contributed to the writing of the manuscript: Akama, Sullivan, Zhou, Jarnagin.

**References**


**Address correspondence to:** Kurt Jarnagin, Anacor Pharmaceuticals, Inc., 1020 E. Meadow Circle, Palo Alto, CA 94303. E-mail: kjarnagin@anacor.com