Discovery of Substituted 4-(Pyrazol-4-yl)-phenylbenzodioxane-2-carboxamides as Potent and Highly Selective Rho Kinase (ROCK-II) Inhibitors

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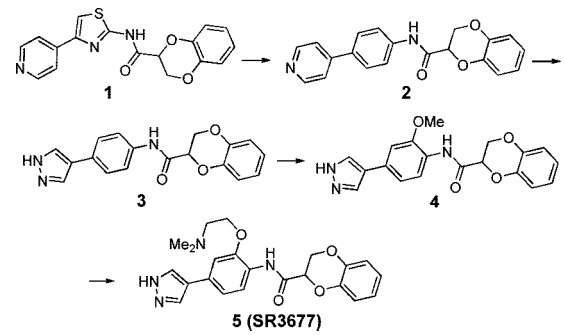
Abstract: The identification of a new class of potent and selective ROCK-II inhibitors is presented. Compound **5** (SR-3677) had an IC_{50} of ~3 nM in enzyme and cell based assays and had an off-target hit rate of 1.4% against 353 kinases, and inhibited only 3 out of 70 nonkinase enzymes and receptors. Pharmacology studies showed that **5** was efficacious in both, increasing *ex vivo* aqueous humor outflow in porcine eyes and inhibiting myosin light chain phosphorylation.

RhoA and its downstream kinase (ROCK) play an important role in the regulation of smooth muscle contraction[1] and neurite growth retraction.[2] The abnormal activation of the ROCK pathway has also been observed in many disorders of the central nervous system.[3] Therefore, the inhibition of ROCK is a promising strategy for the treatment of various diseases such as hypertension,[4] coronary and cerebral vasospasm,[5] erectile dysfunction,[6] glaucoma,[7,8] asthma,[9] multiple sclerosis (MS),[3] atherosclerosis,[10] stroke,[11] and cancer.[12]

Most ROCK inhibitors reported in the literature contain isoquinoline (e.g., Fasudil[a] and H-1152P),[13,14] pyridine (such as Y-27632[15] and Y-39983,[8,16] which is in clinical trials for the treatment of glaucoma[17]), or indazole[18−22] moieties. These compounds are either moderately potent in ROCK biochemical assays (IC_{50} > 100 nM) or have poor cell activities (IC_{50} values in cell-based assays normally in the range of 0.1 μM−10 μM). A promising new class of ROCK inhibitors (ROCK-I) based on aminofurazan-azabenzimidazoles was recently reported.[23] Some compounds in this series were shown to have lower than 100 nM IC_{50} values in cell-based assays.

Our goal was to discover potent ROCK-II inhibitors with IC_{50} values less than 10 nM in biochemical assays and less than 100 nM in cell-based assays. In addition, these inhibitors should be highly selective against other kinases and nonkinase enzymes and receptors (ideally with IC_{50} values >1 μM). An in-house HTS campaign[24] led to the discovery of compound **1** (Chart 1), a pyridine-thiazole based amide compound. Although **1** is a potent ROCK-II inhibitor (Table 1, IC_{50} = 7.2 nM) with good selectivity against a few other kinases, it has a relatively large shift in cell-based potency as assessed by myosin light chain bisphosphorylation (ppMLC) (IC_{50} = 137 nM).[25] The optimization of **1** started by replacing the central thiazole group with a phenyl ring (Chart 1). As shown in Table 1, the potency of the resulting compound **2** was reduced in both enzyme and cell-based assays compared to that of **1**. We reasoned that the pyridine-phenyl system in **2** might have perturbed the favorable geometry that was present in the pyridine-thiazole structure of **1**. A molecule with a 5-membered nitrogen-containing heterocycle linked to the central phenyl ring might mimic the original pyridine-thiazole system. Thus, we prepared compound **3**, which contained a pyrazole to replace the pyridine in **1** as the hinge binding element.

Chart 1. SAR Evolution



The new pyrazole-phenyl scaffold of **3** was even more potent (Table 1) than compound **1**. Introduction of additional functionality on the central aryl ring was used to improve the inhibitor's overall pharmaceutical properties and/or to enhance the selectivity. We thus prepared compound **4**. This methoxy substituted ROCK inhibitor showed good selectivity against PKA (~300-fold) and Akt1 (~2700-fold), and its cell potency was also improved (IC_{50} = 30 nM vs IC_{50} = 72 nM in **3**). However, the selectivity against MRCK (IC_{50} = 367 nM), the most closely related kinase to ROCK that we tested, decreased (110-fold vs 790-fold in **3**). Finally, a dimethylaminoethoxy moiety was introduced to replace the methoxy group to give compound **5**. This novel ROCK-II inhibitor showed high potency in biochemical and cell-based assays as well as high selectivity against all kinases tested (Table 1 and Supporting Information). The IC_{50} value of **5** for ROCK-I was 56 ± 12 nM (n = 4). The equal potency between biochemical and cell-based assays for **5** could be due to cell accumulation, differences in cell permeability, or enzyme kinetics. It should be pointed out that compounds **1**−**5** are all racemates.

The synthesis of **5** (Scheme 1) began from 2-fluoro-4-bromonitrobenzene (**6**). Nucleophilic substitution was used to introduce the side chain dimethylaminoethoxy group to provide the nitrobenzene derivative (**7**). This intermediate was then reduced with $SnCl_2$ to aryl amine (**8**). Amide formation on the newly formed amino group was accomplished in DMF using HATU as the coupling reagent in the presence of DIEA to give the bromide derivative (**9**). A Suzuki coupling reaction using $Pd[P(Ph)_3]_4$ as the catalyst in the presence of K_2CO_3 was then

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[a] Abbreviations: Fasudil, 5-(1,4-diazepan-1-ylsulfonyl)isoquinoline; H-1152P, (*S*)-4-methyl-5-(2-methyl-1,4-diazepan-1-ylsulfonyl)isoquinoline; Y-27632, (*R*)-4-(1-aminoethyl)-*N*-(pyridin-4-yl)cyclohexanecarboxamide; Y-39983, (*R*)-4-(1-aminoethyl)-*N*-(1*H*-pyrrolo[2,3-b]pyridin-4-yl)benzamide; *AUC*, pharmacokinetic area under curve; *Cl*, pharmacokinetic clearance; *Cmax*, pharmacokinetic maximum concentration; *F*, oral bioavailability; HLMS, human liver microsomal stability; hERG, human ether-a-go-go; HTS, high-throughput screening; ppMLC, bis-phospho myosin light chain; TM, trabecular meshwork; *Vd*, volume of distribution.

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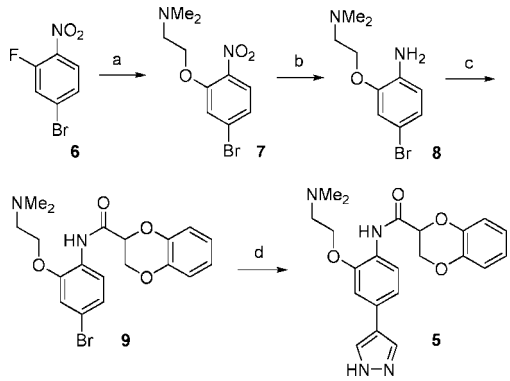
Table 1. Data for Biochemical and Cell-Based Assays

cmpd	ROCK-II IC_{50} (μM)	ppMLC IC_{50} (μM)	kinase IC_{50} (μM)		
			PKA	MRCK	Akt1
1	0.0072 ± 0.0023 ($n = 2$)	0.137 ($n = 1$)[a]	0.637 ± 0.119 ($n = 2$)	4.850 ± 0.212 ($n = 2$)	>20 ($n = 2$)
2	0.046 ± 0.032 ($n = 2$)	0.657 ± 0.119 ($n = 2$)	0.692 ± 0.021 ($n = 2$)	7.050 ± 2.900 ($n = 2$)	6.436 ± 2.834 ($n = 4$)
3	0.0015 ± 0.0007 ($n = 2$)	0.072 ± 0.057 ($n = 4$)	0.186 ± 0.016 ($n = 2$)	1.190 ± 0.057 ($n = 2$)	1.410 ± 0.646 ($n = 4$)
4	0.0033 ± 0.002 ($n = 2$)	0.030 ($n = 1$)[a]	0.940 ± 0.019 ($n = 2$)	0.367 ± 0.188 ($n = 2$)	8.819 ± 2.000 ($n = 2$)
5	0.0032 ± 0.0023 ($n = 9$)	0.0035 ($n = 1$)[a]	3.968 ± 1.680 ($n = 5$)	1.190 ± 0.509 ($n = 2$)	7.491 ± 1.402 ($n = 2$)

[a] The compound was tested as replicates on the plate, and the IC_{50} data was calculated by drawing a curve from both data sets.

Scheme 1. Synthesis of **5**[a]



[a] Reagents and conditions: (a) NaH, THF, 2-(dimethyl-amino)ethanol, rt, 92%; (b) $SnCl_2$, ethanol, 70 °C, 91%; (c) benzodioxane-2-carboxylic acid, HATU/DIEA, DMF, rt, 75%; (d) 4-1*H*-pyrazoleboronic acid, pinacol ester, $Pd[P(Ph)_3]_4$, K_2CO_3, dioxane/H_2O, 95 °C, 35%.

applied to provide **5**. Similar procedures were also used for the preparation of compounds **2**, **3**, and **4**.

The *in vitro* drug metabolism and *in vivo* pharmaco-kinetic properties of these ROCK-II inhibitors were evaluated (Table 2). Four human CYP-450 enzymes (2C9, 2D6, 3A4, and 1A2) were selected for routine screening. The HTS hit compound **1** strongly inhibited all four enzymes. Replacement of the thiazole group with an unsubstituted phenyl ring (compounds **2** and **3**) significantly reduced CYP inhibitions. Addition of a dimethylaminoethoxy group (**5**) to the central phenyl ring eliminated the 3A4 inhibition seen in the original hit **1**. Overall, none of these compounds possessed good *in vitro* drug metabolism and *in vivo* pharmacokinetic properties desired for systemic applications. The inhibitors had either low human liver microsomal stability (HLMS), high clearance (*Cl*), or low *AUC* (i.v.) and *Cmax* (p.o.). In addition, all of the compounds had none or very low oral bioavailability (*%F*). However, as required in a soft drug approach, these properties are desirable for the topical application of a drug for treating glaucoma, which is one of the promising applications for ROCK inhibitors.

In addition to selectivity studies against the kinases listed in Table 1, **5** was also profiled against a large panel of kinases and other nonkinase but therapeutically relevant enzymes and receptors. In the profiling versus 353 kinases (Ambit screen[26]) in single-point inhibition using 3 μM of **5** (Supporting Information), **5** was discovered to hit only 5 other kinases with percentage inhibition greater than 50%: Akt3, Clk1, Clk2, Clk4, and Lats2. All other kinases showed either no or very low (<10%) inhibition. An off-target hit rate of only 1.4% (5 out of 352) is believed to be one among the few best known ATP-competitive kinase inhibitors.[26] In the profiling studies against a panel of 70 nonkinase enzymes and receptors, **5** inhibited only three adrenergic receptors: α_{1a}, α_{1b}, and α_{2a} with 53%, 68%, and 76% inhibition, respectively, at 3 μM inhibitor concentration, which indicates IC_{50} values in the 1 μM range or ~300 times higher than the IC_{50} against ROCK-II in cell-based assays. Furthermore, inhibition of the human-ether-a-go-go (hERG) was only 31% at 3 μM (Supporting Information). All these counter screening results demonstrated that **5** is not only a potent compound but also a highly selective ROCK-II inhibitor.

Cocrystal structures of ROCK with ligands Fasudil and Y-27632 have previously been reported.[27,28] Our docking results of **5** to ROCK-II (Supporting Information) explain well the observed SAR. There are several key binding elements that contribute to the high potency and selectivity of **5**. The best scoring pose (Figure 1) indicated that the pyrazole headgroup binds to the hinge region of the enzyme through two H-bonds: one between the NH of Met-172 and the N of the pyrazole ring and another between the backbone C═O of Glu-170 and the NH of the pyrazole ring. In the phosphate binding region, the amide C═O of **5** forms a hydrogen bond with the side chain NH of Lys-121. A very important H-bonding interaction occurs in the ribose binding region between the protonated tertiary amine of the dimethylaminoethoxy group of **5** and the Asp-176 carboxylate side chain. This interaction is probably the reason for the higher selectivity of **5** compared to that of compounds **1**−**4** (Table 1) in which this H-bond is absent. This specific interaction was observed in the crystal structure of the ROCK-II-Fasudil complex but was not seen in the cocrystal structure of Y-27632 with ROCK-II (bovine) and ROCK-I (human). Thus, we have combined the binding motifs of both Fasudil and Y-27632 into **5.**

It is important to point out that there is a hydrophobic pocket under the P-loop in this enzyme−ligand complex (Supporting Information), and the benzodioxane phenyl ring of **5** is immersed inside this pocket. It is believed that the hydrophobic interaction of the benzodioxane phenyl ring with the hydrophobic surface of this pocket is the dominating factor that contributes to the high potency of **5**. To demonstrate the significance of this interaction, two compounds (**10** and **11**) without the benzodioxane moiety (Figure 2) were synthesized and evaluated. The binding of **10** and **11** to ROCK-II is much weaker (IC_{50} = 920 nM and 760 nM, respectively, compared to 3 nM in **5**). This hydrophobic interaction further explains why the very simple structures **1**, **3**, and **4** have high affinity for ROCK-II.

The effect of **5** on *ex vivo* aqueous humor outflow facility from the trabecular meshwork (TM) of porcine eyes is shown in Figure 3. The results show that continuous exposure of 25 μM **5** increases the outflow facility by 60% at 1 h perfusion, increasing to 70−80% for the 2−5 h time points ($p < 0.01$). This 70−80% increase in outflow is the maximal response that

Table 2. Data for Pharmacokinetics *in Vitro* and *in Vivo*

cmpd	CYP-450s[a] % inh. at 10 μM	HLMS at 2 mg/mL $T_{1/2}$ (min)[b]	*in vivo* rat PK[c] (i.v., 1 mg/kg; p.o., 2 mg/kg)					
			Cl (i.v.) mL/min/kg	$T_{1/2}$ (iv) h	*Vd* (i.v.) L/kg	*AUC* (i.v.) μM-h	*Cmax* (p.o.) μM	*F* (%)
1	96/94/80/90	18	181	0.4	3.7	0.3	0.0	0
2	64/33/51/23	6	161	0.5	5.3	0.3	0.0	0
3	87/40/1/20	20	18	2.2	1.9	2.8	0.02	0.3
4	93/79/36/92	15	11	2.6	1.0	4.3	0.12	0.4
5	80/75/0/46	35	61	0.8	3.0	0.7	0.05	7.0

[a] CYP-450s: 2C9/2D6/3A4/1A2. [b] HLMS: human liver microsomal stability. [c] Data reported is the mean of 3 determinations, and the standard error is within 30% of the mean except for *Cmax*, which is within 50%.

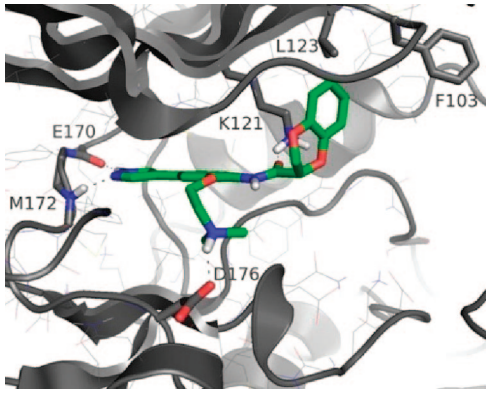


Figure 1. Structure of compound **5** (green) docked in the catalytic domain of the ROCK-II homology model (gray).

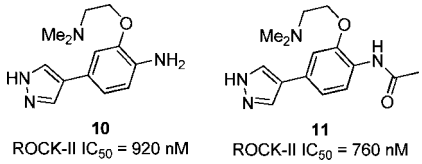


Figure 2. Compounds **10** and **11** and their ROCK-II potency.

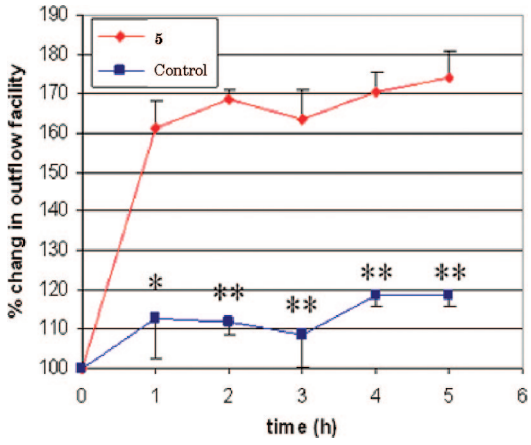


Figure 3. Time course for the aqueous humor outflow facility by 25 μM **5** perfused in enucleated porcine eyes ($N = 3$; average $\pm$ SE; $*p < 0.05$; $**p < 0.01$).

can be attained and is achieved at doses 2−4-fold lower than needed for Y-27632,[29,30] a well studied ROCK inhibitor. The 2−4-fold lower dose of **5** utilized to increase aqueous humor outflow compared to Y-27632 is more modest than one might expect given the difference in biochemical- and cell-based potency between the two compounds and may be due to compound accessibility to the different regions (trabecular meshwork, juxtacanilicular (JCT) area, and Schlemm's canal) of the aqueous outflow pathway. Since lower doses of **5** were not tested, it may be that lower doses of **5** would also be maximally effective in increasing aqueous humor outflow and thus be more reflective of the intrinsic potency differences between **5** and Y-27632. These studies are currently ongoing in our laboratories. These results underscore the important role ROCK plays in aqueous humor outflow and illustrate the promise of this class of compounds for potential intraocular pressure lowering in glaucoma.

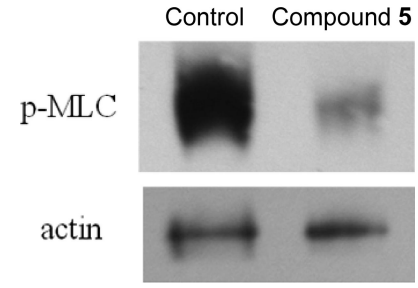


Figure 4. Western blot analysis of p-MLC in the TM tissue derived from the porcine eyes untreated (control) and perfused with 25 μM **5**.

In an effort to ensure that **5** acted through inhibition of ROCK *ex vivo*, we monitored the phosphorylation state of myosin light chain in trabecular meshwork from porcine eyes. Figure 4 presents the Western blot analysis for p-MLC in TM tissue isolated from porcine eyes that were perfused with 25 μM **5** at 15 mmHg for 5 h. The results show a strong cross-reactive band for p-MLC in the control lane indicating large amounts of p-MLC. TM tissue derived from the eyes perfused with 25 μM **5** shows a very faint band for p-MLC suggesting greater than 90% inhibition of Rho kinase in the TM tissue at this dose. Actin immuno-cross-reactivity for both samples showed equal loading of protein between wells. These data strongly support the notion that selective ROCK inhibitors from this scaffold are potent modulators of myosin light chain phosphorylation in TM tissue and correlate nicely with the increased outflow facility seen in these very same porcine eyes (Figure 3).

In summary, we have identified a new class of potent and highly selective ROCK-II inhibitors. These novel compounds possess a pyrazole group that can function as the hinge binding moiety and a side chain with H-bonding capability that can enhance the inhibitor's selectivity. Compound **5** is very potent in both enzyme and cell-based assays with single digit IC_{50} values. Pharmacology studies showed that **5** was efficacious in both increasing aqueous humor outflow and in target modulation−reduction of p-MLC. Further optimizations of this scaffold, including the synthesis and evaluation of each enantiomer of **5**, and more pharmacology studies are underway in our laboratories and will be reported in due course.

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panel screening, and MDS for the nonkinase enzyme and receptor screening. We are also grateful to Professor Patrick Griffin and Professor William Roush for their support. Y. Feng thanks Dr. Yen Ting Chen for reading the manuscript and for the helpful discussion. This work was supported in part by NIH grant R01-EY018590 (P.V.R.).

Supporting Information Available: More docking information for **5**; procedures for biologic testing; and experimental procedures and spectra data for compounds **2**, **3**, **4**, **5**, **10**, and **11**. This material is available free of charge via the Internet at http://pubs.acs.org/instruct/jmcmar.pdf.

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