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Benzothiophene containing Rho kinase inhibitors: Efficacy in an animal model of glaucoma

Robert L. Davis [a,*], Mehmet Kahraman [a,c], Thomas J. Prins [a], Yan Beaver [a,†], Travis G. Cook [a,‡], Jessica Cramp [a,§], Charmagne S. Cayanan [a,¶], Elisabeth M. M. Gardiner [a,‖], Marsha A. McLaughlin [b], Abbot F. Clark [b,††], Mark R. Hellberg [b], Andrew K. Shiau [a,‡‡], Stewart A. Noble [a], Allen J. Borchardt [a]

[a] Kalypsys, Inc., 10420 Wateridge Circle, San Diego, CA 92121, USA
[b] Alcon Research, Ltd, 6201 South Freeway, Fort Worth, TX 76134, USA
[c] Aragon Pharmaceuticals, Inc., 4215 Sorrento Valley Blvd., Suite 215, San Diego, CA 92121, United States

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ABSTRACT

We identified a new benzothiophene containing Rho kinase inhibitor scaffold in an ultra high-throughput enzymatic activity screen. SAR studies, driven by a novel label-free cellular impedance assay, were used to derive **39**, which substantially reduced intraocular pressure in a monkey model of glaucoma-associated ocular hypertension.

Rho-associated coiled-coil containing protein kinases (Rho kinases, or ROCKs) are key effectors of intracellular signaling by the monomeric GTPase RhoA.[1] Activation of RhoA, generally by extracellular stimuli, enables its binding to ROCKs, which in turn stimulates their kinase activity.[2] Active ROCKs then phosphorylate secondary effectors of RhoA signaling, which modulate cell motility, size, division, and differentiation.[3]

ROCKs are pharmaceutical targets based on their roles in apoptosis[4–8] and cytoskeleton dynamics.[9–11] Many structurally diverse small molecule ROCK inhibitors have been identified; a number are in clinical development, and there is one launched drug (Fasudil).[12–19] ROCK inhibition protects cells from apoptotic stimuli, suggesting therapeutic utility for neural injury and neurodegenerative diseases.[20–22] Because ROCK inhibitors can trigger cytoskeleton remodeling and reduce cellular contractility, they can relax vascular smooth muscle and lower blood pressure in models of systemic and pulmonary hypertension.[23–25] Analogous ROCK inhibitor mediated cytoskeleton changes in the aqueous humor outflow tract lead to increased aqueous humor outflow and decreased intraocular pressure. This observation has stimulated the use of ROCK inhibitors as novel agents for the treatment of glaucoma.[15,26–28] Here, we describe optimization of a new series of benzothiophene-based ROCK inhibitors and the in vivo activity of **39** in a model of glaucoma-associated ocular hypertension.

Benzothiophene **1** (Fig. 1) was identified in an ultra high-throughput screen (uHTS) of greater than 850,000 compounds. **1** exhibited an enzymatic IC_{50} of 1.5 μM against ROCK1 and ROCK2 isoforms, showed 5 to 10-fold selectivity over protein kinases A and Cα, and appeared to have an ATP competitive mechanism of

* Corresponding author. Tel.: +1 858 754 3323; fax: +1 858 754 3301.
E-mail address: rdavis@kalypsys.com (R.L. Davis).
† Present address: Dart NeuroScience LLC, 7473 Lusk Blvd., San Diego, CA 92121, United States.
‡ Present address: Oregon Health and Science University, 2525 SW 1st Ave, Suite 120, Portland, OR 97201, United States.
§ Present address: Thermo Fisher Scientific, 10010 Mesa Rim Rd., San Diego, CA 92121, United States.
¶ Present address: Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, CA 92037, United States.
‖ Present address: Genomics Institute of the Novartis Research Foundation, 10675 John Jay Hopkins Dr., San Diego, CA 92121, United States.
†† Present address: University of North Texas Health Science Center, 3500 Camp Bowie Blvd., Fort Worth, TX 76107, United States.
‡‡ Present address: Ludwig Institute for Cancer Research, UCSD, 9500 Gilman Dr., #0660, La Jolla, CA 92093, United States.

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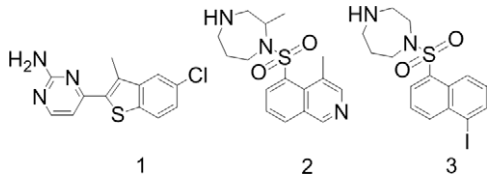


Figure 1. Structure of compound **1** and reference compounds.

action.[29] In a subsequent optimization campaign, substitutions at the benzothiophene 2 and 5-positions were studied to improve potency and solubility.[30] Substitutions at the 3-position will be reported elsewhere. SAR was driven by enzymatic assays of ROCK1 and ROCK2, and a functional cellular assay with a novel label-free readout.[31]

Among the confirmed uHTS hits, cellular activity assessed by visualization of stress fibers in HeLa cells[11,32] generally showed little correlation with enzymatic IC_{50}s (data not shown), likely a result of variable cellular permeability and the non-quantitative nature of the assay. To support cellular SAR, a simple, reliable, easily quantified assay with throughput to support multiple compounds in dose–response was required. Traditional, label-dependent approaches to study ROCK inhibition in cells include manual/automated fluorescence microscopy of cytoskeleton rearrangements[33], or Western blot to detect changes in substrate phosphorylation.[9] None of these existing approaches met our criteria.

Transepithelial electrical impedance has long been used to assess the integrity of cell monolayers in culture.[34,35] More recently, higher throughput impedance platforms have emerged to address the expanding interest in label-free technologies for cellular assays. Since ROCK inhibition profoundly changes cell shape and decreases cell adhesion[32,36], we reasoned that the ACEA/Roche RT-CES® impedance system would enable the quantitative measurement of cellular ROCK inhibition.[37]

Briefly, cells are seeded on 96-well microtiter plates containing microelectrode arrays in each well. As adherent cells either proliferate or increase relative adhesion, and consequently resist current flow, the impedance at constant voltage increases. Conversely, impedance will decrease as adhesion decreases.[38]

The response of GTM-3[39] cells to the known ROCK inhibitor HMN-1152[40] (**2**, Fig. 1 ROCK1 IC_{50} 0.03 ± 0.002 μM) at three concentrations is shown in Figure 2A. There is a rapid, dose dependent decrease in impedance. About one hour after HMN-1152 addition, the impedance differential stabilizes, and the trace follows a parallel path to the DMSO control. These kinetics are consistent with the effects of ROCK inhibition studied by cell imaging.[33]

To correlate the impedance changes with cell morphology changes, GTM-3 cells were treated with HMN-1152, followed by indirect immunofluorescence of vinculin, a cytoskeleton protein associated with cellular focal adhesions (Fig. 2B).[41,42] HMN-1152 treatment decreased the vinculin staining density and intensity, as well as induced a cell shape change, compared to DMSO control-treated cells. Notably, the myosin light-chain kinase inhibitor ML-7, which is structurally similar to HMN-1152 but a very weak ROCK inhibitor (**3**, Fig. 1, ROCK1 IC_{50} 38 ± 4 μM), showed no similar effect in the impedance or immunofluorescence assays (data not shown).

We further validated the impedance assay with a panel of small molecule drugs or drug-like compounds of wide structural diversity and function.[43] Based on the high specificity of the impedance

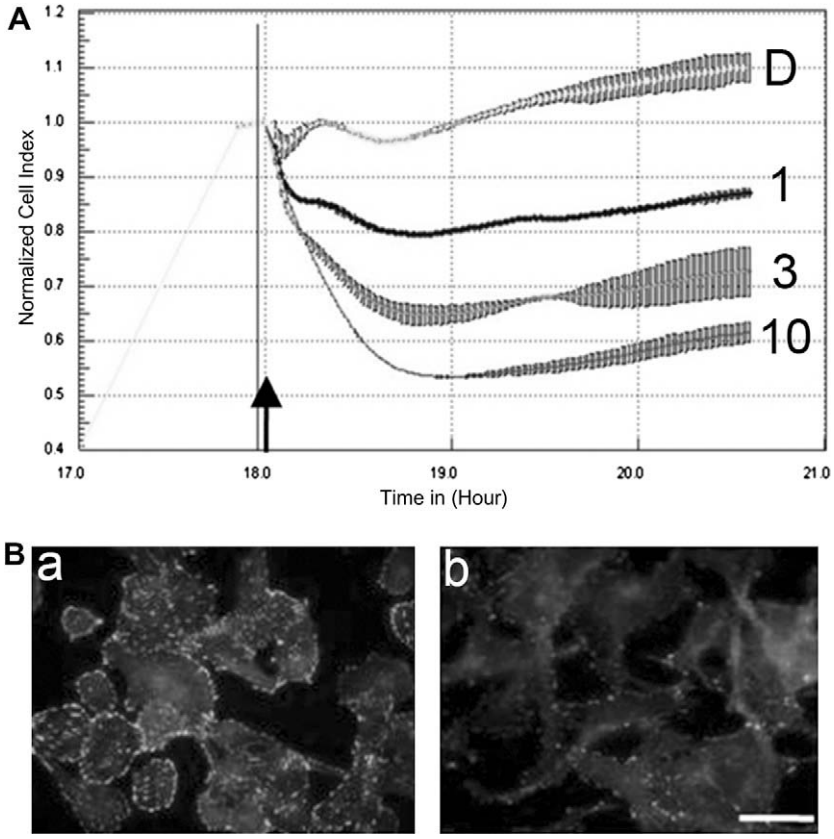
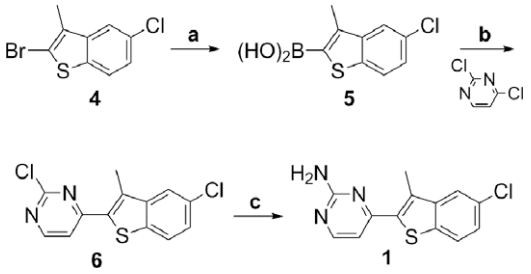


Figure 2. Validation of an impedance assay to study ROCK inhibition in living cells. (A) Impedance (reported as 'Normalized Cell Index', *Y* axis) of GTM-3 cells measured over time (*X* axis) after compound addition at about 18 h (arrow). HMN-1152 in DMSO was added, in duplicate, to a final concentration of 1 μM (1), 3 μM (3), or 10 μM (10), versus DMSO (D) only as negative control. Error bars represent 1 standard deviation. (B) Indirect immunofluorescence of vinculin in GTM-3 cells treated for 3 h with 0.1% DMSO (a) or 0.1% DMSO and 10 μM HMN-1152 (b) in culture media. Bar = 20 μm.



Scheme 1. Synthesis of **1**. Reagents and conditions: (a) *n*-BuLi, B(O*i*-Pr)$_3$, −78 °C; then aq. HCl, rt, 60%; (b) Pd(PPh$_3$)$_2$Cl$_2$, K$_2$CO$_3$, THF, 70 °C, 75%; (c) NH$_4$OH, EtOH, sealed tube, 80 °C, 82%.

effect observed with ROCK inhibitors, and the complete reversibility upon compound washout (data not shown), this assay met our criteria, with sufficient throughput to build cellular SAR. Recently, Schroter et al. developed a 96-well phospho-myosin light-chain indirect immunofluorescence assay for ROCK inhibitor SAR development.[44] The reported EC$_{50}$s of known ROCK inhibitors indicates that their assay is more sensitive, which is an advantage in rank ordering lower potency compounds. By contrast, the complementary impedance assay has the advantage of speed (e.g., most assays completed in one to two hours) and simplicity, requiring no special reagents, fixation, or post-fixation procedures.

Compound **1** was prepared as shown in Scheme 1. Commercially available **4** was converted to boronic acid **5** by lithium halogen exchange of bromine, followed by trapping of the resulting anion with tri-isopropyl borate according to the procedure of Li et al.[45] Suzuki coupling of **5** and 2,4-dichloropyrimidine yielded **6** in 75% yield, which was then treated with aqueous NH$_4$OH in ethanol to give **1** in 82% yield.

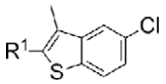
By analogy with the pyridine moiety of ROCK inhibitor Y-27632, compounds **7–13** were prepared to optimize the 2-aminopyrimidine putative donor–acceptor interactions with residues Glu^{154} and Met^{156} in the ATP binding site of ROCK1.[46,47] The 2-position of the benzothiophene was elaborated by Suzuki coupling of the corresponding heteroaryl halides to intermediate **5**.

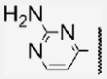
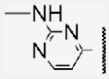
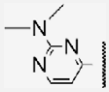
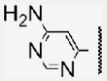
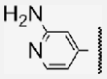
Surprisingly, relatively small changes to the 2-aminopyrimidine were not tolerated (Table 1). For instance, *N*-methyl (**7**) or *N*,*N*-dimethyl (**8**) 2-aminopyrimidines had negligible activity, while 4-aminopyrimidine (**9**) and 2-aminopyridine (**10**) were much weaker than **1**. Even a 4-pyridine (**11**), common to several ROCK inhibitors, was very weak. A 4-azaindole (**12**) showed comparable, albeit less potent, activity, while a 3-azaindole (**13**) was very weak. None of the compounds showed significant cellular activity up to 10 μM. Because the 2-aminopyrimidine of **1** did not tolerate changes, this area of the molecule was not explored further.

To improve potency, we examined potential hydrophobic interactions around the benzothiophene 5-position. Compounds **19–29** were synthesized from **16** as shown in Scheme 2. Intermediate **16** was prepared in five steps, starting with alkylation of **14** with bromoacetone, followed by sulfuric acid mediated cyclization to give **15**. Acylation of **15** under Friedel–Crafts conditions gave the 2-acetyl derivative, which was reacted with DMF/DMA to give **16**. Cyclo-condensation of **16** with guanidine hydrochloride under basic conditions[48] formed the 2-aminopyrimidine ring of **17** in 54% yield. Intermediate **17** was coupled under palladium catalysis with organo-zinc reagents[49], phenol, or aniline[50] to give **19–27**, **28**, and **29**, respectively. Analogous chemistry with **6** was inefficient, due to the lower reactivity at the chlorine.

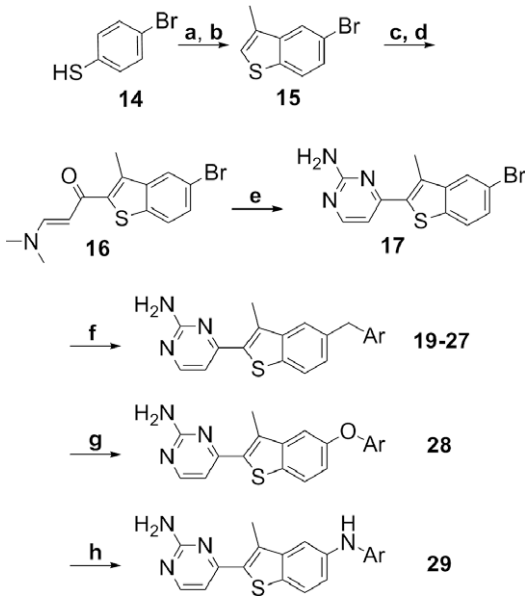
Exploration at the 5-position used the comparative reference **18**[51], with an enzymatic IC$_{50}$ of 2.9 μM (Table 2). Attaching a phenyl directly to the 5-position (**19**) resulted in a loss of potency, possibly due to steric interactions. Moving the phenyl group away

Table 1
Exploration of 2-position SAR



Compound	R^1	ROCK1 IC$_{50}$[a] (μM)
1		1.5 (0.2)
7		1.2 (0.1)[b]
8		>100[c]
9		13 (2)
10		8.3 (2.0)[d]
11		28 (9)
12		7.1 (1.4)
13		>10[e]

[a] Values are means of two or more independent experiments; values for ROCK2 enzymatic assays were comparable. The standard error of the mean is shown in parentheses.
[b] 17% maximum efficacy.
[c] No activity at maximum tested concentration (96 μM).
[d] 44% maximum efficacy.
[e] Not tested above 10 μM (no activity).

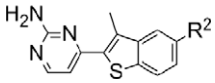


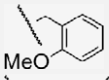
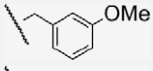
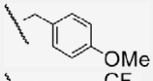
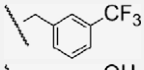
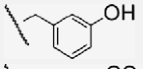
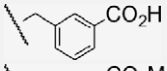
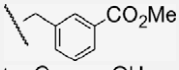
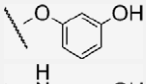
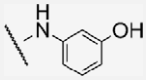
Scheme 2. Synthesis of **19–29**. Reagents and conditions: (a) 1-Bromoacetone, pyridine, Et$_2$O, rt, 80%; (b) H$_2$SO$_4$ (aq), 110 °C, 10 h, 42%; (c) AlCl$_3$, acetyl chloride, CS$_2$, 3 h, 51%; (d) DMF-DMA, 80 °C, 12 h, 59%; (e) guanidine hydrochloride, NaOEt, EtOH, reflux 36 h, 54%; (f) Ar$_2$ZnX, Pd(PPh$_3$)$_2$Cl$_2$, CuI, THF, 70 °C, 50–90%; (g) Pd(OAc)$_2$, Ar-OH, K$_3$PO$_4$, 2-(di-*t*-butylphosphino)biphenyl, toluene, reflux, 24 h, 20–40%; (h) ArNH$_2$, Pd$_2$(dba)$_3$, 1,3-bis(2,6-di-*i*-propylphenyl)-imidazolium chloride, *t*-BuONa, toluene, 100 °C, 40–75%.

from the core with a methylene linker carbon (**20**) improved potency fivefold over **18** and yielded the first compound of this series to show a low but detectable cellular effect at 10 μM. While ortho (**21**) or para (**23**) methoxy groups on the phenyl of **20** reduced enzymatic potency, a meta methoxy group (**22**) was 2 to 3-fold more potent than **20**, although there was no comparable improvement in the cellular assay. Hydroxyl (**25**)[52] or carboxyl (**26**)[53] meta substituents increased enzymatic potency 10 or 4-fold, respectively, beyond that of **22**. Notably, **25** showed significantly greater cellular activity. By contrast, trifluoromethyl (**24**) or carboxymethylester (**27**)[54] meta substituents were very weak. Replacement of the methylene linker carbon in **25** with oxygen (**28**) led to a loss of enzymatic and cellular potency, while substitution with nitrogen (**29**) resulted in activity comparable to **25**. Thus, **25** represented the most optimal analog of **1** yet identified.

The selectivity of compound **25** was determined at 10 μM against a panel of 39 kinases.[55] Only two kinases (Aurora B and MSK1) showed inhibition consistent with low single-digit μM potency, supporting the notion that this series is not especially indiscriminate. However, the modest cellular activity of **25** compared to the benchmark Y-39983[17] (cellular EC_{50} 0.79 ± 0.06 μM) did not support in vivo testing.

Table 2
Exploration of 5-position SAR



Compound	R^2	ROCK1 IC_{50}[a] (μM)	GTM-3 EC_{50}[a] (μM)
18		2.9 (0.1)	>10
19		4.2 (0.4)[b]	>10
20		0.58 (0.13)	>10[c]
21		9.7 (1.2)	>10
22		0.23 (0.04)	>10[d]
23		7.2 (1.1)	>10
24		8.6	>10
25		0.02 (0.002)	2.9 (0.5)[e]
26		0.06 (0.01)	>10
27		10 (0.6)	>10
28		0.36 (0.003)	>10
29		0.02 (0.002)	1.0 (0.2)[f]

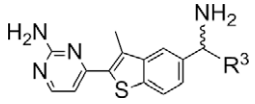
[a] Values are means of two or more independent experiments (except for enzymatic assay of **24**, $n = 1$, $R^2 = 0.99$); values for ROCK2 enzymatic assays were comparable. The standard error of the mean is shown in parentheses.
[b] 35% maximum efficacy at 96 μM.
[c] 11% maximum efficacy at 10 μM.
[d] 15% maximum efficacy at 10 μM.
[e] 50% maximum efficacy at 10 μM.
[f] 35% maximum efficacy at 10 μM.

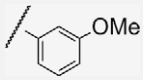
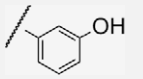
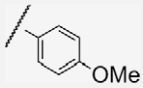
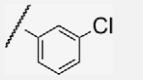
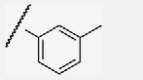
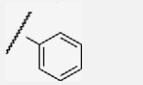
Since solubility is a key determinant for topical ocular bioavailability in our in vivo model, further optimization was directed at increasing solubility while maintaining potency and efficacy in the cellular assay. Initial tests revealed low solubility for compounds such as **22** (0.04 mM), **25** (0.01 mM) and **29** (0.03 mM).[56,57] To improve solubility, amine substituents with a predicted pK_a above 8 were considered. Ideally, the amine would be synthetically accessible, be neutral toward or improve potency, and greatly improve solubility. Initially, we targeted the methylene linker between the benzothiophene core and the 'tail' phenyl group, since this position would place the amine in an environment where it might be less likely to interrupt existing interactions with the binding site.

The first compound in this series, **30**, not only improved solubility (to 0.22 mM), but also improved enzymatic potency 12-fold relative to its des-amino counterpart (**22**), and improved cellular potency by at least threefold (Table 3). The effect of the installed amine on the potency of **22** prompted a re-examination of the 5-position SAR. Compounds **30–35** were prepared by converting intermediate **16** to the nitrile (Scheme 3), followed by reaction with the appropriate Grignard reagent (in excess) to give an imine, which was reduced in situ with sodium borohydride.

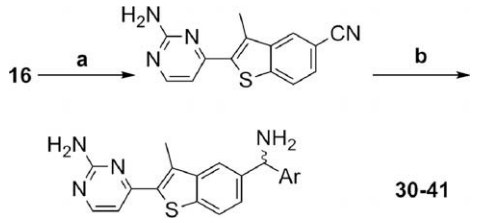
With the aminomethylene linker, there was now much less difference between the IC_{50}s of the meta hydroxyl (**31**)[58] and methoxy (**30**) groups. While the rank difference in cellular EC_{50}s was preserved (compare to **25** and **22**, respectively), there was a major overall increase in potency. Remarkably, the poor activity of the para methoxy group improved 80-fold with the aminomethylene linker (compare **32** and **23**), and gained appreciable cellular activity where there was none detected before. The potency of compounds **33** and **34** provided further evidence of "flat" SAR for phenyl substituents when combined with the aminomethylene linker. In fact, the unmodified phenyl of **35** was just as potent, in dramatic contrast to **20**.

Table 3
Exploration of tail group SAR with aminomethylene linker



Compound	R^3	ROCK1 IC_{50}[a] (μM)	GTM-3 EC_{50}[a] (μM)
30		0.02 (0.001)	3.6 (1.1)[b]
31		0.01 (0.001)	0.9 (0.04)
32		0.09 (0.01)	2.6 (0.6)[c]
33		0.03 (0.002)	2.7 (0.7)
34		0.05 (0.01)	1.9 (0.1)[d]
35		0.02 (0.003)	1.4 (0.4)

[a] Values are means of two or more independent experiments; values for ROCK2 enzymatic assays were comparable. The standard error of the mean is shown in parentheses.
[b] 46% maximum efficacy.
[c] 56% maximum efficacy.
[d] 67% maximum efficacy.



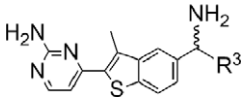
Scheme 3. Synthesis of **30–41**. Reagents and conditions: (a) $Zn(CN)_2$, Zn, Pd[P(*t*-Bu)$_3$]$_2$, DMAC, 90%; (b) ArMgX, THF, 70 °C, 16 h; then $NaBH_4$, MeOH 0 °C to rt, 29–90%.

In silico docking of **30** into the ATP binding site of ROCK2 (PDB: 2F2U) suggested an explanation for the altered SAR relative to compounds without the aminomethylene linker. With the 2-aminopyrimidine positioned in the ATP binding site to make hydrogen bonds with Glu^{170} and Met^{172}, the methylene linker amine could be positioned within hydrogen bonding distance of the carboxyl side chain of Asp^{232} (analogous to Asp^{216} of ROCK1). This strong polar interaction could reduce the importance of the phenyl substituents and account for the shift in SAR (model not shown).

We introduced 5-position tail groups smaller than phenyl with the goal of retaining potency in the context of the aminomethylene linker, and possibly improving corneal permeability.[59] While substitution of the phenyl group with hydrogen (**36**) greatly reduced enzymatic and cellular potency compared to **35,** increasing the size of an attached alkyl group from methyl (**37**) to ethyl (**38**) to *n*-propyl (**39**) lead to corresponding improvements in enzymatic potency of about fivefold, 15-fold, and 100-fold relative to **36** (Table 4). In addition, the rank order of cellular activity showed a similar effect. Branched alkyl groups were well tolerated, both alpha (**40**) and beta (**41**) to the aminomethylene linker.

Based on their low μM (**37, 38**, **40**, **41**) or sub-μM (**39**) cellular EC_{50}s, low molecular weight, and improved solubility (>0.45 mM), these compounds have attractive properties comparable to other advanced ROCK inhibitors, such as the clinical candidate Y-39983.[15,17] To demonstrate in vivo activity, we tested compound **39** by topical application to the eyes of conscious cynomolgus monkeys with laser-induced trabeculoplasty and ocular hypertension (Table 5).[60] Statistically significant reduction of intraocular pressure was achieved by 1 h after dosing. The IOP reduction was sustained in the hypertensive eye through at least six hours. The efficacy of **39** (250 μg/eye) was comparable to the efficacy of Y-39983 (30 μg per eye), providing in vivo proof-of-activity.

Table 4
Exploration of alkyl tail groups with aminomethylene linker



Compound	R^3	ROCK1 IC_{50}[a] (μM)	GTM-3 EC_{50}[a] (μM)
36	H	2.2 (0.5)	>10
37	Me	0.40 (0.04)	4.2 (0.6)
38	Et	0.14 (0.014)	1.7 (0.3)
39	nPr	0.02 (0.003)	0.6 (0.1)
40		0.06 (0.016)	2.1 (0.3)
41		0.03 (0.003)	1.4 (0.2)

[a] Values are means of two or more independent experiments; values for ROCK2 enzymatic assays were comparable. The standard error of the mean is shown in parentheses.

Table 5
In vivo activity of compound **39**

Time (h)	Δ IOP (%, L)	Δ IOP (%, NL)
0	0	0
1	−19.6 ± 4.4	−20.0 ± 6.1
3	−24.1 ± 6.0	−13.9 ± 6.2
6	−26.0 ± 5.5	−12.0 ± 7.2

Mean and standard error of percentage change in intraocular pressure [IOP; lasered eye (L), non-lasered eye (NL)] for treated animals [2 × 25 μl of a 0.5% (w/v) suspension, 250 μg/eye total] were measured up to 6 h after topical instillation. For reference, Y-39983 [1 × 30 μl of a 0.1% (w/v) suspension, 30 μg/eye total] yielded a 28.1% and a 20.1% maximum IOP reduction in the lasered and non-lasered eye, respectively. Mean baseline measurements for the lasered and non-lasered eyes were 35.8 mm Hg and 26.1 mm Hg, respectively.

In conclusion, we have discovered a new benzothiophene containing series of ROCK inhibitors based on an uHTS hit. The development of a novel medium throughput label-free live-cell assay for ROCK inhibition allowed us to optimize cellular SAR in parallel with enzymatic assays, and guided compound selection for in vivo pharmacology. In principle, this application of electrical impedance should translate to other targets with dose-proportional effects on cell adhesion, such as $G_{12/13}$ coupled G-protein receptors.

Installation of an aminomethylene linker between the benzothiophene core and the tail group had a profound impact on both the enzymatic and cellular SAR, leading to compounds suitable for topical application to the eye. A compound with in vitro activity comparable to the clinical candidate Y-39983 significantly lowered intraocular pressure in vivo, validating this series for further medicinal chemistry optimization.

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29. *Enzymatic activity was measured by ATP consumption*: Purified ROCK1 or ROCK2 (Invitrogen, cat. PR7028A or PV4048, respectively), peptide substrate (S6: KEAKEKRQEQIAKRRRLSSLRASTSKSGGS-QKOH, Biopeptide, Inc.) and ATP (final concentrations 0.82 μg/ml, 100 μg/ml, and 3 μM, respectively) were added to reaction mix (20 mM Tris–HCl [pH 7.5], 10 mM $MgCl_2$, 0.4 mM $CaCl_2$, 0.15 mM EGTA, 0.1 mg/ml bovine serum albumin) just prior to dispensing 25 μl/well to 384-well plates. Immediately thereafter, 0.24 μl of test compounds, previously arrayed in 11 point, 1/2 log dilution dose–response, were added by passive pin transfer (final top concentration 96 μM, 2–4 replicate wells per concentration, 1% DMSO [vol/vol] in all assay wells). After 2 h at 30 °C, 15 μl of Easylite (Perkin–Elmer) was added, and the resulting luminescence was read on a Molecular Devices Acquest plate reader. Raw luminescence data were normalized to negative (DMSO) and positive (HMN-1152, **2**) controls. Data analysis was performed using Spotfire (Spotfire, Inc.) and Kalypsys proprietary software. Some compounds were also tested in kinase assays measuring ADP production (ADPQuest, DiscoverX), under similar conditions and according to the manufacturer's instructions. Increasing the concentration of ATP in these assays led to an increase in IC_{50} values, consistent with an ATP-competitive mechanism of action.
30. All synthesized compounds displayed spectral data (MS, NMR) consistent with their assigned structures. Compounds were tested as racemic mixtures when present as such. Synthetic methods can be found in Kahraman, M. et al., U.S. patent application 20080021026.
31. Cellular activity was measured with the xCelligence system (ACEA Biosciences/Roche Applied Sciences). Briefly, 96-well E-plates were coated with fibronectin (US Biological, cat. C2605) by adding 35 μl undiluted reagent to each well for 30 min at 37 °C. After aspiration, 50 μl of cell media (DMEM, Pen/Strep, 1% fetal bovine serum) was added to each well, and background impedance was measured. Then 100 μl of GTM-3 cells in media (0.25×10^6/ml) was dispensed to each well, and the cells were allowed to settle and spread for 30 minutes at rt. The detector was mounted in a culture incubator, and impedance was measured every hour until the following day, when test compounds were added to each well (final top concentration 10 μM [0.1% DMSO] or 15 μM [0.15% DMSO], duplicate wells per concentration). Impedance was then measured every minute for 2–3 h, followed by every 15 min for several hours more, or up to 2 days to monitor long-term effects. The average minimal impedance per compound per concentration was used to generate a dose–response curve. Data normalization and analysis were similar to that for the enzymatic assay. The positive control was Y-39983 (ROCK1 IC_{50} 0.03 ± 0.002 μM, GTM-3 EC_{50} 0.79 ± 0.06 μM). Since DMSO at a final concentration of 0.2% or greater induced impedance effects without test compound, we were limited to a top concentration of about 10 μM for test compounds, based on a standard 10 mM stock in 100% DMSO. This limitation and the overall sensitivity limit of the assay often yielded less than 100% efficacy at the highest tested concentration, where we report an EC_{50} but less than 100% efficacy. In separate experiments, reference compounds dissolved in water at 100 mM allowed us to test concentrations up to 100 μM and confirm 100% efficacy compared to the most potent control, Y-39983. We tested reversibility by aspirating compound-containing media and replacing with compound-free media after multiple washes. Reversibility was tested up to 24 h after compound addition, and was complete about 3 h after media replacement. In parallel 24 h cytotoxicity assays, there was no significant effect of compounds up to at least 30 μM.
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42. GTM-3 cells were plated on glass coverslips one day before use, treated with compound or DMSO negative control for 3 h, fixed with 2% paraformaldehyde in PBS for 10 min at rt, and immuno-stained with an anti-vinculin mAb (Sigma) and goat anti-mouse $(Fab')_2$ Alexa-488 secondary antibody (Invitrogen) under conventional conditions. Images were acquired on a Zeiss Axiovert 200 M equipped with a Hamamatsu ORCA ER cooled CCD camera and OpenLab software (Improvision). Images were imported into Adobe Photoshop (v.10) for labeling and minor alterations in brightness only.
43. The cell impedance assay was validated for ROCK inhibition by testing the following kinase inhibitors at 10 μM (targets in parentheses): sorafenib (VEGFR, PDGFR, Raf), PD-184352 (Mek), gefitinib (EGFR), imatinib (PDGFR, Abl, Kit), VX-745 (p38α), Ro-31-7549 (PKCs), indirubin-3′-oxime (GSK3β, some CDKs), STO-609 (CAMKK), SU-6656 (Src, Fyn), LY-294,002 (PI3-kinase), and H-89 (PKA, ROCK). Only H-89 had a similar but weaker effect than HMN-1152, consistent with its IC_{50} of 0.36 ± 0.08 μM against ROCK. The other compounds had dramatically different effects quantitatively, qualitatively, or both. For example, imatinib caused a rapid, small magnitude increase in impedance, followed by a slow decrease to baseline over 3 h. Other compounds with different effects than ROCK inhibitors included 6-anilinoquinoline-5,8-quinone (guanylate cyclase inhibitor), 2′,5′-dideoxyadenosine (adenylate cyclase inhibitor), and nocodazole (microtubule polymerization inhibitor, tested at 2 μM). Treatment with blebbistatin (myosin II inhibitor, 10 or 1 μM) showed a dose-dependent effect similar to ROCK inhibitors, but with one-third the magnitude, while taxol (microtubule stabilizer, 0.1 or 0.01 μM) showed a similar low magnitude, non dose-dependent effect. Only cytochalasin D (actin polymerization inhibitor) or an RGD containing peptide with sequence GRGDTP (integrin inhibitor; a control peptide with sequence GRADSP was inactive) showed dose dependent effects at 10 and 1 μM strikingly similar to that of ROCK inhibitors. The effects of the last four, and especially the last two, compounds are consistent with their targets and expected effects on cell adhesion. Sorafenib, PD-184352, gefitinib, imatinib, and VX-745 were synthesized by Kalypsys; all other compounds in impedance validation experiments were purchased from commercial vendors.
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51. Prepared as described in Scheme 1; des-chloro **4** was used as an intermediate.
52. Prepared from **22** by treatment with BBr_3 in DCM.
53. Prepared from **17**: *n*-BuLi, THF, −78 °C; then solid CO_2, 3 h, 20%.
54. Prepared from **26**: (trimethylsilyl)diazomethane (2 M in Et_2O), THF/MeOH (1:1), rt, 43%.
55. Compound **25** was tested at 10 μM for inhibition in functional assays of the following kinases (Invitrogen,% inhibition in parentheses if ⩾50%): ALK4, AMPK, Aurora B (82), B-Raf, CaMK2α, CaMK4, CDK2/cyclinA, CDK5/p35, CK1α1, CK1ε1, CK2α1, DAPK3, EGFR, EphB4, Erk1, FGFR1, GSK3β, INSR, Jak2, Jnk1, Lck, Mek1, MKK6, MLK1, MAPKAPK2, MSK1 (94), p38α, p70S6K (55), Pak6, PDGFRα, PKA (67), PKCα, PKCε, PKN1 (53), RSK1, SGK1 (61), Src, TrkA, ZAP70.
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