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Substituted 2*H*-isoquinolin-1-one as potent Rho-Kinase inhibitors. Part 1: Hit-to-lead account

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ABSTRACT

Two closely related scaffolds were identified through an uHTS campaign as desirable starting points for the development of Rho-Kinase (ROCK) inhibitors. Here, we describe our hit-to-lead evaluation process which culminated in the rapid discovery of potent leads such as **22** which successfully demonstrated an early in vivo proof of concept for anti-hypertensive activity.

Rho-Kinase (ROCK) is a member of the serine–threonine protein kinase family. ROCK exists in two isoforms, ROCK 1 and ROCK 2.[1] ROCK has been identified as an effector molecule of RhoA, a small GTP-binding protein (G protein) that is upregulated in a variety of pathological conditions. RhoA/ROCK signaling pathway plays an important role in signal transduction initiated by several vasoactive factors, for example angiotensin II,[2a] urotension II,[2b] endothelin-1,[2c] serotonin,[2d] norepinephrine[2e] and platelet-derived growth factor (PDGF).[2f] Many of these factors are implicated in the pathogenesis of cardiovascular disease. One well established contributing role ROCK plays in cardiovascular disease is hypertension via increased smooth muscle contraction.[3] Mechanistically ROCK catalyzes an inactivating phosphorylation of the myosin-binding subunit of myosin light chain phosphatase, which leads to prolonged smooth muscle contraction and increased vascular tone.[1,3] Therefore, ROCK inhibition represents a new approach to treat the cardiovascular diseases such as hypertension.

A number of ROCK inhibitors have been reported in recent years,[4] the most cited being the isoquinoline derivative Fasudil and the pyridine-based Y-27632 (Fig. 1). Here, we wish to report our discovery and hit-to-lead evaluation of a novel series of substituted 2*H*-isoquinolin-1-one and 3*H*-quinazolin-4-one ROCK inhibitors (Fig. 1). These compounds provide desirable levels of potency and reasonable kinase selectivity profiles to serve as tools for further therapeutic target validation and a starting point for lead optimization.

Boehringer Ingelheim's compound collection was screened in a homogenous luciferase assay using ROCK 2, substrate AKRRRLSSLRA, and a luciferin–luciferase detection reagent to quantify residual ATP.[4] Included in the screening collection was a library designed as potential ROCK inhibitors based in part on the work of Takami et al.[5] This consisted of bicyclic cores with hydrogen bonding moieties that could potentially bind to the hinge region of the kinase. The cores were attached through an amide linker to a set of hydrophobic fragments. Two members of this library were the quinazolinone inhibitor **1** and the isoquinolone **2** with modest potency of 2.9 and 1.7 μM, respectively. Several related heterocyclic cores containing potential H-bond donor and acceptor

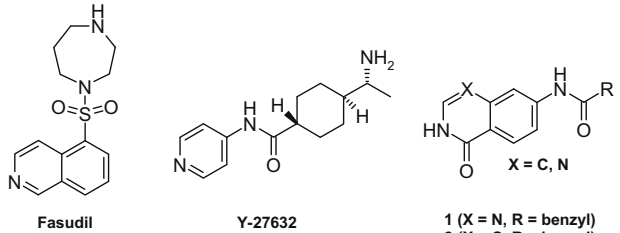

Figure 1. Structures of Fasudil, Y-27632 and compound **1** and **2**.

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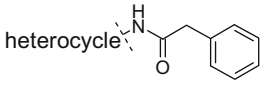
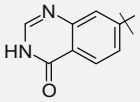
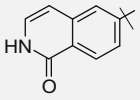
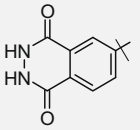
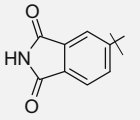
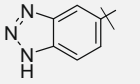
motifs were not tolerated (Table 1). The phthalazindione **3**, phthalimide **4** and benzotriazole **5** showed no activity up to 10 μM. Our initial goal was to demonstrate tractable SAR and evaluate the opportunity to improve potency. To this end, results of exploration of the SAR of **1** are summarized in Tables 2–4.

Table 2 shows the results of phenyl ring amide regioisomer SAR using both quinazolinone isoquinolone cores as templates. These studies showed an absolute requirement for attachment at the b position suggesting a strong spatial requirement of the hinge binding moiety with regard to a proper orientation of the benzyl amide moiety.

We next explored replacements of the exocyclic amide linker. Observed in Table 3, carbamate **12** and urea **13** were three- to four-

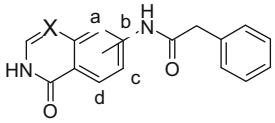
Table 1
SAR of heterocycle core



Compound	Heterocycle	ROCK 2 IC_{50} (nM)
1		2900
2		1700
3		>10,000
4		>10,000
5		>10,000

Cambrex PKLight ATP Detection Reagent using luciferin–luciferase to quantify residual ATP. Assay values reported as the mean of $n \geqslant 2$ independent replicates, and standard error (SE) typically <20%.

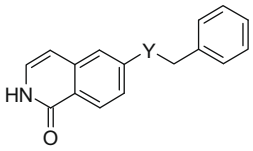
Table 2
SAR studies of regioisomers

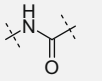
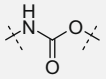
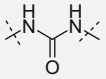


Compound	X	Position	ROCK 2 IC_{50} (nM)
1	N	b	2900
6	N	a	>10,000
7	N	c	>10,000
8	N	d	>10,000
2	C	b	1700
9	C	a	>10,000
10	C	c	>10,000
11	C	d	>10,000

Cambrex PKLight ATP Detection Reagent using luciferin–luciferase to quantify residual ATP. Assay values reported as the mean of $n \geqslant 2$ independent replicates, and standard error (SE) typically <20%.

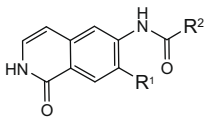
Table 3
SAR studies of linker



Compound	Y	ROCK 2 IC_{50} (nM)
2		1700
12		360
13		520
14		>10,000

Cambrex PKLight ATP Detection Reagent using luciferin–luciferase to quantify residual ATP. Assay values reported as the mean of $n \geqslant 2$ independent replicates, and standard error (SE) typically <20%.

Table 4
SAR studies of R^2 amide of molecule



Compound	R^1	R^2	ROCK 2 IC_{50} (nM)
2	H	$PhCH_2$–	1700
15	Cl	$PhCH_2$–	320
16	Cl	CH_3–	1300
17	Cl	Ph	>10,000
18	Cl	$PhCH_2CH_2$–	280
19	Cl	Cyclohexylmethyl–	430
20	Cl	$NH_2(Ph)CH$–	57
21	Cl	MeNH(Ph)CH–	12
22	Cl	Cyclopropylamino(Ph)CH–	19
23	Cl	$Me_2N(Ph)CH$–	26
24	Cl	$PhCH_2NH(Ph)CH$–	5100
25	Cl	$PhCH_2NH(Me)CH$–	210
26	Cl	$PhCH_2NHCH_2$–	110
27	Cl	(*S*)-NH_2(PhCH2)CH–	48
28	Cl	(*R*)-NH_2(PhCH2)CH–	160

Cambrex PKLight ATP Detection Reagent using luciferin–luciferase to quantify residual ATP. Assay values reported as the mean of $n \geqslant 2$ independent replicates, and standard error (SE) typically <20%.

fold more potent than **2**; however, sulfonamide **14** did not show significant activity up to 10 μM. The loss of activity of **14** may be due to the fact that, unlike amide bonds, sulfonamides adopt predominantly a cis-bond rotamer, resulting in an unfavorable orientation of the molecule.

A summary of amide substituent R_2 SAR is presented in Table 4. We used 7-chloro substituted isoquinolone **15** as a starting reference as it provides a fivefold improvement in potency than non-substituted core (**15** vs **2**).

Replacing the benzyl group with a methyl group resulted in a fourfold loss of potency (**15** vs **16**), while replacement of the benzyl group with phenyl was detrimental (**15** vs **17**). These data implied

that there exists an important hydrophobic interaction in the region, which requires a proper orientation of the phenyl group. Compound **17** failed to achieve this conformation. Increasing linker length (phenethyl **18** vs **15**) had no significant effect on the activity, and a cyclohexylmethyl replacement of the benzyl group (**19** vs **15**) was tolerated. More interestingly a significant increase of activity was observed in **20** with the incorporation of an alpha-amino group. Modulation with *N*-substitution with methyl and cyclopropyl groups further improved the potency to 12 and 19 nM, respectively (**21**–**22**). Compound **23** with a *N,N*-dimethyl group had a slight decrease of activity relative to **21**. These data suggested that the basic amino group, most likely protonated at physiological pH, participates in a specific interaction with the protein as a salt bridge. A significant loss of activity (>200-fold) was observed when further increasing size of N-substitution group (e.g., with benzyl group **24**). It should be noted that this loss of potency was able to be mostly restored by decreasing the size of the group substituted on the alpha carbon as was seen in **25**–**26**. These results hinted that this portion of molecule cannot tolerate the two bulky/hydrophobic moieties simultaneously. Compound **20** was originally synthesized as a racemic mixture as phenylglycine **20** was known to racemize readily. In order to examine the chiral preference, we synthesized the respective L- and D-phenylanine analogues (**27** and **28**) as surrogates, and noticed that there was a moderate stereochemical preference of (*S*)-configuration for the biological activity.

The series was confirmed being ATP competitive in biochemical experiments. The crystal structure of ROCK was not available when this work was started. Among the kinases whose three-dimensional structures were known, PKA was chosen as a template for homology modeling based on its reasonable homology (56%, 39% identity) throughout the kinase domain to Rho-kinase.[6] Additionally, co-crystal structures of ROCK inhibitors in PKA had been published.[6] We used Schrödinger's PRIME software to build the homology model and GLIDE for the docking studies.[7] Shown in Figure 2 is a docking model of compound **23** (preferred enantiomer) in the ATP binding site. In the modeling the isoquinolinone moiety has specific interactions with the protein, forming two essential H-bonds in the hinge region, one with the backbone NH of Met172 and the other being to the backbone carbonyl of Glu170. The isoquinolinone ring appears to enjoy favorable π interactions with the protein, and the isoquinolone-substituted chlorine interacts with Phe384 from the C-terminal loop. The exocyclic amide linker appears to serve as a spacer group without direct interaction with the backbone of the protein. The phenyl group, which orients itself nearly perpendicular to the isoquinolinone ring, sits nicely in a hydrophobic groove underneath the glycine-rich loop. The basic amino group inserts into the ATP phosphate binding region pocket, interacting with acid residue of Asp232 and the side chain carbonyl of Asn219. This model was instrumental in guiding our early SAR work, and proved to be consistent with X-ray crystallography work, which became available later.[8]

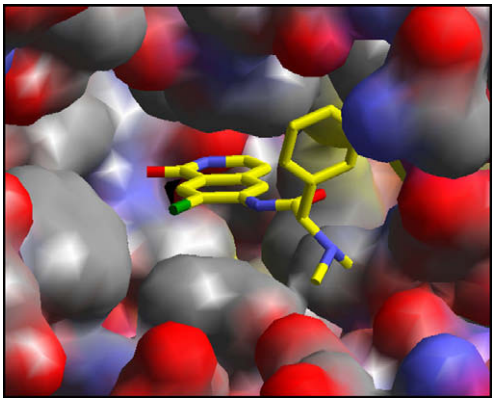


Figure 2. A docking pose of compound **23** in the ATP pocket.

The ATP binding sites of kinase proteins are relatively conserved which poses a significant challenge discovering of highly selective inhibitors. In this program a two-stage kinase selectivity testing strategy was implemented. Compounds were first tested against a panel of counter screen kinases Prkce, DMPK, cdc42, Prkcl2, which were chosen as representatives based on their similarity to Rho-Kinase in terms of sequence homology, phylogenetic relationship, biological relationship and chemical susceptibility, respectively. The most promising compounds were then profiled against an extended panel of about 50 kinases. Several representative inhibitors are shown in Table 5 which demonstrate improved selectivity relative to Fasudil and Y-27632. The SAR trends indicated a balance between potency and selectivity could be achieved by modulating the amino substitution of molecule (e.g., compound **22**). These compounds were found to be dual inhibitors of ROCK 1 and ROCK 2.

Table 5
Selectivity profile in counter screening kinases

Compound	ROCK 2 IC_{50} (nM)	ROCK 1 IC_{50} (nM)	Prkce IC_{50} (nM)	DMPK IC_{50} (nM)	Cdc42 IC_{50} (nM)	Prkcl2 IC_{50} (nM)
15	320	180	>8000	>8000	5400	1400
20	57	59	>8000	>8000	1700	510
21	12	14	>8000	>8000	1300	110
22	19	39	>8000	>8000	>8000	>8000
23	26	19	>8000	>8000	>8000	2300
26	110	53	>8000	>8000	>8000	3000
Fasudil	400	660	3000	>8000	>8000	780
Y-27632	61	46	335	>8000	>8000	70

Cambrex PKLight ATP Detection Reagent using luciferin–luciferase to quantify residual ATP. Assay values reported as the mean of $n \geqslant 2$ independent replicates, and standard error (SE) typically <20%.

Table 6
An extended kinase panel profiling of **22**

Kinase	Percent of control @3 μM	Kinase	Percent of control @3 μM
ROCK 2	0.55	JNK3 alpha1	84
PKBdeltaPH	17	GSK3B	103
S6K1	15	CAMK-1	100
PKB-beta	39	PBK	100
PRK2	16	MAPKAPK2	95
PKA (rec)	31	MST2	86
MAPKAPK1A	31	MAPKAP-K3	94
MSK1	35	CDK2/ cyclinA	89
ERK8	54	MAPK2/ ERK2	103
MAPKAP-K1b	74	SGK	111
MKK1	70	PKD1	73
smMLCK	76	NEK7	90
PKCA	85	MNK2 alpha	90
MARK3	68	NEK6	104
AuroraB	41	CK2	98
CHK2	76	AMPK	86
SRPK1	82	CHK1	90
NEK2A	91	JNK1A1	93
CSK	94	EF2K	105
PRAK	63	Src	88
PDK1	94	PIM2	87
CK1	90	PLK1	93
DYRK1A	69	MNK1	89
SAPK2AP38	95		

Table 7
Results of functional activity and profiling

Compound	22	23	Fasudil	Y-27632
ROCK 2 IC_{50} (nM)	19	26	440	260
Aortic ring IC_{50} (nM)	570	90	7500	4750
Cytotoxic (μM)	>10	>10	>10	>10
Solubility pH 7 μg/ml	16	6	>60	>32
Caco-2 (10 cm/s) (A to B/B to A)	20/23	32/20	10/10	nd
HLM/RLM[a] $t_{1/2}$ min	21/7	15/3	73/nd	>90/nd
Cl (ml/min/kg)	82	123	nd	nd
AUC p.o. (ng h/mL)	1293	30	nd	nd
$T_{1/2}$ p.o. (h)	2.7	2.5	nd	nd
% F[b]	64	2	nd	nd
Cyp inh. (μM) 1A2/2D6/3A4	>10	>10	>10/3.6/>10	>10/3.2/>10
hERG inhibition (μM)	>30	nd	nd	nd

[a] Compounds were incubated with liver microsomes at a concentration of 1 mg protein/ml.
[b] In vivo rat pk data was generated at dose of 1.0 mg/kg iv and 10 mg/kg po

Illustrated as an example in Table 6, compound **22** showed no significant inhibitory activity at 3 μM against other kinases in the panel except PKB delta, S6K1, PRK2 (less than 30% of control).

In addition to kinase selectivity screening, representative compounds were further profiled in a general selectivity testing. Compound **22** demonstrated a good selectivity profile against a panel of 53 receptors, ion channels, and enzymes and exhibited <40% inhibition of all of them at 10 μM (data not shown).

Inhibition of ROCK has been reported to relax blood vessels ex vivo.[9] Shown in Table 7, these compounds produce a dose dependent relaxation effect in phenylephrine pre-constricted rat aortic rings. The relaxation effect of these compounds was reversible, as contractility could be restored by washing the treated aortic rings. The difference between enzymatic and tissue activities may partially be due to the higher ATP concentration in the cellular level as well as compound permeability. In comparison with Fasudil and Y-27632, compound **22** demonstrated greater than 10-fold improvement in potency in both the enzymatic and functional tissue assays. Furthermore, compounds did not induce cytotoxic effects in HeLa cells up to 10 μM concentration.

In order to assess the key areas for further optimization of this series, compounds were profiled in a battery of target independent drug-like properties assays. These assays include solubility, permeability, CYP450 inhibition, hERG channel inhibition, in vitro metabolic stability as well as in vivo rat pk studies. This exercise highlighted an overall promising profile as starting point for optimization and focused our attention on metabolic stability as a key parameter. In spite of low in vitro metabolic stability, **22** was found to have sufficient oral exposure in rat pk studies to be advanced as an early proof of concept tool compound in a disease-related spontaneously hypertensive rat (SHR) in vivo model.[10] Compound **22** demonstrated a moderate but statistically significant activity in attenuating hypertension at the highest dose tested, 40 mg/kg (shown in Fig. 3).

Albeit with marginal effect, compound **22** dose dependently decreased mean arterial pressure (MAP). The maximum response was 14% decrease in blood pressure at 4 h with a 40 mg/kg p.o. dose and the duration of the response was approximately 8 h.

The general synthetic procedure for our compounds is illustrated in Schemes 1–3. Schemes 1 and 2 depict the synthesis of the amino isoquinolinone and quinazolinone intermediates.[10] Synthesis of **22** is provided as representative example in Scheme 3.

A nitro substituted cinnamic acid **29** was reduced to the corresponding amine using tin II chloride in hydrochloric acid and was subsequently acetylated The resulting cinnamic acid **30** was then converted to the corresponding acyl azide **31**, which was converted in a Curtius rearrangement to isoquinolinone by heating in diethylene glycol dibutyl ether at a temperature of 230–260 °C. In the case where R_1 is H, a mixture of regioisomeric products was formed (**32a** or **32b**), which was separated by chromatography. The acetyl group was removed by hydrolysis under acidic conditions using concentrated hydrochloric acid, generating the desired amino isoquinolinone intermediate **32** (Scheme 1).

Heating the nitro anthranilic acid **33** with formamidine at a temperature of 200 °C provided nitro **34**, which was reduced to amino quinazolinone intermediate **35** by catalytic hydrogenation over palladium (Scheme 2).

Shown in Scheme 3, intermediate **32** was treated with 2-chlorophenylacetyl chloride in DMF in the presence of triethylamine, and the resulting chloro **36** was converted to the final compound **22** by reacting with cyclopropyl amine.

In summary, a novel series of potent ROCK inhibitors with an improved kinase selectivity profile were discovered. These inhibitors have demonstrated potent functional tissue activity and in vivo activity, suggesting their potential therapeutic utility in cardiovascular diseases such as hypertension. Further drug-like prop-

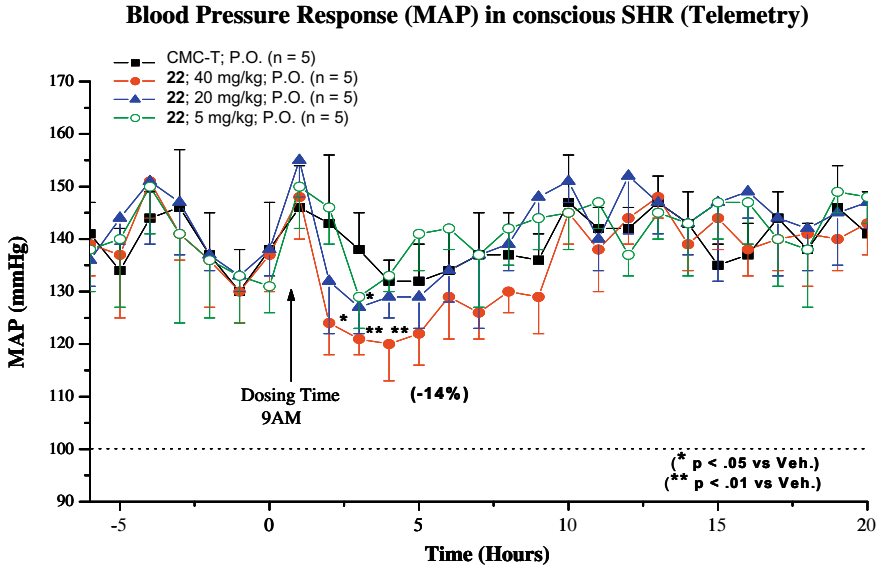
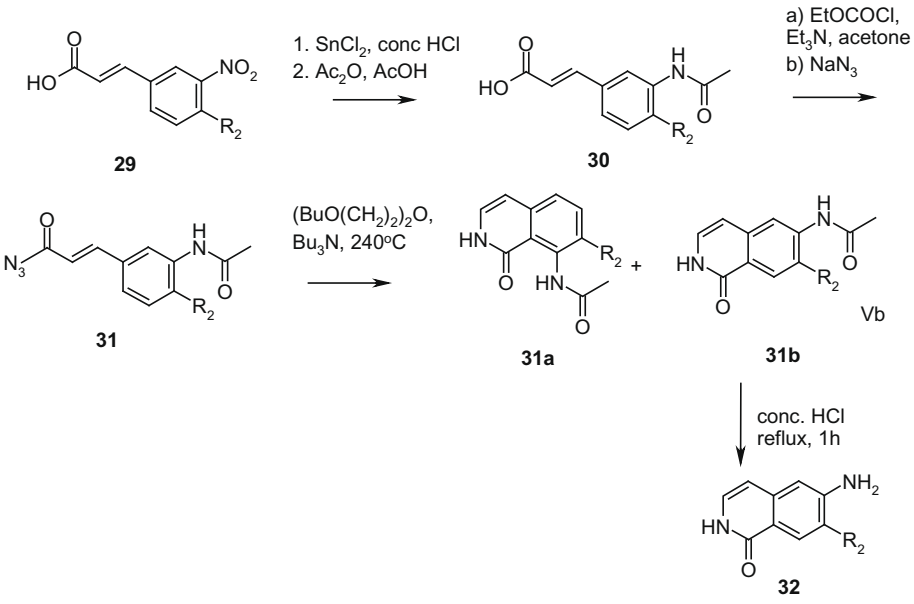
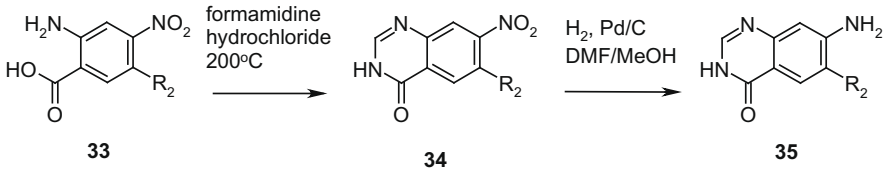


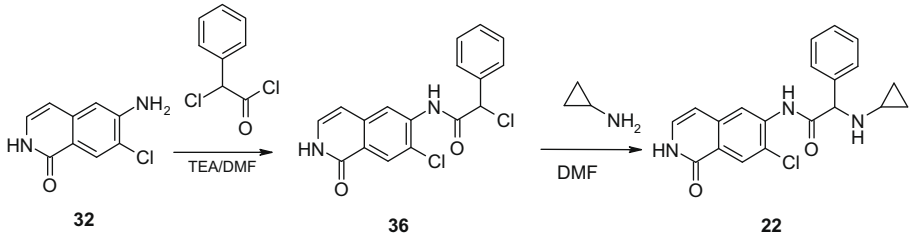
Figure 3. Effect of **22** on the mean arterial pressure (MAP) in conscious SHR. Compound was administered as a single dose oral gavage. Reported results represent the mean arterial pressure for three treated animals.



Scheme 1. Synthesis of intermediate **32**.



Scheme 2. Synthesis of intermediate **35**.



Scheme 3. Synthesis of **22** as an example.

erty profiling qualified this series of compounds, represented by **22**, as a starting point for lead optimization.

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