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Structure–activity relationships, and drug metabolism and pharmacokinetic properties for indazole piperazine and indazole piperidine inhibitors of ROCK-II

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Abstract—ROCK has been implicated in many diseases ranging from glaucoma to spinal cord injury and is therefore an important target for therapeutic intervention. In this study, we have designed a series of 1-(4-(1H-indazol-5-yl)piperazin-1-yl)-2-hydroxy(or 2-amino) analogs and a series of 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-hydroxy(or 2-amino) inhibitors of ROCK-II. SR-1459 has IC_{50} = 13 nM versus ROCK-II while the IC_{50}s for SR-715 and SR-899 are 80 nM and 100 nM, respectively. Many of these inhibitors, especially the 2-amino substituted analogs for both series, are modest/potent CYP3A4 inhibitors as well. However, a few of these inhibitors (SR-715 and SR-899) show strong selectivity for ROCK-II over CYP3A4, but the overall potency of the 2-amino analogs (SR-1459) on CYP3A4 and the high clearance and volume of distribution of these compounds makes the in vivo utility of these analogs undesirable.

Rho kinase (ROCK) is a serine/threonine kinase from the AGC kinase family and is activated by the GTP-bound form of the small GTPase RhoA.[1,2] Two isoforms of Rho-kinase have been described. Human ROCK-I (also referred to as p160 ROCK or ROKβ)[3] and human ROCK-II (ROKα)[4] are approximately 160-kDa proteins containing an N-terminal Ser/Thr kinase domain, followed by a coiled-coil structure, a pleckstrin homology domain, and a cysteine-rich region at the C-terminus.[3,4] The two isoforms share approximately 60% overall amino acid identity and approximately 90% identity within the N-terminal kinase domain.[4] Other closely related family members include myotonic dystrophy kinase (DMPK) and myotonic dystrophy kinase-related CDC42-binding kinase (MRCK).[5] Interestingly, human ROCK-I and ROCK-II are both ubiquitously expressed in most tissues, with the exception of brain, in which ROCK-I has negligible expression.[3] ROCK-II, on the other hand, is abundantly expressed in brain,[4] suggesting the intriguing notion of a brain-specific functional role for ROCK-II.

Fasudil, a ROCK inhibitor, has been shown to be clinically safe and efficacious in a number of vascular disorders such as stable angina,[6,7] and acute ischemic stroke,[8] and has been marketed as a treatment for cerebral vasospasm[9] in Japan since 1995. In addition to the clinical applications, ROCK inhibition has been shown to be efficacious in a number of preclinical models for a variety of diseases ranging from erectile dysfunction,[10–12] to glaucoma (intraocular pressure),[13,14] to multiple sclerosis[15] and spinal cord injury.[16,17] Collectively, these data suggest inhibition of ROCK may have many therapeutic benefits.

Three primary structural classes have emerged as the most well-studied ROCK inhibitor series. The isoquinoline series is represented by fasudil (IC_{50} = 150–550 nM), the clinically utilized compound,[6–9] and H-1152P (IC_{50} = 6–12 nM), also known as dimethyl-fasudil, the most widely published upon potent inhibitor of this class.[18–20] The 4-aminopyridine series represented by Y-27632 has a biochemical IC_{50} = 140–260 nM.[19,21,22]

Keywords: Rho-kinase; ROCK-II; Kinase; Inhibitor; Pharmacokinetics.
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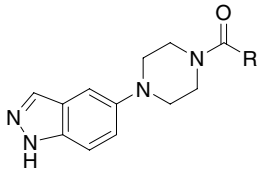
This compound is one of the most widely characterized ROCK inhibitors and has shown efficacy in both erectile dysfunction[11] and spinal cord injury[16] preclinical models. The indazole series[5,23–25] contains perhaps the most potent ROCK inhibitors, but is the least well characterized both in vitro and in vivo.

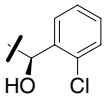
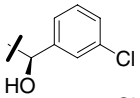
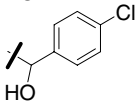
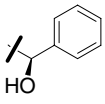
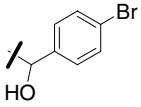
Given the paucity of biochemical and pharmacokinetic data for indazole-based ROCK inhibitors, we tested a series of 13 1-(4-(1H-indazol-5-yl)piperazin-1-yl)-2-hydroxy(or 2-amino) analogs and a series of 10 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-hydroxy(or2-amino) inhibitors of ROCK-II, and measured the IC_{50} for both ROCK-II and CYP3A4 inhibition. Moreover, we tested the in vivo pharmacokinetic parameters in rat for four of the most potent ROCK inhibitors. The data showed that SR-715 and SR-899 had good oral bioavailability, but the high clearance and high volume of distribution made them poor leads for further development.

Because there was only limited SAR information available for the 1-H-indazole series,[23–25] we made two libraries consisting of 37 analogs in the 1-(4-(1H-indazol-5-yl)piperazin-1-yl)-2-hydroxy(or 2-amino) series and 43 analogs in the 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-hydroxy(or 2-amino) series. Table 1 presents the IC_{50} values for inhibition of ROCK-II and CYP3A4 for six 1-(4-(1H-indazol-5-yl)piperazin-1-yl)-2-hydroxy analogs. All of the compounds had $IC_{50} \geqslant 3.0\ \mu M$ for CYP3A4. Two compounds in the 2-hydroxy series, SR-899 and SR-890, had IC_{50}s for ROCK-II of 100 nM, and 190 nM, respectively. Both of these compounds had para-substituted halogens on the phenyl group suggesting the importance of para substitution for increasing potency of ROCK inhibition but not CYP3A4 inhibition. The importance of the para substitution on the phenyl group for ROCK-II was further highlighted by the decrease in potency for the *ortho*-(SR-887) and *meta*-(SR-889) substituted analogs (Table 1).

In contrast to the modest CYP3A4 inhibition observed with the 2-hydroxy analogs, the 2-amino analogs were much more potent CYP3A4 inhibitors (Table 2). Table 2 presents the IC_{50} values for inhibition of ROCK-II and CYP3A4 for seven 1-(4-(1H-indazol-5-yl)piperazin-1-yl)-2-amino analogs. SR-1362, the 2-amino analog of SR-899, maintained its ROCK potency ($IC_{50} = 90$ nM), but also showed a 10-fold increase in potency versus CYP3A4 ($IC_{50} = 410$ nM). These data suggest that the 2-amino substitution, especially when directly linked to halo-phenyl (see SR-1351, SR-1353, and SR-1362), confers much of the CYP3A4 specificity in this series indicating the preference of 2-hydroxy substitution for ROCK inhibitors. Finally, the phenyl substitution in both the 2-hydroxy and 2-amino analogs increased potency of ROCK inhibition compared to mere alkyl substitution (compare SR-897 vs SR-895; Table 1, and SR-1349 vs SR-1360; Table 2).

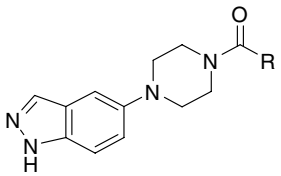
Table 1. IC_{50} values for ROCK II and CYP3A4 inhibition for1-(4-(1H-indazol-5-yl)piperazin-1-yl)-2-hydroxy analogs

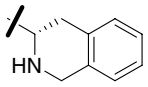
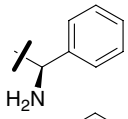
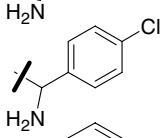
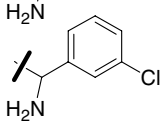
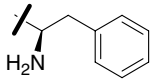
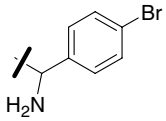


SR #	R	ROCK II inhibition IC_{50}[a] (μM)	3A4 inhibition IC_{50}[a] (μM)	Ratio (3A4/ ROCKII)
SR-887		1.2 ± 0.29	8.0	6.7
SR-889		1.0 ± 0.36	3.5	3.5
SR-890		0.19 ± 0.01	3.0	15.8
SR-895		3.9 ± 1.9	10.0	2.6
SR-897		0.80 ± 0.28	10.0	12.5
SR-899		0.10 ± 0.07	4.0	40

[a] Values are means of two or more experiments ± SD. ROCK-II was assayed with 10 μM S6-peptide, 10 μM ATP, and 4 nM ROCK-II.

Table 2. IC_{50} values for ROCK II and CYP3A4 inhibition for 1-(4-(1H-indazol-5-yl)piperazin-1-yl)-2-amino analogs



SR #	R	ROCK II inhibition IC_{50}[a] (μM)	3A4 inhibition IC_{50}[a] (μM)	Ratio (3A4/ROCKII)
SR-902		5.3 ± 0.76	10.0	1.9
SR-1349		1.3 ± 0.3	0.47	0.36
SR-1351		0.19 ± 0.01	0.53	2.8
SR-1353		0.76 ± 0.06	0.25	0.33
SR-1357		0.38 ± 0.08	7.0	18.4
SR-1360		>10.0	10.0	<1.0
SR-1362		0.09 ± 0.006	0.41	4.6

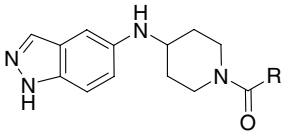
[a] Values are means of two or more experiments ± SD.

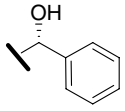
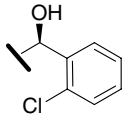
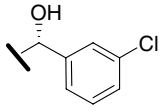
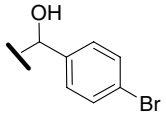
A similar SAR was seen for the 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-hydroxy(or 2-amino) series. Table 3 presents the IC_{50} values for inhibition of ROCK-II and CYP3A4 for five 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-hydroxy analogs. All of the compounds had $IC_{50} \geqslant 1.0$ μM for CYP3A4. Again, the para-substituted bromo-phenyl analog (SR-715) was the most potent ROCK-II inhibitor ($IC_{50} = 80$ nM) and had the best selectivity over CYP3A4 (44-fold) (Table 3). When the 2-amino para-bromo-phenyl analog was synthesized (SR-1459), the potency of ROCK inhibition was increased to 13 nM, but this came at a 7-fold increase in the potency of CYP3A4 inhibition (Table 4). While the overall selectivity of ROCK versus CYP3A4 was maintained in SR-1459 (38-fold) (Table 4), the absolute potency vs CYP3A4 (490 nM) (Table 4) makes this compound less than desirable for further development unless further SAR can be developed that maintains the ROCK potency while eliminating the CYP3A4 activity.

Table 5 shows the human microsomal stability and the in vivo pharmacokinetic parameters measured in rats after intravenous (iv) and oral (po) dosing for the four most potent ROCK-II inhibitors tested. The human microsomal stability for the four compounds ranged from 9.8 min to 36.3 min, with SR-715 having the longest $t_{1/2}$ at 36.3 min (Table 5). Two of the four compounds (SR-715 and SR-899) showed good oral bioavailability (F% between 20 and 30), while the two amino analogs (SR-1459 and SR-1362) showed poorer oral bioavailability (Table 5). The major in vivo liability of these series is the fact that both the 1-(4-(1H-indazol-5-yl)piperazin-1-yl)-2-hydroxy(or 2-amino) analogs and the 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-hydroxy(or 2-amino) inhibitors of ROCK-II have very large volume of distribution (V_d) and extremely high clearance (Table 5). Moreover, the $t_{1/2}$ for all four compounds is fairly low (although higher than expected based on the high clearance) and can most likely be attributed to the high V_d. Indeed, the $t_{1/2}$ is likely attributed to the equilibria of exchange from the tissues of distribution.

Table 6 presents the selectivity data versus other AGC-kinase family members and the cell-based activity in neurite retraction assays for the most potent compounds from each structural class. All four compounds (SR-715, SR-1459, SR-899, and SR-1362) show greater

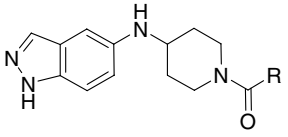
Table 3. IC_{50} values for ROCK II and CYP3A4 inhibition for 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-hydroxy analogs

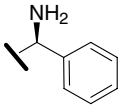
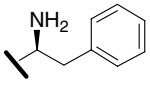
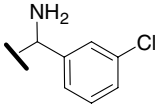
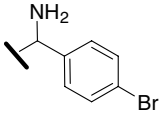


SR #	R	ROCK II inhibition IC_{50}[a] (μM)	3A4 inhibition IC_{50}[a] (μM)	Ratio (3A4/ROCKII)
SR-705		10.0	1.0	0.1
SR-706		0.77 ± 0.006	1.0	1.3
SR-709		1.7 ± 0.2	4.0	2.4
SR-710		0.35 ± 0.002	2.0	5.7
R-715		0.08 ± 0.01	3.5	43.8

[a] Values are means of two or more experiments ± SD. Inhibition of 1′-hydroxy midazolam formation from incubation of 5 μM midazolam was measured by LC–MS/MS.

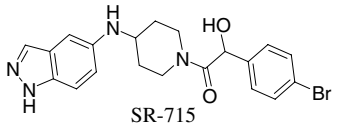
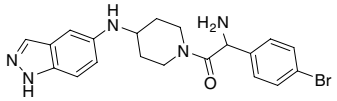
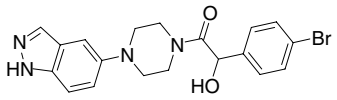
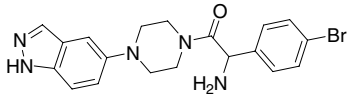
Table 4. IC_{50} values for ROCK II and CYP3A4 inhibition for 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-amino analogs



SR #	R	ROCK II inhibition IC_{50}[a] (μM)	3A4 inhibition IC_{50}[a] (μM)	Ratio (3A4/ROCKII)
SR-1448		6.0 ± 2.2	>10.0	>1.7
SR-1450		0.83 ± 0.1	0.45	0.54
SR-1452		0.47 ± 0.07	1.0	2.1
SR-1457		0.15 ± 0.04	0.17	1.1
SR-1459		0.013 ± 0.001	0.49	37.7

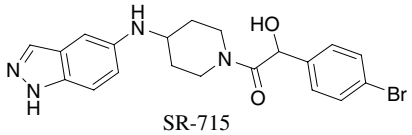
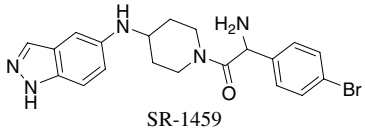
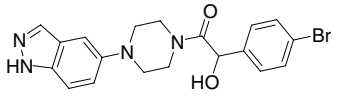
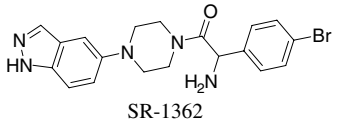
[a] Values are means of two or more experiments ± SD.

Table 5. Drug metabolism and pharmacokinetic parameters (Rat)

Compound	Microsomal stability, $t_{1/2}$ (min)	IV (1 mg/kg)				PO (2 mg/kg)			
		$t_{1/2}$ (h)	Cl (ml/min/kg)	V_d (L)	AUC ∞ (μM h)	AUC ∞ (μM h)	C_{max} (μM)	T_{max} (h)	F%
 SR-715	36.3	1.1	30.3	1.9	1.35	0.71	0.2	0.4	26
 SR-1459	10.8	0.6	41.4	1.7	0.95	0.15	0.1	0.7	8
 SR-899	19.8	0.7	108.8	3.7	0.38	0.21	0.1	0.4	28
 SR-1362	9.8	0.6	75.5	2.7	0.54	0.09	0.4	0.4	8

2 mg/mL human liver microsomes were used in stability studies.

Table 6. IC_{50} values for MRCK, PKA inhibition, and N2A neurite retraction

Compound	MRCK inhibition IC_{50}[a] (μM)	Ratio (MRCK/ROCK II)	PKA inhibition IC_{50}[a] (μM)	Ratio (PKA/ROCK II)	N2A neurite retraction inhibition IC_{50}[a] (μM)
 SR-715	>20	>250	4.2 ± 0.6	53	3.5 ± 0.9
 SR-1459	7.2 ± 3.9	554	0.94 ± 0.04	72	5.7 ± 2.9
 SR-899	>20	>200	2.7 ± 0.6	27	2.4 ± 0.2
 SR-1362	>20	>222	0.45 ± 0.05	5	>15

For the PKA biochemical assay, kemptide was used at a final concentration of 30 μM together with 10 μM ATP and 0.25 nM PKA. MRCK biochemical assay utilized 20 μM S6 peptide, 5 μM ATP, and 6 nM MRCK. All biochemical assays were run in 384-well format using a 10-point dose–response curve. Residual ATP levels after kinase reaction were determined using an enzyme-coupled chemiluminescence readout technique. IC_{50} values were calculated using a 4-parameter logistic. N2A neuroblastoma cells were serum-starved for 3 days to induce differentiation. Following compound addition (7 different doses), neurite retraction was induced with 5 μM lysophosphatidic acid (LPA). Cells were fixed and stained with an antibody against α-tubulin. Images were acquired on the InCell 1000 workstation and neurite length was quantitated using the developer toolbox software.

[a] Values are means of two or more experiments ± SD.

than 200-fold selectivity versus MRCK, the most closely related kinase to ROCK-II (Table 6). Only SR-1362 had minimal selectivity over protein kinase A (PKA) (Table 6). The data suggest that the selectivity for ROCK-II displayed by SR-1459 over CYP3A4 (38-fold; Table 4) and PKA (72-fold; Table 6) compared to the poor selectivity for ROCK-II displayed by SR-1362 over CYP3A4 (4.6-fold; Table 2) and PKA (fivefold; Table 6) can be attributed to the 5-indazole amino group in SR-1459. When the 5-indazole amino group is incorporated into the piperizinyl ring as in SR-1362, the ROCK-II selectivity over CYP3A4 and PKA becomes less, especially when the α-amino group is also present. Without having the crystal structure for CYP3A4 with bound SR-1362 it is difficult to suggest why selectivity would decrease. It is interesting to speculate however that if SR-1362 bound to PKA in a similar fashion as does H-1152P, a ROCK inhibitor whose structure has been determined for both PKA and human ROCK-I,[18,20] then the decrease in selectivity of SR-1362 may be attributed to potential hydrogen bond or van der Waals interactions between the α-amino group of SR-1362 with glutamate 127 in PKA given the shortened distance necessitated by incorporating the 5-indazole amino group into the piperizinyl ring in SR-1362. Further SAR extending the distance between the indazole ring and the piperizinyl ring as well as substitutions of the α-amino acid portion of SR-1362 could be used to test this hypothesis. Synthesis of more analogs and X-ray crystallography studies should help elucidate the exact structural features in both the enzyme and inhibitors that give rise to the selectivity. In addition to suffering from poor selectivity over PKA and CYP3A4, SR-1362 does not appear to have any activity in the N2A neurite retraction assay (Table 6) suggesting that the cell permeability of the compound is poor. Coupled with the high clearance and poor oral bioavailability, the poor cell permeability makes further advancement of this compound challenging.

The 1H-indazole series of ROCK inhibitors is of great interest because the biochemical potency on ROCK for this series is 10–30-fold more potent than that reported for fasudil and for Y-27632.[19,21–25] Despite the low nanomolar potency of this series, there have been no SAR studies that have been expanded beyond ROCK activity. To fill this void, and explore the potential in vivo utility of this series, we expanded the SAR around both the 1-(4-(1H-indazol-5-yl)piperazin-1-yl)-2-hydroxy(or 2-amino) analogs and the 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-hydroxy(or 2-amino) inhibitors, and found that the 2-amino analogs were modest/potent inhibitors of CYP3A4 and had large volume of distribution and high clearance (exceeding that of hepatic blood flow in rat which is generally accepted to be ~60 ml/min/kg).[26] Given the CYP3A4 inhibition activity and the poor in vivo pharmacokinetic parameters of this series it is unlikely that these compounds, without extensive further modifications to improve upon these properties, will be useful for pharmacological studies detailing the potential efficacy of ROCK in the many indications cited above.

References and notes

1. Somlyo, A. P.; Somlyo, A. V. *J. Physiol.* **2000**, *522*, 177.
2. Somlyo, A. P.; Somlyo, A. V. *Physiol. Rev.* **2003**, *83*, 1325.
3. Ishizaki, T.; Maekawa, M.; Fujisawa, K.; Okawa, K.; Iwamatsu, A.; Fujita, A.; Watanabe, N.; Saito, Y.; Kakizuka, A.; Morii, N.; Narumiya, S. *EMBO J.* **1996**, *15*, 1885.
4. Takahashi, N.; Tuiki, H.; Saya, H.; Kaibuchi, K. *Genomics* **1999**, *55*, 235.
5. Mueller, B. K.; Mack, H.; Teusch, N. *Nat. Rev. Drug Discov.* **2005**, *4*, 387.
6. Shimokawa, H.; Hiramori, K.; Iinuma, H.; Hosoda, S.; Kishida, H.; Osada, H.; Katagiri, T.; Yamauchi, K.; Yui, Y.; Minamino, T.; Nakashima, M.; Kato, K. *J. Cardiovasc. Pharmacol.* **2002**, *40*, 751.
7. Vicari, R. M.; Chaitman, B.; Keefe, D.; Smith, W. B.; Chrysant, S. G.; Tonkon, M. J.; Bittar, N.; Weiss, R. J.; Morales-Ballejo, H.; Thadani, U. *J. Am. Coll. Cardiol.* **2005**, *46*, 1803.
8. Shibuya, M.; Hirai, S.; Seto, M.; Satoh, S.; Ohtomo, E. *J. Neurol. Sci.* **2005**, *238*, 31.
9. Shibuya, M.; Suzuki, Y.; Sugita, K.; Saito, I.; Sasaki, T.; Takakura, K.; Nagata, I.; Kikuchi, H.; Takemae, T.; Hidaka, H., et al. *J. Neurosurg.* **1992**, *76*, 571.
10. Chitaley, K.; Wingard, C. J.; Clinton Webb, R.; Branam, H.; Stopper, V. S.; Lewis, R. W.; Mills, T. M. *Nat. Med.* **2001**, *7*, 119.
11. Mills, T. M.; Chitaley, K.; Wingard, C. J.; Lewis, R. W.; Webb, R. C. *J. Appl. Physiol.* **2001**, *91*, 1269.
12. Rees, R. W.; Ralph, D. J.; Royle, M.; Moncada, S.; Cellek, S. *Br. J. Pharmacol.* **2001**, *133*, 455.
13. Honjo, M.; Inatani, M.; Kido, N.; Sawamura, T.; Yue, B. Y.; Honda, Y.; Tanihara, H. *Arch. Ophthalmol.* **2001**, *119*, 1171.
14. Waki, M.; Yoshida, Y.; Oka, T.; Azuma, M. *Curr. Eye Res.* **2001**, *22*, 470.
15. Sun, X.; Minohara, M.; Kikuchi, H.; Ishizu, T.; Tanaka, M.; Piao, H.; Osoegawa, M.; Ohyagi, Y.; Shimokawa, H.; Kira, J. I. *J. Neuroimmunol.* **2006**, *180*, 126.
16. Chan, C. C.; Khodarahmi, K.; Liu, J.; Sutherland, D.; Oschipok, L. W.; Steeves, J. D.; Tetzlaff, W. *Exp. Neurol.* **2005**, *196*, 352.
17. Hara, M.; Takayasu, M.; Watanabe, K.; Noda, A.; Takagi, T.; Suzuki, Y.; Yoshida, J. *J. Neurosurg.* **2000**, *93*, 94.
18. Breitenlechner, C.; Gassel, M.; Hidaka, H.; Kinzel, V.; Huber, R.; Engh, R. A.; Bossemeyer, D. *Structure* **2003**, *11*, 1595.
19. Hu, E.; Lee, D. *Expert Opin. Ther. Targets* **2005**, *9*, 715.
20. Jacobs, M.; Hayakawa, K.; Swenson, L.; Bellon, S.; Fleming, M.; Taslimi, P.; Doran, J. *J. Biol. Chem.* **2006**, *281*, 260.
21. Ishizaki, T.; Uehata, M.; Tamechika, I.; Keel, J.; Nonomura, K.; Maekawa, M.; Narumiya, S. *Mol. Pharmacol.* **2000**, *57*, 976.
22. Turner, M. S.; Fen Fen, L.; Trauger, J. W.; Stephens, J.; LoGrasso, P. *Arch. Biochem. Biophys.* **2002**, *405*, 13.
23. Iwakubo, M.; Takami, A.; Okada, Y.; Kawata, T.; Tagami, Y.; Ohashi, H.; Sato, M.; Sugiyama, T.; Fukushima, K.; Iijima, H. *Bioorg. Med. Chem.* **2007**, *15*, 1022.
24. Takami, A.; Iwakubo, M.; Okada, Y.; Kawata, T.; Odai, H.; Takahashi, N.; Shindo, K.; Kimura, K.; Tagami, Y.; Miyake, M.; Fukushima, K.; Inagaki, M.; Amano, M.; Kaibuchi, K.; Iijima, H. *Bioorg. Med. Chem.* **2004**, *12*, 2115.
25. Takami, A.; Iijima, H.; Iwakubo, M.; Okada, Y. Nitrogen-containing Compounds Having Kinase Inhibitory Activity and Drugs Containing the Same. 2001, US 2004/0102437 A1.
26. Davies, B.; Morris, T. *Pharm. Res.* **1993**, *10*, 1093.