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Rho Kinase (ROCK) Inhibitors

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Abstract

The Rho kinase (ROCK) isoforms, ROCK1 and ROCK2, were initially discovered as downstream targets of the small GTP-binding protein Rho. Because ROCKs mediate various important cellular functions such as cell shape, motility, secretion, proliferation, and gene expression, it is likely that this pathway will intersect with other signaling pathways known to contribute to cardiovascular disease. Indeed, ROCKs have already been implicated in the regulation of vascular tone, proliferation, inflammation, and oxidative stress. However, it is not entirely clear how ROCKs are regulated, what some of their downstream targets are, and whether ROCK1 and ROCK2 mediate different cellular functions. Clinically, inhibition of ROCK pathway is believed to contribute to some of the cardiovascular benefits of statin therapy that are independent of lipid lowering (ie, pleiotropic effects). To what extent ROCK activity is inhibited in patients on statin therapy is not known, but it may have important clinical implications. Indeed, several pharmaceutical companies are already actively engaged in the development of ROCK inhibitors as the next generation of therapeutic agents for cardiovascular disease because evidence from animal studies suggests the potential involvement of ROCK in hypertension and atherosclerosis.

Keywords

actin cytoskeleton; Rho GTPase; hypertension; inflammation; atherosclerosis

INTRODUCTION

The small GTP-binding proteins belonging to the Rho family regulate various aspects of cell shape, motility, proliferation, and apoptosis.^{1,2} Rho kinases (ROCKs), which were the first downstream effectors of Rho to be discovered,^{3–5} were found to mediate RhoA-induced actin cytoskeletal changes through effects on myosin light chain phosphorylation.^{6,7} ROCKs are protein serine/threonine kinases that share 45% to 50% homology to other actin cytoskeletal kinases such as myotonic dystrophy kinase (DMPK), myotonic dystrophy-related cdc42-binding kinase (MRCK), and citron kinase.¹ ROCKs consist of an amino-terminal kinase domain, followed by a mid coiled-coil-forming region containing a Rho-binding domain (RBD), and carboxy-terminal cysteine-rich domain (CRD) located within the pleckstrin homology (PH) motif. Two ROCK isoforms have been identified in mammalian system. *ROCK1*, which is also known as ROK β and p160ROCK, is located on chromosome 18 and encodes a 1354-amino acid protein.^{5,6} *ROCK2*, which is also known as ROK α and sometimes

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confusingly called Rho-kinase, is located on chromosome 12 and contains 1388 amino acids.^{3,4,8} ROCK1 and ROCK2 share an overall 65% homology in amino-acid sequence and 92% homology in their kinase domains (Figure 1).

The carboxy-terminal regions of ROCKs serve as an autoregulatory inhibitor of the amino-terminal kinase domain.⁹ The interaction of the active GTP-bound form of Rho to ROCK's RBD increases ROCK activity through *derepression* of the carboxyl-terminal RBD-PH domain on the amino-terminal kinase domain, leading to an active "open" kinase domain. The open conformation could also be caused by the binding of arachidonic acid to the PH domain¹⁰ or cleavage of the carboxyl-terminus in ROCK1 by caspase-3^{11,12} and that in ROCK2 by granzyme B or caspase-2.^{13,14} This closed-to-open conformation of ROCK activation is similar to that of DMPK and MRCK activation^{9,15} and is consistent with studies showing that overexpression of various carboxyl-terminal constructs of ROCK or kinase-defective forms of full-length ROCK, functions as dominant-negative ROCK mutants.^{5,6,16} ROCKs can also be activated independently of Rho through amino-terminal transphosphorylation^{15,17} or inhibited by other small GTP-binding proteins such as Gem and Rad.¹⁸

Downstream Targets of ROCK

In response to activators of Rho, such as lysophosphatidic acid (LPA) or sphingosine-1 phosphate (S1P), which stimulate Rho guanine nucleotide exchange factor (GEF) and lead to the formation of active GTP-bound Rho, ROCKs mediate a broad range of cellular responses that involve the actin cytoskeleton. For example, they control assembly of the actin cytoskeleton and cell contractility by phosphorylating a variety of proteins, such as myosin light chain (MLC) phosphatase, LIM kinases, adducin, and ezrin-radixin-moesin (ERM) proteins (Figure 2). These actin cytoskeletal proteins are also phosphorylated by other serine-threonine kinases such as protein kinase A, protein kinase C, and G-kinase.^{19,20} The consensus amino acid sequences for phosphorylation are R/KXS/T or R/KXXS/T (R: arginine; K: lysine; X: any amino acid; S: serine; T: threonine).^{21,22} ROCKs can also be auto-phosphorylated,^{3,5} which might modulate their function.

Despite having similar kinase domain, ROCK1 and ROCK2 may serve different functions and may have different downstream targets. Specifically, ROCK2 phosphorylates Ser¹⁹ of MLC, the same residue that is phosphorylated by MLC kinase (MLCK). Thus, ROCK2 can alter the sensitivity of SMC contraction to Ca²⁺ since MLCK is Ca²⁺-sensitive.²³ In addition, ROCKs regulate MLC phosphorylation indirectly through the inhibition of MLC phosphatase (MLCP) activity. MLCP holoenzyme is composed of 3 subunits: a catalytic subunit (PP1 δ), a myosin-binding subunit (MBS) composed of a 58-kD head and 32-kD tail region, and a small non-catalytic subunit, M21. Depending on the species, ROCK2 phosphorylates MBS at Thr⁶⁹⁷, Ser⁸⁵⁴, and Thr⁸⁵⁵.²² Phosphorylation of Thr⁶⁹⁷ or Thr⁸⁵⁵ attenuates MLCP activity¹⁰ and in some instances, the dissociation of MLCP from myosin.²⁴ ROCK2 also phosphorylates ERM proteins, namely Thr⁵⁶⁷ of ezrin, Thr⁵⁶⁴ of radixin, and Thr⁵⁵⁸ of moesin.²⁵ ROCK-mediated phosphorylation leads to the disruption of the head-to-tail association of ERM proteins and actin cytoskeletal reorganization. In contrast, ROCK1 phosphorylates LIM kinase-1 at Thr⁵⁰⁸ and LIM kinase-2 at Thr^{505,21,26} which enhance the ability of LIM kinases to phosphorylate cofilin.²⁷ Since cofilin is an actin-binding and actin-depolymerizing protein that regulates the turnover of actin filaments, the phosphorylation of LIM kinases by ROCKs inhibits cofilin-mediated actin filament disassembly and leads to an increase in the number of actin filaments. Further studies concerning the physiological role of these downstream targets of ROCKs are expected with great respects.

Cellular Functions of ROCK

ROCKs are important regulators of cellular growth, migration, metabolism, and apoptosis through control of the actin cytoskeletal assembly and cell contraction.¹ Although there is no evidence that ROCK isoforms have different functions, they are differentially expressed and regulated in various tissues. For example, only ROCK1 is cleaved by caspase-3 during apoptosis,^{11,12} while smooth muscle-specific basic calponin is phosphorylated only by ROCK2.²⁸ Furthermore, ROCK1 expression tends to be more ubiquitous, while ROCK2 is most highly expressed in cardiac and brain tissues.^{8,29,30} Indeed, homozygous deletion of ROCK1 and ROCK2 leads to differing causes of embryonic lethality.^{31,32} Thus, it is likely that using a genetic approach to dissecting the roles of ROCK isoforms (ie, conditional ROCK deletion), distinct and novel cellular functions will be uncovered, which could be specifically ascribed to either ROCK1 or ROCK2.

Stimulation of tyrosine kinase and G-protein–coupled receptors leads to activation of Rho, the direct upstream activator of ROCKs, via recruitment and activation of RhoGEF.^{33,34} ROCKs are important effectors of Rho in regulating the actin cytoskeleton. Inhibitors of ROCKs, such as Y27632 and fasudil, or overexpression of dominant-negative mutants of ROCKs lead to the loss of stress fibers and focal adhesion complexes.^{5,35} This is due predominantly to the phosphorylation and inhibition of MLCP by ROCK, which increases MLC phosphorylation and cellular contraction, by facilitating interaction of myosin with F-actin (Figure 2). Thus, ROCKs regulate cell polarity and migration predominantly through enhancing actomyosin contraction and focal adhesions. This would increase cellular contraction as well as mediate cellular migration and chemotaxis. Indeed, increased ROCK activity is observed in tumor metastasis³⁶ and overexpression of constitutively activated ROCK promotes tumor invasion.³⁷ ROCK also regulates leukocyte chemotaxis, possibly by altering the localization and activation of phosphatase and tensin homologue (PTEN).^{38,39} Conversely, invasion of rat hepatoma cells and migration of metastatic breast cancer cells are inhibited by overexpression of dominant-negative ROCK constructs or by the ROCK inhibitor Y-27632.⁴⁰ Treatment with Y-27632 reduces tumor-cell dissemination in vivo, suggesting its potential use in cancer therapy.⁴⁰ In addition, ROCKs could also regulate macrophage phagocytic activity via actin cytoskeletal membrane protrusions and mediate endothelial cell permeability via effects on tight and adheren junctions.^{41,42} ROCKs could inhibit insulin signaling via phosphorylation of insulin receptor substrate (IRS)-1, which uncouples the insulin receptor to phosphatidylinositol-3 kinase.⁴³ Conversely, it could also regulate cell size via enhancing insulin-like growth factor (IGF)-induced cAMP response element binding protein (CREB) phosphorylation.⁴⁴ Indeed, this may be the underlying mechanism by which ROCK inhibitors reduce cardiac hypertrophy.^{45,46} Finally, ROCKs may be involved in tissue differentiation from adipocytes to myocytes. In p190-B Rho GTPase-activating protein (GAP)-deficient mice, which have high basal Rho/ROCK activity because there is no “off switch” for Rho, there is a defect in adipogenesis, with a predilection toward myogenesis.^{44,47} Treatment of p190-B RhoGAP-deficient mice with Y27632 restores normal adipogenesis,⁴⁷ suggesting that ROCKs are involved in the myogenesis differentiation program.

Role of ROCK in Cardiovascular Disease

Many cholesterol-independent or so-called “pleiotropic” effects 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors or statins are due to their ability to block the synthesis of isoprenoid intermediates, which serve as important lipid attachments for a variety of intracellular signaling molecules.⁴⁸ In particular, the inhibition of small GTP-binding proteins Rho, Ras, and Rac, whose proper membrane localization and function are dependent on isoprenylation,⁴⁸ may play an important role in mediating the biological effects of statins. For example, statins increase the expression of endothelial nitric oxide synthase (eNOS) via inhibition of RhoA/ROCK-mediated actin cytoskeletal changes, leading to the stabilization of

eNOS mRNA.^{49,50} Indeed, a recent report suggests that binding of G-actin to the 3'-untranslated region of eNOS mRNA decreases eNOS mRNA expression.⁵¹ Furthermore, inhibition of Rho/ROCK pathway leads to the rapid phosphorylation and activation of eNOS via the phosphatidylinositol (PI)-3 kinase/protein kinase Akt pathway.^{52,53} Thus, Rho/ROCKs negatively regulate endothelial function at the level of both eNOS expression and activation via 2 distinct mechanisms.

There is growing evidence that abnormal ROCK function contributes to cardiovascular disease.⁵⁴ In the vascular wall, ROCK mediates vascular smooth muscle contraction, actin cytoskeleton organization, cell adhesion, and motility.⁵⁵ Thus, abnormal ROCK activity may contribute to abnormal smooth muscle contraction observed in cerebral and coronary vasospasm,^{56,57} hypertension,⁵⁸ and pulmonary hypertension.⁵⁹ In addition, ROCK could also regulate vascular tone and blood flow indirectly through negative effects on eNOS expression and activity^{52,60} or via direct effects on the central nervous system.^{61,62} Inhibition of ROCK leads to increase in cerebral blood flow and decrease in cerebral infarct size via upregulation of eNOS.⁶³ ROCK is also involved in vascular inflammation and remodeling,⁶⁴ restenosis after balloon injury,^{65–67} ischemia-reperfusion injury,^{52,68,69} and atherosclerosis.^{70,71} Recent studies also suggest that long-term treatment with a ROCK inhibitor, fasudil, improved monocrotaline-induced fatal pulmonary hypertension in rats⁵⁹ and suppresses cardiac allograft vasculopathy in mice.⁷² ROCK has also been implicated in the expression of a variety of genes, which are pertinent to vascular function, such as monocyte chemoattractant protein-1 (MCP-1),⁷³ plasminogen activator inhibitor-1 (PAI-1),⁷⁴ and osteopontin.⁷⁵ Indeed, ROCK is up-regulated by inflammatory stimuli, such as angiotensin II and interleukin-1 β , in cultured cells⁷⁶ and by lipopolysaccharide (LPS) in vivo.⁷⁷

Rationale for the Development of ROCK Inhibitors

Despite an increasing number of reports showing that ROCK activity is increased under a variety of pathological conditions, little is known about the molecular mechanisms that contribute to increased ROCK activity or what the downstream targets for ROCK are. Furthermore, determining the precise role of ROCK in the vascular wall is limited by pharmacological inhibitors, which cannot discriminate between ROCK isoforms or the role of ROCKs in individual component cells. Hence, a genetic approach with tissue-specific gene targeting of specific ROCK deletion to individual components of the vascular wall offers the greatest likelihood of success in dissecting the role of pathophysiological role of ROCKs. Because ROCKs are critical for cardiovascular and central nervous system (CNS) development, embryonic lethality occurs in both ROCK1^{-/-} and ROCK2^{-/-} mice.^{31,78} However, the phenotypes of the lethality are quite different. ROCK1^{-/-} mice die soon after birth due to development of omphalocele caused by a defect in umbilical ring closure from impairment of filamentous actin accumulation. ROCK1^{-/-} mice also exhibit eyes open at birth (EOB) due to disorganization of actin filaments in the epithelial cells of the eyelid. In contrast, ROCK2^{-/-} mice die embryonically due to dysfunction and intrauterine growth retardation caused by the manifest thrombus formation in the labyrinth layer of the placenta. These findings suggest distinct tissue distribution and downstream targets of ROCK1 and ROCK2. Indeed, although previous studies suggest that ROCK inhibitors prevent the development of cardiac hypertrophy,^{78–80} ROCK1^{-/-} mice develop cardiac hypertrophy, but not fibrosis.^{78,81} It is possible that ROCK2 but not ROCK1 is involved in the development of cardiac hypertrophy. Furthermore, nonselective ROCK inhibitors have been shown to decrease systemic blood pressure.^{58,82} However, neither the haploinsufficient ROCK1 nor ROCK2 mouse, show any differences in basal or angiotensin II-induced increase in systemic blood pressure compared with that of wild-type mice.⁷⁸

Nevertheless, because ROCK is involved in various aspects of vascular function and inflammatory conditions, the development of selective and nonselective ROCK inhibitors has gained considerable interest in the pharmaceutical industry. Presently, Y-27632 and fasudil are non-isoform-selective ROCK inhibitors that target their ATP-dependent kinase domains and are therefore equipotent in terms of inhibiting both ROCK1 and ROCK2. Neither fasudil nor Y27632 can distinguish between ROCK1 and ROCK2. Furthermore, at higher concentrations, these ROCK inhibitors could also inhibit other serine-threonine kinases such as PKA and PKC.⁶³ Nevertheless, compared with the other kinases, fasudil and its active metabolite, hydroxyfasudil, are relatively more selective for ROCKs, with hydroxyfasudil being slightly more selective than fasudil and Y27632.⁶³ Compared to ROCKs, the IC₅₀ value for PKA was approximately 5-fold higher for fasudil and 50-fold higher for hydroxyfasudil. With the exception of PKC isoforms, which have IC₅₀ values ranging from 20 to 100 μ mol/L, all of the other protein kinases tested such as Raf1, ERK, and p38, have IC₅₀ values that were > 100 μ mol/L for fasudil and hydroxyfasudil.⁶³

ROCK Inhibitors in Cardiovascular Disease

Non-isoform-selective ROCK inhibitors such as fasudil have been shown to prevent cerebral vasospasm after subarachnoid hemorrhage.^{56,83} Similarly, animal studies with Y-27632 showed that it could inhibit the development of atherosclerosis and arterial remodeling following vascular injury.^{65,70} ROCK activity is involved in the expression of PAI-1 mediated by hyperglycemia, indicating that ROCK may function as a key regulator of cardiovascular injury in patients with diabetes mellitus.⁸⁴ Furthermore, RhoA/ROCK pathway has been reported to be involved in angiogenesis,^{85,86} cerebral ischemia,⁸⁷ erectile dysfunction,⁸⁸ glomerulosclerosis,⁹⁰ hypertension,³⁵ myocardial hypertrophy,⁷⁹ myocardial ischemia-reperfusion injury,^{52,69} neointima formation,^{65,91} pulmonary hypertension,⁵⁹ and vascular remodeling.⁷¹ Moreover, ROCK inhibitors have shown benefits in Alzheimer's disease,⁹² bronchial asthma,⁹³ cancers,⁹⁴ demyelinating diseases,⁹² glaucoma,⁹⁵ and osteoporosis.⁹⁶ Although most of the previous studies have shown that inhibition of both isoforms by ROCK inhibitors results in the beneficial effect, whether the effects are mediated by inhibition of ROCK1, ROCK2, or both, remains to be determined.

Development of ROCK Inhibitors in Cardiovascular Disease

Inhibition of ROCK by fasudil leads to beneficial effects in patients with systemic hypertension,⁵⁸ pulmonary hypertension,⁹⁷ vasospastic angina,⁵⁷ stable effort angina,⁹⁸ stroke,⁹⁹ and chronic heart failure.¹⁰⁰ Indeed, perhaps many of the so-called "pleiotropic" effects of statins may be mediated by ROCK inhibition,^{49,50,52,60,101,102} while the extent of inhibitory effect of ROCK by statins remains to be cleared, especially in humans. However, despite the potential clinical importance of ROCK inhibition, fasudil is the only ROCK inhibitor approved for human use.

The development of fasudil began with functional research on calmodulin inhibition by quinoline or isoquinoline derivatives. Fasudil was obtained by chemical screening of isoquinoline sulfonamide derivatives using a bioassay for vasodilatory activity of normal and spastic arteries. The isoquinoline derivatives are metabolized to their hydroxyl forms in animals and humans. The hydroxyl form of fasudil, hydroxyfasudil, has the same ROCK inhibitory activity as fasudil. Although the half-life of fasudil in humans is extremely short (eg, less than 0.5 hours), the half-life of hydroxyfasudil, which is >5 hours, is of sufficient duration for the efficacy of ROCK inhibition to be observed with twice-daily fasudil administration. Furthermore, the ratios of the protein-unbound form of fasudil and hydroxyfasudil are greater than 50% in human plasma. The large amount of protein-unbound form allows the drug to easily distribute to target organs and may contribute to its efficacy. In 1995, fasudil was approved in Japan and China for prevention and treatment of cerebral vasospasm following

surgery for subarachnoid hemorrhage and has since been used in over 124,000 patients in Japan. Although several adverse effects such as hepatic function abnormal, intracranial hemorrhage, and hypotension have been reported, previous investigators have also revealed that no serious adverse events have been seen not only in fasudil-treated patients of subarachnoid hemorrhage but also of acute ischemic stroke compared with those in placebo-treated patients.^{99,103,104} Hence, as a selective ROCK inhibitor, fasudil is currently being developed for treating acute stroke and pulmonary artery hypertension.

Given the breadth of preclinical data suggesting benefits of ROCK inhibition in hypertension and cardiovascular diseases, many biotechnology and pharmaceutical companies are developing other selective and nonselective ROCK inhibitors (Table 1). Many of these compounds are still in the discovery phase. So, it will be some time before we know whether ROCK isoforms are viable targets in humans at risk for cardiovascular disease.

CONCLUSIONS

There is growing evidence that RhoA/ROCK pathway plays an important pathophysiological role in cardiovascular diseases and that inhibition of ROCKs by ROCK inhibitors or statins may be beneficial. To date, a great number of cellular and physiological functions are mediated by ROCK, and ROCK activity is often elevated in disorders of the cardiovascular system. Thus, inhibition of ROCK may be an attractive therapeutic target in reducing cardiovascular disease. However, a greater understanding of the physiological role of each ROCK isoforms in the cardiovascular system and the development of isoform-specific inhibitors are needed to resolve the specificity and safety of ROCK inhibitors.

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FIGURE 1.

Structures of ROCK isoforms. Both ROCKs consist of an amino-terminal kinase domain followed by a coiled-coil forming region containing a Rho-binding (RB) domain and a carboxy-terminal cysteine-rich domain (CRD) located within the pleckstrin-homology (PH) domain. ROCK1 and ROCK2 share overall 65% homology in amino acid sequence and 92% homology in their kinase domains.

FIGURE 2.

Regulation of cellular function by ROCK. Stimulation of G-protein-coupled receptors (GPCR) leads to an increase in intracellular calcium/calmodulin (CaM)-mediated activation of myosin light chain kinase (MLCK). MLCK phosphorylates MLC, leading to actin-myosin interaction and cellular contraction, migration, proliferation, and survival. Stimulation of GPCR also leads to ROCK activation via Rho guanine exchange factor (GEF). Activated ROCK, mediated through, phosphorylates various downstream targets, such as ezrin-radixin-moesin (ERM), a 17-kDa PKC-potentiated inhibitory protein of protein phosphatase-1 (CPI17), and the myosin-binding subunit (MBS) of MLC phosphatase. Phosphorylation of MBS inhibits MLC phosphatase activity leading to increase MLC phosphorylation and actomyosin activation. ILK, integrin-linked kinase.

ROCK Inhibitors

Table 1

Compound	Isoform Selectivity	Therapeutic Area	Company	Development Status
Fasudil (Intravenous and oral formulation)	None K _i 0.40 μM for ROCK	Cerebral Vasospasm, acute stroke, angina, pulmonary hypertension	Asahi-Kasei Pharmaceuticals, Inc.	Approved in Japan for cerebral vasospasm and ischemia; Phase 2 for other indications
Y27632	None K _i 0.22 μM for ROCK1; K _i 0.30 μM for ROCK2	Reperfusion injury, hypertension, stroke, asthma, cancer	Mitsubishi Pharmaceuticals	Discontinued, research tool
Y39983 (Ophthalmic liquid formulation)	Unknown	Glaucoma	Mitsubishi Pharmaceuticals, Senju Pharmaceuticals, Novartis AG	Phase I
Wf-536	Unknown	Cancer	Mitsubishi Pharmaceuticals	Discovery phase
SLx-2119	ROCK2 K _i 25 nM for ROCK1; K _i 5 nM for ROCK2	Atherosclerosis, fibrosis, solid tumors	Surface Logix, Inc.	Discovery phase
Azabenzimidazole-aminofurazans (ROCK inhibitor, GSK)	None GSK-269962A, IC ₅₀ 1.6 nM; SB772077, IC ₅₀ 5.6 nM	Hypertension, antiinflammatory	Glaxo-Smith-Kline	Discovery phase
DE-104, Olefins, Isoquinolines, Indazoles, pyridinealkene derivatives	None	Glaucoma	Santen Pharmaceuticals, UBE Industries	Discovery phase
H-1152P	None K _i 6 nM for ROCK1; IC ₅₀ 12 nM for ROCK2	Glaucoma	Kowa Pharmaceuticals	Discovery phase
ROKα inhibitor (ROCK inhibitor, BF)	? ROCK2 BF 66851, K _i 140 nM for ROCK; BF66852, K _i 86 nM for ROCK; BF66853, K _i 52 nM for ROCK	Asthma, CV disease, erectile dysfunction, glaucoma, HIV infection, osteoporosis	BioFocus plc	Discovery phase
XD-4000	ROCK2	Atherosclerosis, fibrosis, solid tumors	Xcellsysz, Ltd	Discovery phase
HMN-1152	Unknown	Hearing loss, tinnitus, hypertension, cerebrovascular disease	Nagoya University	Discovery phase
4-(1-aminoalkyl)-N-(4-pyridyl)cyclohexane-carboxamides (Oral formulation)	None	Neurodegenerative diseases	BioAzone Therapeutics	Discovery phase
Rhostatin (Drug implant, recombinant protein)	Unknown	Solid tumors, brain tumors	BioAzone Therapeutics	Discovery phase
BA-210	Unknown	Macular degeneration, spinal cord injury, glaucoma, cancer	BioAzone Therapeutics	Phase 2
BA-207, BA-215, BA-285, BA-1037	Unknown	Cardiovascular disease, macular degeneration, cancer	BioAzone Therapeutics	Discovery phase
Ki-23095	Unknown	Cardiovascular and renal disease	Kirin Brewery Co.	Discovery phase
VAS-012	Unknown	Cancer	VasGene Therapeutics	Discovery phase
ROCK Inhibitor (quinazoline)	Unknown	Cancer	Bayer AG	Discovery phase