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Rho-kinase inhibitors as therapeutics: from pan inhibition to isoform selectivity

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Abstract The emerging critical implications of Rho/Rho-kinase (ROCK) signaling in neurodegenerative diseases, glaucoma, renoprotection, diabetes and cancer have sparked growing interest in the pharmacological potential of ROCK inhibitors beyond their current application in cardiovascular disease. This article discusses the therapeutic benefits of novel ROCK inhibitors in development, and highlights the recent advances in the current understanding of disease-dependent and isoform-specific functions of ROCK and their potential impact on future therapeutic strategies.

Keywords Rho-kinase · ROCK · ROCK1 · ROCK2 · Fasudil · Y-27632

Rho-associated kinase (ROCK) belongs to the AGC (PKA/PKG/PKC) family of serine-threonine protein kinases and is a major downstream effector of the small GTPase RhoA [1–3]. To date, two ROCK isoforms have been described; ROCK1 (ROKβ, p160ROCK) and ROCK2 (ROKα). ROCK1 and ROCK2 are highly homologous, sharing 65% homology in amino acid sequence and 92% homology in their kinase domains. Although both isoforms are ubiquitously expressed, ROCK1 expression is enriched in lung, liver, spleen, kidney, and testis, whereas ROCK2 is more prominent in the brain and heart [4]. ROCK activity leads to the phosphorylation of downstream targets including myosin light chain (MLC) [5, 6], MLC phosphatase (MYPT1) [7, 8], ezrin/radixin/moesin (ERM) [9], adducin [10], and LIM kinases (LIMK) [11–13], thereby modulating actin cytoskeletal organization, stress fiber formation and cell contraction [14]. ROCK controls vascular smooth muscle contraction and endothelial barrier function [15–22]. This key role as a master regulator of the vascular bed explains why cardiovascular diseases were the primary indication in early ROCK drug discovery projects and these remain ongoing efforts.

ROCK1$^{-/-}$ and ROCK2$^{-/-}$ knockout mice develop a similar phenotype resulting in eyes-open at birth and omphalocele [23–25], demonstrating that the biological functions of ROCK1 and ROCK2 isoforms are in many cases redundant and cannot be separated. For example, both isoforms phosphorylate the same major downstream substrates such as smooth muscle MLC and MYPT1 in vitro [26]. Given the pathway redundancy and the high degree of homology within the kinase domain of both isoforms, it was believed that isoform selectivity could not be achieved, and early drug discovery efforts concentrated on the development of non-isoform selective ROCK inhibitors. To date, the only clinically approved ROCK inhibitor from these efforts is Fasudil, which was approved in Japan in 1995 for the treatment of vasospasm following subarachnoid haemorrhage. Besides regulating the cardiovascular bed, ROCKs are involved in a myriad of biological functions such that inhibition of ROCK activity could be of potential benefit for the treatment of diseases ranging from glaucoma to neurodegenerative diseases to cancer. For an overview see Table 1.

Most of the studies were performed using chemical inhibitors, and one has to be careful how to interpret these data, especially when using Fasudil and Y-27632, considering their selectivity profile and potency. Y-27632 was non-selective against 4 out of 25 tested kinases [27] and

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Table 1 Effects of pharmacological ROCK inhibitors on disease biology

Therapeutic area	Compounds	Pharmacological effects	Species	References
Hypertension	Fasudil	Decrease in blood pressure and vascular resistance	Clinical study	[66, 67]
	AD	Decrease in blood pressure	Rat, mouse, dog	[59, 60]
	AA	Decrease in blood pressure	Rat	[34]
	SAR407899	Decrease in blood pressure	Rat, mouse	[31]
Glaucoma	Fasudil	Decrease in IOP	Rabbit	[68]
	Y-27632	Decrease in IOP, increase in aqueous outflow	Rabbit, mouse	[37, 69, 70]
		Increase in aqueous outflow	Monkey, pig	[71, 72]
	Y-39983	Decrease in IOP	Mouse, rabbit, monkey	[36, 37]
Neurodegeneration	Fasudil, Y-27632	Axonal sprouting and functional recovery after SCI	Rat	[73–76]
	Y-27632	Decreased amount of amyloidogenic Aβ42	Mouse	[77]
	Fasudil, Y-27632, H-1152	Inhibition of mutant huntingtin aggregation	Mouse (in vitro)	[78]
Oncology	Wf-536 (Y-32885)	Inhibition of tumor metastasis	Mouse	[79]
		Inhibition of tumor growth, angiogenesis and metastasis	Human lung and prostate cancer xenograft	[32, 80]
	Y-27632	Inhibition of tumor metastasis (in vivo), angiogenesis (in vitro)	Human hepatocellular and prostate cancer xenograft	[81, 82]
	Fasudil	Inhibition of tumor progression and metastasis	Human lung and breast cancer xenograft	[83]
Renoprotection	Y-27632	Macrophage infiltration and interstitial fibrosis	Mouse	[84]
	Fasudil	Improved proteinuria, glomerulosclerosis, renal interstitial fibrosis and macrophage infiltration	Rat	[85–89]
		Improved progression of diabetic nephropathy	Mouse	[90]
Diabetes	Fasudil	Prevention of diabetes development	Rat	[86]
		Corrected glucose and lipid metabolism, improved insulin signaling	Rat	[91]
Erectile dysfunction	Y-27632	Improved erectile function	Rat	[92–94]
	H-1152, Y-27632	Improved erectile function	Rat	[95]

All data are derived from in vivo experiments unless otherwise noted

AA Aminofurazan azabenzimidazoles, *AD* azaindole derivative, *IOP* intraocular pressure, *SCI* spinal cord injury

Fasudil exhibited non-selectivity for 8 out of 27 tested kinases [27]. Both inhibitors have biochemical IC_{50}s in the hundreds of nanomolar, but they are typically used at concentrations of 10–50 μM in cell-based studies, opening up the possibility that observed phenotypes could be off-target effects. However, based on the overall promising studies showing efficacy of ROCK inhibitors in a variety of animal disease models, there are significant efforts aimed at developing more potent and selective ROCK inhibitors. Recent activities within the pharmaceutical industry and academia have led to a series of novel ROCK inhibitors with enhanced potency within the low nanomolar range and improved kinase selectivity [28–34]. In addition, as of July 2009, two compounds, RKI983 (Novartis) and INS117548 (Inspire), are in phase I/II studies for the treatment of glaucoma (NCT00846989, NCT00767793). Moreover, phase II trials for the treatment of glaucoma investigating DE-104 (Santen) as well as phase I/II trials for the treatment of spinal cord injury investigating BA-210 (Alseres Pharmaceuticals) are completed (NCT00650338, NCT00500812). Recently published data from a phase I clinical trial for the treatment of glaucoma

using SNJ-1656 [35] showed no adverse systemic effects and only minor side effects in this 7-day repeated-instillation trial when dosed either once or twice a day, i.e., the occurrence of ocular hyperemia was reported, which is in accordance with previous animal studies using Y-39983 [36]. Consistent with the old paradigm that both ROCK isoforms show similarity in their downstream targets, recent studies of the role of ROCK in glaucoma suggest that neither isoform is predominant in modulating intraocular pressure (IOP), since mice deficient for either ROCK1 or ROCK2 exhibit a significant decrease in IOP compared to wild-type littermates [37]. Off-target effects and systemic toxicity might play a minor role for applications such as for the treatment of glaucoma, where pharmacokinetic and pharmacodynamic parameters of inhibitors can be optimized to reduce or eliminate systemic exposure. However, little is known about side effects and toxicity of ROCK inhibitors for systemic and long-term applications and these need to be further investigated. From the knockout mouse models, it is known that inhibition of both ROCK isoforms results in embryonic death due to placental malfunction, and ROCK inhibitors might be counter-indicated for pregnant women. In addition, given ROCK's involvement in many biological functions, especially the regulation of blood pressure, isoform-selective ROCK inhibitors may be preferential as systemic drugs. For any disease, blood pressure changes will need to be monitored with respect to the efficacious dose of ROCK inhibitor for the indicated disease, and it has to be decided what level of blood pressure decrease is acceptable or even advantageous and what level would be a health concern.

Recent genetic studies as well as animal disease models, along with short interfering RNA (siRNA)-based gene silencing experiments in vitro, provide significant insights into ROCK isoform biology. Cell type-specific cases, in which either ROCK1 or ROCK2 appear to have distinct non-redundant functions, have been shown in fibroblasts and vascular smooth muscle cells. Whereas knockdown of ROCK1 but not ROCK2 leads to disassembly of stress fibers in fibroblasts [26], in smooth muscle cells this phenotype is mediated by knockdown of ROCK2 [38]. These findings could be explained by the fact that both isoforms are expressed to different levels in individual cell types and have unique interaction partners. ROCK1 but not ROCK2 is regulated by RhoE [39–41], which competes with RhoA for interaction with ROCK1. In addition, ROCK1 is cleaved by caspase-3 at a conserved sequence in the C-terminus that does not exist in ROCK2, leading to constitutively active ROCK1, MLC2 phosphorylation, and membrane blebbing during apoptosis [42, 43]. In contrast, during granzyme B-induced cell death, granzyme B specifically cleaves and activates ROCK2 but not ROCK1, resulting in downstream MLC2 phosphorylation and membrane blebbing [44]. ROCK1 and ROCK2 activity and signaling, however, are not only regulated differentially by their upstream modulators but both ROCK isoforms also utilize selective downstream partners to mediate their biological functions. For example, ROCK2 induces degradation of transforming growth factor β type I receptor [45], thereby regulating mesoderm induction. In vascular smooth muscle cells, both ROCK isoforms modulate MYPT1 activity but have different effects on smooth muscle cell morphology. Only ROCK2 binds directly to and phosphorylates MYPT1 [38]. In contrast, upon UV-induced stress, ROCK1 activates c-Jun N-terminal kinase (JNK) and induces apoptosis through binding and phosphorylating JNK-interacting protein 3 (JIP-3) [46]. In addition, cell spreading of breast cancer cells during mesenchymal-mode of 3D-migration depends upon specific ROCK1-LIMK2 interaction [47]. The functional and regulatory significance of each isoform is further highlighted during pathological conditions. Induction of pressure overload cardiac hypertrophy in mice by aortic banding shows that, at the state of stable hypertrophy, after 3 weeks the levels of ROCK1 expression and ERM phosphorylation are increased, while ROCK2 expression levels remain unaltered. Even though the disruption of ROCK1 did not affect the development of cardiac hypertrophy, the development of fibrosis in the myocardium was significantly reduced in the ROCK1$^{-/-}$ [48] and ROCK1$^{+/-}$ mice [49]. ROCK isoforms also have distinct contributions in cancer progression. Increased ROCK2 levels have been reported in hepatocellular [50], colon [51], and bladder [52] cancer. A study of 41 pairs of hepatocellular carcinomas revealed that ROCK2 is frequently overexpressed as compared to non-tumorous livers, while ROCK1 expression is unaltered. Silencing of ROCK2 by short-hairpin RNA reduces stress fiber formation, phosphorylation of MYPT1, migration and invasion in vitro, and lung metastasis in vivo [50]. In contrast, ROCK1 expression levels, but not ROCK2, are significantly higher in human mammary tumors and are associated with poor clinical outcome and overall survival of patients [53], and elevated ROCK1 levels were recently reported to be involved in the transformation of hormone-refractory prostate cancer [54]. Overexpression of ROCK isoforms during disease progression could be the cause for or the effect of the disease. Recent advances in siRNA delivery should allow researchers to answer those questions not only in vitro but also in vivo. It is now possible to not only administer siRNA systemically but also to target specific cells or organs. β1,3-D-glucan-encapsulated siRNA nanoparticles were successfully used to target specifically macrophages [55], and magnetic siRNA nanoparticles were used to target solid tumors [56]. Others successfully applied siRNA coupled to an antibody or cell surface receptor ligand such as transferrin [57] or rabies

virus glycoprotein [58] to target a specific cell type. In addition, Alcon Research filed a patent application to treat glaucoma using siRNA-mediated knockdown of ROCK (WO/2007/076367), demonstrating that siRNA treatment in vivo cannot only be used as a tool to investigate pathway biology but also shows the potential for the use of siRNA as therapeutic agent.

Despite the significant progress and the increasing understanding of the functions of ROCK isoforms in disease progression, it is notable that recent patents on ROCK inhibitors [30, 34, 59, 60] do not address isoform selectivity. This might be due to the fact that both isoforms show such a high degree in amino acid homology within the kinase domain that it is not believed to be possible to achieve isoform selectivity. In fact, prior to 2008, all published ROCK inhibitors are equally potent against both isoforms. Only recently has one isoform-selective ROCK inhibitor been described. SLx-2119 is a ROCK2 selective compound that has shown promise in cancer xenograft models [61] and in preclinical models of fibrosis [62]. SLx-2119 has further been reported to attenuate arterial plaque formation in apolipoprotein-E-deficient mice. At the same time, this inhibitor avoids unwanted hemodynamic side effects compared with non-selective ROCK inhibitors [63]. Interestingly, these data are in conflict with the findings that ROCK2 polymorphism [64] and a haplotype block consisting of 4 SNPs within the ROCK2 allele [65] are associated with changes in systemic blood pressure.

In conclusion, ROCK inhibition has shown promise as a therapeutic target for a variety of human diseases, and several small molecule inhibitors are in development. For some diseases, such as glaucoma, ROCK isoform selectivity is not required, and for such topical applications the compound can be easily dosed directly to the target organ. A systemic inhibition of ROCK, however, bears the risk of unwanted side effects such as drop of blood pressure and it has to be carefully evaluated if the benefit is to outweigh the risk in a disease-dependent context. Even though ROCKs are expressed ubiquitously, there is rationale for future efforts towards isoform-selective ROCK inhibitors. A growing body of evidence clearly indicates that both ROCK1 and ROCK2 have distinct expression levels and unique interaction partners in individual tissue types, suggesting that these functional differences could be of therapeutic benefit. Yet, further investigation is required to elucidate the regulatory networks of both isoforms in a disease context, especially in their native in vivo environment. This task may be accomplished by recent advances in siRNA delivery technologies allowing targeting to specific organs or cells. Ultimately, this will not only lead to a deeper understanding of the relative importance of each isoform for the pathophysiology of many human diseases, but will also help the next generation of effective ROCK inhibitors to avoid unwanted systemic side effects by targeting each isoform.

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