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Targeting ROCK2 isoform with its widely used inhibitors for faster post-stroke recovery

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Recovery after ischemic stroke is slow and highly variable. Activated ROCK (Rho-associated coiled-coil kinase) pathway hampers recovery of impaired neurons. Though inhibiting ROCK pathway has shown therapeutic effects *in vitro*, the selectivity of most of the ROCK inhibitors is still not investigated. Present study aims to investigate the binding affinity *in silico* of nine widely used ROCK inhibitors with brain–specific ROCK2 isoform. Three-dimensional structures of ROCK2 and eight drugs were taken from Protein Data Bank and PubChem Chemical Compound Database, respectively, whereas, FSD-C10 structure was generated based on Xin *et al*., 2015. In docking, ROCK2 was set to be rigid and drugs were free to rotate. All simulations were carried out using AutoDock 4.2. This study demonstrated strong complexation between all ligands and ROCK2. All ROCK inhibitors, except FSD-C10, were able to bind to ROCK2 more strongly [Binding constant (K_a) between 2.6 – 36.7 × 10^5 M^{-1}] than fasudil (Ka = 2.5 × 10^5 M^{-1}). SLx-2119 (KD-025) had the highest binding constant (K_a = 36.7 × 10^5 M^{-1}) thus succeeding as a better ROCK2 specific inhibitor. Selectivity of ROCK inhibitors (*in silico*) towards ROCK2 can be an indicative measure to estimate therapeutic benefits or adverse effects prior to *in vitro* study.

Keywords: Binding affinity, Ischemic stroke, Molecular docking, Rho kinase inhibitors

Stroke is the second most common cause for mortality among diseases of cardiovascular origin and has varying incidence, case-fatality, and mortality in different countries[1,2]. Years lived with disability is high among stroke patients with high morbidity even in developed countries[3]. Current managements aim to limit brain injury by immediate medical intervention and post-stroke rehabilitation measures to enhance clinical recovery[4,5]. Rehabilitation training alone is a major post–stroke treatment strategy[6]. Hence therapeutic intervention that can complement the on-going rehabilitative measures can hasten recovery and subsequently improve the quality of life. The injured axons of the central nervous system (CNS), as seen in stroke, have poor regenerative capacity due to a spurt of axonal growth inhibitors in the surrounding neuro-astroglial environment[7]. Activated ROCK (Rho-associated coiled-coil containing protein kinase/Rho-kinase) pathway in the injured neuronal tissue contributes to post-injury regeneration of axons significantly[8,9].

The ROCKs are serine/threonine kinases and are major downstream targets of GTPase, RhoA. GTP-bound RhoA activates ROCKs to phosphorylate a variety of substrates viz. myosin light chain neurofilament protein, myristylated alanine-rich C-kinase *etc*[10]. The ROCK2 isoform is specifically expressed in the CNS and heart[11,12]. Anomalous behaviour of ROCK in ischemic stroke and breakdown of blood– brain barrier is well established in the literature. For example, high ROCK activity has been reported within 48 h of acute ischemic stroke in humans[13] and it’s high expression (>2 folds) in the ischemic region was reported in a mouse model of middle cerebral artery occlusion[14]. Elevated levels of phosphorylated myosin in ischemic brain wall[15] and reduced expression of endothelial nitric oxide synthase in endothelial cells[16] are all resultants of high ROCK expression. Recently, expression of ROCK-2 isoform is reported in brain arterioles performing a

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major role in proinflammatory cell adhesion molecule expression[17,18]. Hence, all these findings speculate the crucial role of ROCK-2 isoform in ischemic stroke and provide a vital therapeutic target. Several ROCK inhibitors, for example, fasudil and its derivatives have been investigated *in vitro* to investigate their role in neuronal regeneration.

Many ROCK inhibitors (Rho– kinase inhibitors) have also been explored for their high therapeutic potentials in cancer[19], glaucoma[20], insulin resistance[21] *etc*. In glaucoma, ROCK inhibitors such as K-115 and SNJ-1656 lowered intra-ocular pressure[22,23]. It is important to note that most of the ROCK inhibitors are not target specific in their action and may bind to ROCK2 or other similar kinases. This non-specificity leads to several kinds of adverse effects like hypotension, intracranial haemorrhage, and abnormal hepatic and renal function, conjunctival hyperaemia, sporadic punctate sub-conjunctival haemorrhage. Therefore, identifying the target specificity of drug molecules is a fundamental step to determine their usefulness.

The present study aims to find out the binding affinity of selected inhibitors with ROCK2, the highly expressed ROCK isoform in CNS. We selected pharmacological ROCK inhibitors from a range of non-selective (fasudil), analogues of fasudil (hydroxy-fasudil and dimethyl-fasudil), and selective (SLx-2119) ROCK2 inhibitors to demonstrate their binding affinity using molecular docking simulations.

Materials and Methods

Sequence retrieval and protein two/three-dimensional structure

The amino acid sequence of ROCK2 protein (ID: O75116; 1388 amino acids) of *Homo sapiens* was retrieved from the UniProt protein database (http://www.uniprot.org). The sequence was used for the prediction of the secondary structure of the protein by using the online tool SAS-sequence annotated by structure (http://www.ebi.ac.uk/thornton-srv/databases/sas/). The three-dimensional X-ray structure with 2.93 Å resolution of ROCK2 protein (PDB ID: 4WOT) was downloaded from the structure database protein data bank (PDB) (www.rcsb.org/) which was further refined and energy minimized using Swiss-PDB Viewer (https://spdbv.vital-it.ch/). At last, the protein structure was validated using the RAMPAGE web-tool (mordred.bioc.cam.ac.uk/~rapper/rampage.php).

Ligand preparation

The three-dimensional structures of therapeutic molecules (ligands) namely, dimethyl fasudil, fasudil, FSD-C10, K-115, SNJ-1656, Y-27632, hydroxy fasudil, SAR407899, SLx-2119 were generated by Marvinsketch (https://www.chemaxon.com/products/marvin/marvinsketch/) and converted into the PDB format. It differentiates between drug– like and non-drug like molecules by predicting their possibilities of success or failure on interacting with the target protein. Our study evaluated the characteristics based on five parameters namely: mass of the ligand (less than 500 daltons), hydrogen bond donor (≤5), hydrogen bond acceptor (≤10), Log P (Octanol-water partition coefficient ≤5), and molar refractivity ranging between 40-130[24]. Complying with two or more rules reflects success in achieving major drug-target protein interaction.

Molecular docking

A rigid docking methodology present in the AutoDock 4.2 software was followed while docking the filtered compounds against the ROCK2 (PDB ID: 4WOT) target protein. The Autodock consist of two main programs, (1) autogrid, pre-calculates these grids, and (2) it performs the docking of the ligand to a set of grids describing the target protein. In addition to using them for docking, the atomic affinity grids can be visualized. A graphical user interface called auto dock tools (ADT) was utilized to generate grids, calculate dock score, and evaluate the conformers. All ligands under study (Suppl. Fig. 1) were docked to the model of the ROCK2 protein, using the Lamarckian genetic algorithm (LGA)[25]. The active site in the 3D structure was not defined and the blind docking procedure for the interaction study was performed in the study. Before performing the docking, the receptor was prepared using the MGL tool package. The grid size for the receptor for docking was given as 126 Å, 126 Å, and 126 Å on X, Y & Z coordinates respectively, which makes sure that the search space covers the whole protein as a binding site and large enough for the ligand to rotate and find appropriate binding conformation. In addition to returning the docked structure, AutoDock also calculates free binding energy for each ligand-receptor configuration. The best ligand-receptor structure from the docked structures was chosen based on the lowest free binding energy.

Results

The toxicity profile of the selected nine (ligands) was analysed based on the Lipinski rule of five. The QSAR (quantitative structure-activity relationship) analysis showed that every ligand complied with all rules for drug–likeness (Table 1) and therefore could be processed further for docking studies.

The protein-ligand interaction between the ROCK2 and ligands was assessed using AutoDock 4.2 software. High binding or association constant (K_a) and high negative free energy (-ΔG) resulting from non-covalent interaction between respective ligand and ROCK2 demonstrate the drug's potential in inhibiting the enzyme activity. The docked views of drug-enzyme interactions are shown in (Fig. 1) while (Table 2) depicts the association constants (K_a) and free energies (ΔG).

Docking study showed that all nine ligands bind to ROCK2 and could be possible inhibitors of ROCK2 at a different strengths. The SLx-2119 and ROCK2 complex demonstrated the highest binding constant and lowest ΔG values than other ligand-ROCK2 complexes (Table 2). The polar contacts between ROCK2 and the respective ligands are shown in (Table 3). We observed a maximum number of polar contacts for SLx-2119 and hydroxy fasudil (five each) while SNJ-1656 having four polar contacts with target ROCK2 showing their different degree of interactions.

Discussion

The present study demonstrates the differential binding efficiency of nine potential Rho kinase inhibitors with ROCK2 enzyme *in silico*. The SLx-2119 showed the highest binding efficiency among all ligands studied and FSD-C10 possessed the weakest interaction with ROCK2 (Table 2). ROCK2, which is highly expressed in brain endothelial cells, is one of the lead molecules responsible for poor regeneration of neurons[26]. Inhibition of ROCK2 is a promising way which can promote axonal regeneration and functional recovery. Several ROCK inhibitors have been proven beneficial by increasing neurite regeneration, neuroprotective, and altering inflammation. Few ROCK inhibitors, for *e.g.*, SNJ-1656[27], Slx-2119[26,28] have been shown high specificity for ROCK2 isoform *in vitro*. However, their interaction with ROCK2 is still unknown. Our *in silico* docking analysis shows that all the nine inhibitors can bind and inhibit ROCK2 but with variable selectivity (Fig. 1 & Table 2). The utility of the selected inhibitors is currently limited to preclinical studies except for fasudil, which is approved in Japan and China for human use[29,30].

Fasudil, in a double-blinded study, has been shown to improve clinical outcomes in acute ischemic stroke with no significant adverse effects[29] and also demonstrated a vasodilator effect in several studies. Analogues of fasudil, hydroxy fasudil (active metabolite of fasudil), and dimethyl fasudil have also shown similar ROCK inhibition properties in reducing cerebral infarction and inflammation[31] and restoring neurite regeneration *in vitro*[32], respectively. The FSD-C10, another fasudil analogue, too have shown similar effects on neuronal regeneration but with significantly much lower toxicity than fasudil[33]. Considering fasudil as a starting molecule from which several ROCK inhibitors have been developed; almost all its derivatives demonstrated higher binding affinity with ROCK2 in our *in silico* study (Table 2 & Fig. 1). Among the four drugs (fasudil, hydroxy fasudil, dimethyl fasudil and FSD-C10), hydroxy fasudil surpassed the binding

Table 2 — Docking analysis of ligands-ROCK2 association

Drug (Ligand)	Association constant ($Ka \times 10^5\ M^{-1}$)	Free energy (ΔG) (Kcal/mol)
SLx-2119 (KD-025)	36.7	−9.07
SNJ-1656	23	−8.79
Hydroxy fasudil	13.7	−8.48
Dimethyl fasudil	9.2	−8.24
K-115	7.9	−8.15
SAR407899	4.7	−7.83
Y27632	2.6	−7.47
Fasudil	2.5	−7.46
FSD-C10	2.2	−7.39

Table 1 — The QSAR description of ligands under study

S. No.	Ligand	Mass	Hydrogen Bond Donor	Hydrogen Bond Acceptor	Log P	Molar Refractivity
1	Dimethyl Fasudil	320	2	4	1.97	86.15
2	Fasudil	292	2	4	1.27	76.82
3	FSD-C10	290	0	4	3.88	78.99
4	K-115	308	4	5	−0.12	75.57
5	SNJ-1656	281	5	3	2.12	81.79
6	Y-27632	248	4	3	1.46	70.66
7	Hydroxy Fasudil	308	3	5	0.44	78.02
8	SAR407899	245	3	3	0.51	68.25
9	SLx-2119	452	3	7	4.63	132.46

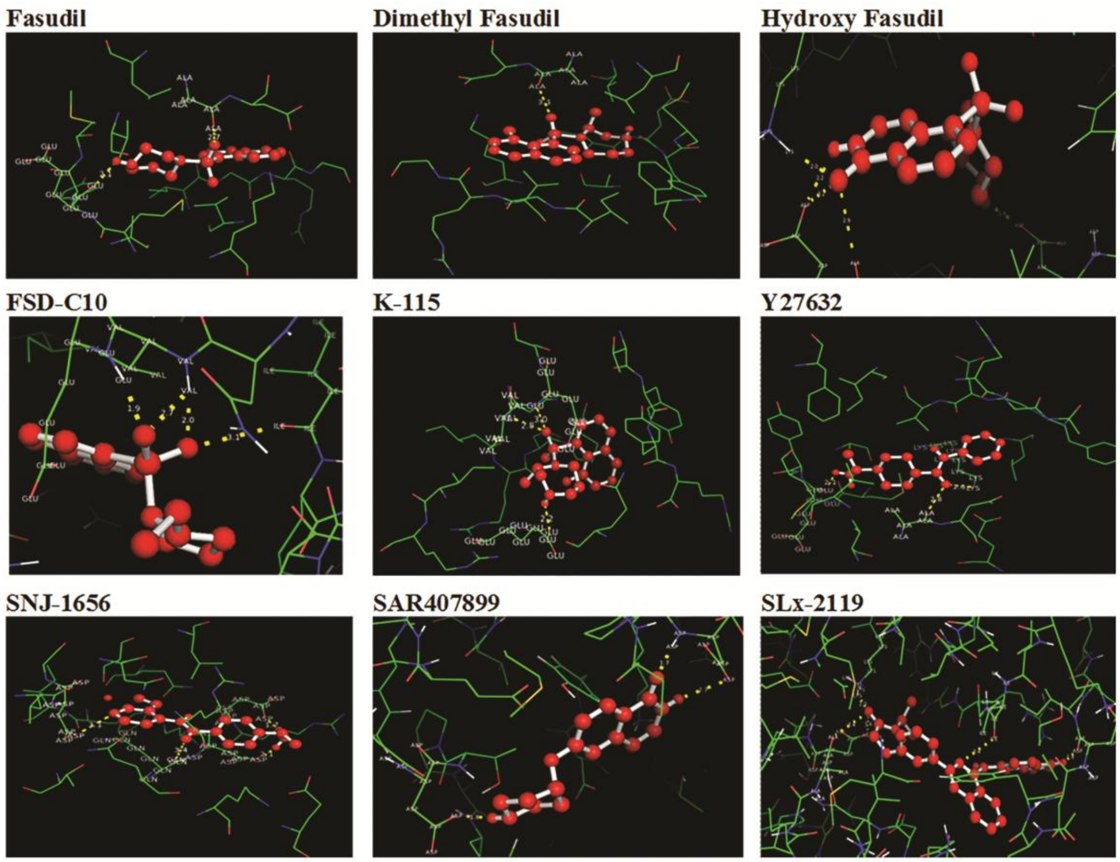

Fig. 1 — Molecular docking poses of various ROCK inhibitors (ligands) with ROCK2

Table 3 — Polar contacts between ROCK2 and various ligands

Ligand	Polar Contacts		Distance (Å)
	Receptor Residue	Ligand Atoms	
Y27632	116 O GLU A	196 H UNK	2.1
	76 NZ LYS A	195 O UNK	2.6
	155 O ALA A	195 O UNK	2.8
Fasudil	72 O GLU A	142 H UNK	2.1
	111 O ALA A	137 O UNK	2.7
FSD-C10	43 N VAL D	12 O UNK	2.0
	33 O LEU D	12 O UNK	3.1
	43 N VAL D	13 O UNK	2.7
	44 H GLU D	13 O UNK	1.9
Dimethyl Fasudil	64 N VAL D	140 O UNK	2.9
	71 N GLU D	140 O UNK	3.2
	103 O TYR D	144 N UNK	3.4
K-115	19 OE2 GLU B	172 H UNK	2.2
	83 N VAL D	164 O UNK	2.8
	90 N GLU D	164 O UNK	3.0
SNJ-1656	121 OD1 ASP A	195 H UNK	1.7
	114 OD2 ASP A	194 H UNK	2.1
	15 OD1 ASP A	190 N UNK	3.1
	101 O GLN A	193 O UNK	3.4
Hydroxy Fasudil	231 O ALA A	11 O UNK	2.9

(*Contd*.)

Table 3 — Polar contacts between ROCK2 and various ligands

Ligand	Polar Contacts		Distance (Å)
	121 HZ2 LYS A	11 O UNK	2.0
	232 OD2 ASP A	11 O UNK	3.2
	232 OD2 ASP A	12 H UNK	2.2
	176 OD2 ASP A	21 H UNK	1.9
SAR407899	218 OD2 ASP A	12 H UNK	2.7
	218 H ASP A	11 O UNK	1.7
	291 OD1 ASP A	21 H UNK	1.8
SLx-2119	98 O ILE A	1 N UNK	3.4
(KD-025)	349 O ARG A	34 H UNK	1.8
	121 H LYS A	20 O UNK	2.0
	232 OD2 ASP A	20 O UNK	3.4
	231 O ALA A	20 O UNK	3.4

efficiency with ROCK2 (K_a = 13.7 × 10^5 M^{-1}, ΔG = −8.48 Kcal/mol) while FSD-C10 showed least tendency to bind with ROCK2 (K_a = 2.2 × 10^5 M^{-1}, ΔG = −7.39 Kcal/mol), almost equal to fasudil (K_a = 2.5 × 10^5 M^{-1}, ΔG = −7.46 Kcal/mol). High K_a and high negative ΔG depict strong binding and hence more potent inhibition of the target enzyme. These results demonstrate hydroxy fasudil could be a more potent inhibitor of ROCK2 than fasudil, dimethyl fasudil, and FSD-C10.

The ROCK inhibitors, SAR407899 and SLx-2119 (KD-025) have also shown the potential to lower blood pressure and relieve vascular occlusion in focal cerebral ischemic cases[28,34]. The SAR407899 is a potent vasodilator and reduces blood pressure in experimental animals[34,35] and also have been reported to reduce phosphorylation of MYPT (Myosin-associated phosphatase) *in vitro* and *ex vivo*[34]. The SLx-2119 is more specific to ROCK2 and has been shown to enhance cerebral perfusion in local cerebral ischemic regions of the mouse brain and protects from rt-PA (recombinant plasminogen activator) thrombolysis induced cerebrovascular damage[26,28]. Our *in silico* binding analysis found that SLx-2119 has the strongest binding (K_a= 36.7 × 10^5 M^{-1}, ΔG= −9.07 Kcal/mol) (Table 2 & Fig. 1) with ROCK2 as compared to eight other drugs tested and hence possess high potency to inhibit ROCK2 isoform.

The optic nerve is an integral part of the CNS has shown distinctive regeneration potential with the instillation of ROCK inhibitors[23,36]. In ocular diseases, inhibition of Rho kinase/ROCK pathway has been shown to reduce intra-ocular pressure and promote optic nerve regeneration[32]. In our study, we included ROCK inhibitors, K-115 (ripasudil; a fasudil derivative), SNJ-1656, and Y27632, which has been explored earlier in an ocular disorders like glaucoma[23,24,37] but their therapeutic benefits in ischemic stroke is not yet explored in human subjects. The K-115 has been shown to enhance the survival of retinal ganglion cells after optic nerve crush and reduced *Nox1* expression[23]. Recently, the clinical trial of K-115 has led to avoid glaucoma surgery in 35 patients by lowering of intraocular pressure with well tolerability up to three months[38]. Similarly, Y27632 induced optic nerve regeneration beyond the crush site in a dose– dependent manner in adult cats[39]. The SNJ-1656, an ocular ROCK inhibitor that reduced the intraocular pressure with minimal side effects has been shown to enhance axonal regeneration in rat retinal ganglion cells[27]. In our molecular docking study, we found that SNJ-1656 also establishes thermodynamically favourable interactions with ROCK2 due to its high K_a and low ΔG (K_a= 23.0 × 10^5 M^{-1}, ΔG= −8.79) (Table 2 & Fig. 1). Further *in vitro* studies are needed to prove their therapeutic potential in stroke.

An earlier clinical trials with ROCK inhibitor fasudil did not establish any significant adverse effects[29]. Moreover, ROCK inhibitors, *viz*. fasudil have also been tried in myeloproliferative disorder[40], pulmonary hypertension[41], amyotrophic lateral sclerosis[42] and have shown beneficial effects. Similarly, preclinical studies in mouse stroke models have shown SLx-2119 to be relatively safe with no substantial hypotensive events[28]. However, blood pressure fluctuation, systemic vasodilation, hypotension and hepatotoxicity are few adverse effects that should be specially gauged and monitored during a clinical trials involving ROCK inhibitors[33]. Therefore, identifying the selectivity of ROCK inhibitors is necessary to reduce toxicity. No ROCK inhibitors, other than fasudil (only in Japan and China)[29,30] are approved for human use due to their adverse effects. Selectivity towards a particular ROCK isoform is the prime step towards the reduction of toxicity and subsequently, their delivery to target tissues/organs can further reduce adverse effects and raise their bioavailability.

In our novel *in silico* molecular docking study, we observed all the nine ROCK inhibitors can potentially bind with ROCK2 which is highly expressed in the brain and during CNS injury. Our study also shows that SLx-2119, SNJ-1656, and fasudil analogues, hydroxy fasudil, and dimethyl fasudil bind more strongly to ROCK2 and may be better ROCK inhibitors than fasudil itself. The high selectivity of SLx-2119 towards ROCK2 has already been shown[28,43]. Moreover, anti-glaucoma drug SNJ-1656 has shown higher potency as compared to fasudil in terms of ROCK2 interaction (Tables 2 & 3). We got very little binding of FSD-C10 with ROCK2 which has shown more ROCK2 selective *in vitro*[33,44]. This requires more investigations in different types of conditions. Thus, from *in silico* perspective, this study highlights the interaction of widely studied nine ROCK inhibitors with ROCK2 which can facilitate early neuronal regeneration by impeding ROCK2 activity following stroke.

Strength and limitation

The *in silico* work is quick and does not require an animal or cell line model to evaluate the efficiency of any drug/ligand. Hence, such studies are cost-effective, safe and time– saving. In addition, docking studies are easy-to-use workflows of systems biology that utilizes every detail of the data (of drugs/proteins/DNA) and obtain consensus predictions of small molecule activities and their off-target interactions[45]. However, further clinical studies are required to ascertain its efficacy, safety, and outcome in animal and human subjects so that they can further be used in clinics.

Conclusion

Following a stroke, the management currently emphasizes secondary prevention and rehabilitation measures. Molecular analysis reveals that certain cellular pathways impair the neural regeneration process. Rho kinase/ROCK pathway is one such molecular signalling mechanism. While numerous potent ROCK inhibitors are under trial, selectivity towards ROCK isoforms is always a challenging task. In this maiden *in silico* molecular docking study, we have assessed the strength of interaction of nine ROCK inhibitors against ROCK2. We found SLx-2119 to possess the highest propensity to bind ROCK2 which is in concordance with its *in vitro* studies elsewhere in the neural tissue enhancing regenerative potential. The safety, efficacy, and pharmacokinetics of this drug in human subjects need to be further established.

Conflict of interest

All authors declare no conflict of interest.

References

1 Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, de Ferranti SD, Floyd J, Fornage M, Gillespie C, Isasi CR, Jiménez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Mackey RH, Matsushita K, Mozaffarian D, Mussolino ME, Nasir K, Neumar RW, Palaniappan L, Pandey DK, Thiagarajan RR, Reeves MJ, Ritchey M, Rodriguez CJ, Roth GA, Rosamond WD, Sasson C, Towfighi A, Tsao CW, Turner MB, Virani SS, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS & Muntner P, Heart disease and stroke statistics-2017 Update: A Report from the American Heart Association. *Circulation*, 135 (2017) e146.

2 Thrift AG, Thayabaranathan T, Howard G, Howard VJ, Rothwell PM, Feigin VL, Norrving B, Donnan GA & Cadilhac DA, Global stroke statistics. *Int J Stroke*, 12 (2017) 13.

3 Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Després JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jiménez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW & Turner MB, Heart disease and stroke statistics-2016 Update: A Report From the American Heart Association. *Circulation*, 133 (2016) e38.

4 de Athayde Costa E Silva A, Viana da Cruz Júnior AT, Cardoso do Nascimento NI, Andrade Candeira SR, do Socorro Soares Cardoso Almeida A, Santana de Castro KJ, Costa de Lima R, Generoso Campos Pinho Barroso T, da Silva Souza G & Callegari B, Positive balance recovery in ischemic post-stroke patients with delayed access to physical therapy. *Biomed Res Int*, (2020) 9153174.

5 Goldstein LB, Modern medical management of acute ischemic stroke. *Methodist Debakey Cardiovasc J*, 10 (2014) 99.

6 Bernhardt J, Urimubenshi G, Gandhi DBC & Eng JJ, Stroke rehabilitation in low-income and middle-income countries: a call to action. *Lancet*, 6 (2020) 1452.

7 Yiu G & He Z, Glial inhibition of CNS axon regeneration. *Nat Rev Neurosci*, 7 (2006) 617.

8 Kishima K, Tachibana T, Yamanaka H, Kobayashi K, Okubo M, Maruo K & Noguchi K, Role of Rho-associated coiled-coil containing protein kinase in the spinal cord injury induced neuropathic pain. *Spine J*, S1529-9430 (2020) 31051.

9 Bell M, Sopko NA, Matsui H, Hannan JL & Bivalacqua TJ, RhoA/ROCK activation in major pelvic ganglion mediates caspase-3 dependent nitrergic neuronal apoptosis following cavernous nerve injury. *Neural Regen Res*, 12 (2017) 572.

10 Feng Y, LoGrasso PV, Defert O & Li R, Rho Kinase (ROCK) inhibitors and their therapeutic potential. *J Med Chem*, 9 (2016) 2269.

11 Liu J, Gao HY & Wang XF, The role of the Rho/ROCK signaling pathway in inhibiting axonal regeneration in the central nervous system. *Neural Regen Res*, 10 (2015) 1892.

12 Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K & Narumiya S, ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett*, 392 (1996) 189.

13 Feske SK, Sorond FA, Henderson GV, Seto M, Hitomi A, Kawasaki K, Sasaki Y, Asano T & Liao JK, Increased leukocyte ROCK activity in patients after acute ischemic stroke. *Brain Res*, 1257 (2009) 89.

14 Rikitake Y, Kim HH, Huang Z, Seto M, Yano K, Asano T, Moskowitz MA & Liao JK, Inhibition of Rho kinase (ROCK) leads to increased cerebral blood flow and stroke protection. *Stroke*, 36 (2005) 2251.

15 Fu Z, Chen Y, Qin F, Yang S, Deng X, Ding R, Feng L, Li W & Zhu J, Increased activity of Rho kinase contributes to hemoglobin-induced early disruption of the blood-brain barrier *in vivo* after the occurrence of intracerebral hemorrhage. *Int J Clin Exp Pathol*, 7 (2014) 7844.

16 Noma K, Kihara Y & Higashi Y, Striking crosstalk of ROCK signaling with endothelial function. *J Cardiol*, 60 (2012) 1.

17 De Silva TM, Kinzenbaw DA, Modrick ML, Reinhardt LD & Faraci FM, Heterogeneous Impact of ROCK2 on carotid and cerebrovascular function. *Hypertension*, 68 (2016) 809.

18 Shimada H & Rajagopalan LE, Rho kinase-2 activation in human endothelial cells drives lysophosphatidic acid-mediated expression of cell adhesion molecules via NF-kappaB p65. *J Biol Chem*, 285 (2010) 12536.

19 Meng F, Su Y & Xu B, Rho-associated protein kinase-dependent moesin phosphorylation is required for PD-L1 stabilization in breast cancer. *Mol Oncol*, 14 (2020) 2701.

20 Tanna AP & Johnson M, Rho kinase inhibitors as a novel treatment for glaucoma and ocular hypertension. *Ophthalmology*, 125 (2018) 1741.

21 Lee DH, Shi J, Jeoung NH, Kim MS, Zabolotny JM, Lee SW, White MF, Wei L & Kim YB, Targeted disruption of ROCK1 causes insulin resistance *in vivo*. *J Med Chem*, 59 (2016) 2269.

22 Yamamoto K, Maruyama K, Himori N, Omodaka K, Yokoyama Y, Shiga Y, Morin R & Nakazawa T, The novel Rho kinase (ROCK) inhibitor K-115: a new candidate drug for neuroprotective treatment in glaucoma. *Invest Ophthalmol Vis Sci*, 55 (2014) 7126.

23 Inoue T, Tanihara H, Tokushige H & Araie M, Efficacy and safety of SNJ-1656 in primary open-angle glaucoma or ocular hypertension. *Acta Ophthalmol*, 93 (2015) e393.

24 Sliwoski G, Kothiwale S, Meiler J & Lowe EW Jr, Computational methods in drug discovery. *Pharmacol Rev*, 66 (2013) 334.

25 Agrawal A, Kulkarni GT & Lakshmayya, Molecular docking study to elucidate the antipruritic mechanism of selected natural ligands by disentisizing TRPV3 ion channel in Psoriasis: An *in silico* approach. *Indian J Biochem Biophys*, 57 (2020) 57.

26 Niego B, Lee N, Larsson P, De Silva TM, Au AE, McCutcheon F & Medcalf RL, Selective inhibition of brain endothelial Rho-kinase-2 provides optimal protection of an *in vitro* blood-brain barrier from tissue-type plasminogen activator and plasmin. *PLoS One*, 12 (2017) e0177332.

27 Tanihara H, Inatani M, Honjo M, Tokushige H, Azuma J & Araie M, Intraocular pressure-lowering effects and safety of topical administration of a selective ROCK inhibitor, SNJ-1656, in healthy volunteers. *Arch Ophthalmol*, 126 (2008) 309.

28 Lee JH, Zheng Y, von Bornstadt D, Wei Y, Balcioglu A, Daneshmand A, Yalcin N, Yu E, Herisson F, Atalay YB, Kim MH, Ahn YJ, Balkaya M, Sweetnam P, Schueller O, Poyurovsky MV, Kim HH, Lo EH, Furie KL & Ayata C, Selective ROCK2 Inhibition in focal cerebral ischemia. *Ann Clin Transl Neurol*, 1 (2014) 2.

29 Shibuya M, Hirai S, Seto M, Satoh S & Ohtomo E, Effects of fasudil in acute ischemic stroke: results of a prospective placebo-controlled double-blind trial. *J Neurol Sci*, 238 (2005) 31.

30 Suzuki Y, Shibuya M, Satoh S, Sugimoto Y & Takakura K, A postmarketing surveillance study of fasudil treatment after aneurysmal subarachnoid hemorrhage. *Surg Neurol*, 68 (2007) 126.

31 Satoh S, Utsunomiya T, Tsurui K, Kobayashi T, Ikegaki I, Sasaki Y & Asano T, Pharmacological profile of hydroxy fasudil as a selective rho kinase inhibitor on ischemic brain damage. *Life Sci*, 69 (2001) 1441.

32 Lingor P, Teusch N, Schwarz K, Mueller R, Mack H, Bähr M & Mueller BK, Inhibition of Rho kinase (ROCK) increases neurite outgrowth on chondroitin sulphate proteoglycan *in vitro* and axonal regeneration in the adult optic nerve *in vivo*. *J Neurochem*, 103 (2007) 181.

33 Xin YL, Yu JZ, Yang XW, Liu CY, Li YH, Feng L, Chai Z, Yang WF, Wang Q, Jiang WJ, Zhang GX, Xiao BG & Ma CG, FSD-C10: A more promising novel ROCK inhibitor than Fasudil for treatment of CNS autoimmunity. *Biosci Rep*, 35 (2015) e00247.

34 Löhn M, Plettenburg O, Ivashchenko Y, Kannt A, Hofmeister A, Kadereit D, Schaefer M, Linz W, Kohlmann M, Herbert JM, Janiak P, O'Connor SE & Ruetten H, Pharmacological characterization of SAR407899, a novel rho-kinase inhibitor. *Hypertension*, 54 (2009) 676.

35 Löhn M, Plettenburg O, Kannt A, Kohlmann M, Hofmeister A, Kadereit D, Monecke P, Schiffer A, Schulte A, Ruetten H & Ivashchenko Y, End-organ protection in hypertension by the novel and selective Rho-kinase inhibitor, SAR407899. *World J Cardiol*, 7 (2015) 31.

36 Hove IV, Lefevere E & Moons L, ROCK inhibition as a novel potential strategy for axonal regeneration in optic neuropathies. *Neural Regen Res*, 10 (2015) 1949.

37 Inoue T & Tanihara H, Ripasudil hydrochloride hydrate: targeting Rho kinase in the treatment of glaucoma. *Expert Opin Pharmacother*, 18 (2017) 1669.

38 Inazaki H, Kobayashi S, Anzai Y, Satoh H, Sato S, Inoue M, Yamane S & Kadonosono K, Efficacy of the additional use of ripasudil, a rho-kinase inhibitor, in patients with glaucoma inadequately controlled under maximum medical therapy. *J Glaucoma*, 26 (2017) 96.

39 Ichikawa M, Yoshida J, Saito K, Sagawa H, Tokita Y & Watanabe M, Differential effects of two ROCK inhibitors, Fasudil and Y-27632, on optic nerve regeneration in adult cats. *Brain Res*, 1201 (2008) 23.

40 William BM, An W, Feng D, Nadeau S, Mohapatra BC, Storck MA, Band V & Band H, Fasudil, a clinically safe ROCK inhibitor, decreases disease burden in a Cbl/Cbl-b deficiency-driven murine model of myeloproliferative disorders. *Hematology*, 21 (2016) 218.

41 Zhang Y & Wu S, Effects of fasudil on pulmonary hypertension in clinical practice. *Pulm Pharmacol Ther*, 46 (2017) 54.

42 Günther R, Balck A, Koch JC, Nientiedt T, Sereda M, Bähr M, Lingor P & Tönges L, Rho kinase inhibition with fasudil in the sod1g93a mouse model of amyotrophic lateral sclerosis-symptomatic treatment potential after disease onset. *Front Pharmacol*, 8 (2017) 17.

43 Diep DTV, Hong K, Khun T, Zheng M, Ul-Haq A, Jun HS, Kim YB & Chun KH, Anti-adipogenic effects of KD025 (SLx-2119), a ROCK2-specific inhibitor, in 3T3-L1 cells. *Sci Rep*, 8 (2018) 2477.

44 Li YH, Xie C, Zhang Y, Li X, Zhang HF, Wang Q, Chai Z, Xiao BG, Thome R, Zhang GX & Ma CG, FSD-C10, a Fasudil derivative, promotes neuroregeneration through indirect and direct mechanisms. *Sci Rep*, 7 (2017) 41227.

45 Zloh M & Kirton SB, The benefits of in silico modeling to identify possible small-molecule drugs and their off-target interactions. *Future Med Chem*, 10 (2018) 423.