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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 $2.6 - 36.7 \times 10^5 M^{-1}$] than fasudil ($K_a = 2.5 \times 10^5 M^{-1}$). SLx-2119 (KD-025) had the highest binding constant ($K_a = 36.7 \times 10^5 M^{-1}$) 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 countries³. 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 strategy⁶. Hence therapeutic intervention that can complement the ongoing 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 environment⁷. 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 humans¹³ and its high expression (>2 folds) in the ischemic region was reported in a mouse model of middle cerebral artery occlusion¹⁴. Elevated levels of phosphorylated myosin in ischemic brain wall¹⁵ and reduced expression of endothelial nitric oxide synthase in endothelial cells¹⁶ are all resultants of high ROCK expression. Recently, expression of ROCK-2 isoform is reported in brain arterioles performing a

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Suppl. Data available on respective page of NOPR

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 cancer¹⁹, glaucoma²⁰, insulin resistance²¹ 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 subconjunctival 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 40-130²⁴. 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 ($-\Delta G$) 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 neurons²⁶. 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-1656²⁷, 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 effects²⁹ 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 inflammation³¹ 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 fasudil³³. 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 ($K_a \times 10^5 M^{-1}$)	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

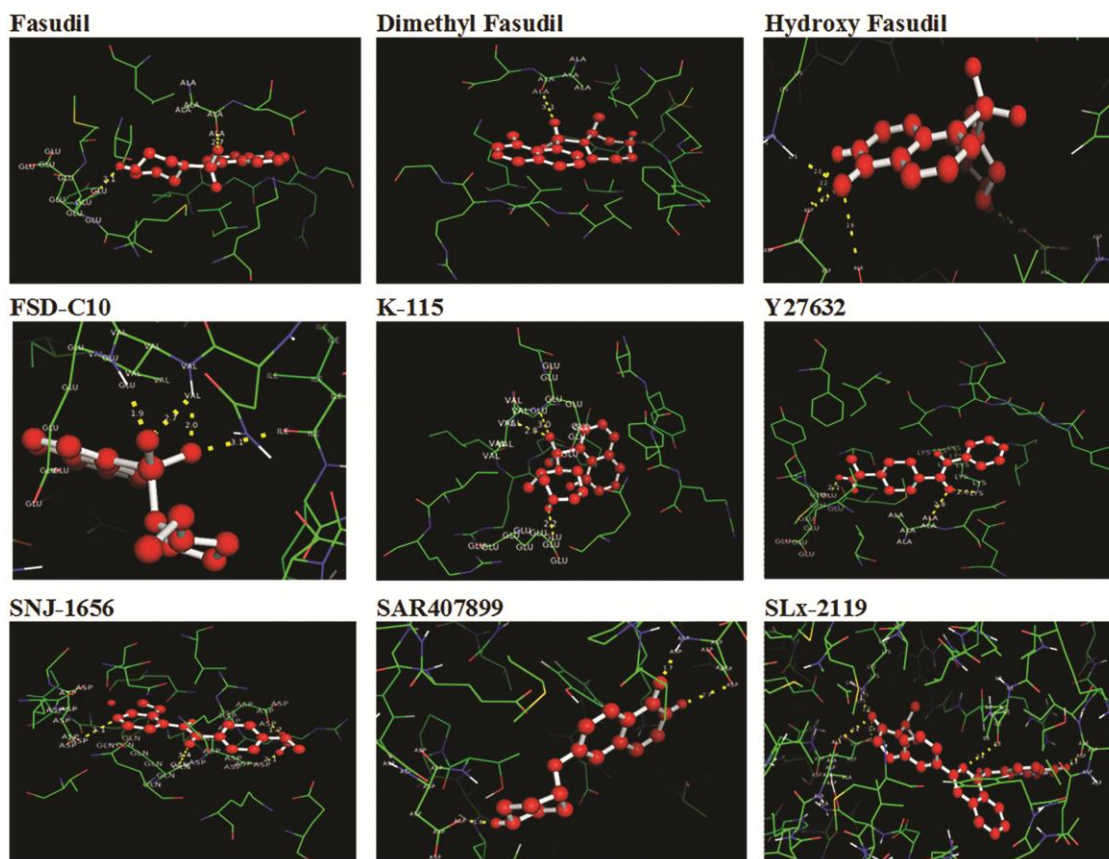


Fig. 1 — Molecular docking poses of various ROCK inhibitors (ligands) with ROCK2

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)
SAR407899	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
	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 (KD-025)	98 O ILE A	1 N UNK	3.4
	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 ($K_a = 13.7 \times 10^5 \text{ M}^{-1}$, $\Delta G = -8.48 \text{ Kcal/mol}$) while FSD-C10 showed least tendency to bind with ROCK2 ($K_a = 2.2 \times 10^5 \text{ M}^{-1}$, $\Delta G = -7.39 \text{ Kcal/mol}$), almost equal to fasudil ($K_a = 2.5 \times 10^5 \text{ M}^{-1}$, $\Delta G = -7.46 \text{ Kcal/mol}$). 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 ($K_a = 36.7 \times 10^5 \text{ M}^{-1}$, $\Delta G = -9.07 \text{ Kcal/mol}$) (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 regeneration³². 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* expression²³. 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 months³⁸. Similarly, Y27632 induced optic nerve regeneration beyond the crush site in a dose-dependent manner in adult cats³⁹. 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 cells²⁷. 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 ($K_a = 23.0 \times 10^5 \text{ M}^{-1}$, $\Delta G = -8.79$) (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 effects²⁹. Moreover, ROCK inhibitors, *viz.* fasudil have also been tried in myeloproliferative disorder⁴⁰, pulmonary hypertension⁴¹, amyotrophic lateral sclerosis⁴² and have shown beneficial effects. Similarly, preclinical studies in mouse stroke models have shown SLx-2119 to be relatively safe with no substantial hypotensive events²⁸. 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 inhibitors³³. 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 interactions⁴⁵. 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.

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