MLSCN Probe Summary

Project: Rho Kinase II
Grant Number: N/A
Screening Center: The Scripps Research Institute, TSRI
Principal Investigators: Thomas Schroeter, Philip LoGrasso, Yangbo Feng
Assay Submitter & Institution: The Scripps Research Institute, TSRI
Assay Target: Rho Kinase 2
Probe PubChem compound Identifier (SID): 26657388

Assay Provider Information:

Rho-Kinase is a serine/threonine kinase involved in the regulation of smooth muscle contraction and cytoskeletal reorganization of nonmuscle cells. Its inhibition is known to promote the smooth muscle relaxation. Thus, small-molecule inhibitors of Rho-Kinase may be effective probes for treatment of cerebral vasospasm and potentially effective for treatment of angina, hypertension, arteriosclerosis, and erectile dysfunction.

This experiment's specific aim was to find potent inhibitors for Rho-Kinase. "Kinase-Glo", an ATP depletion assay was used to find inhibitors that are specific to the ATP binding site.

Screening Center Information:

PubChem Bioassay Name: Dose-response biochemical assay of Rho kinase 2 (Rock2) inhibitors
PubChem Bioassay Identifier (AID): 791

Assay description:

The assay is based on ability of Rhok2 to phosphorylate a specific peptide sequence derived from its substrate - ribosomal protein S6 (amino acid residues 229-239). Rhok2 uses ATP as a donor of phosphate for the phosphorylation of the substrate, which leads to the depletion of ATP in the reaction mix. An assay kit ("Kinase-Glo", Promega) was used to quantify enzyme activity. Using this kit, residual amounts of ATP are measured by a secondary enzymatic reaction, through which luciferase utilizes the remaining ATP to produce luminescence. Luminescent signal is directly proportional to ATP concentration and inversely proportional to Rhok2 activity.

This dose response assay was conducted in 384 well plate formats. Each concentration was tested nominally in triplicate.

Protocol Summary:

5 microliters of solution containing 20 micromolar ATP and 20 micromolar S6 peptide (substrate) in assay buffer (50 millimolar HEPES pH 7.3, 10 millimolar MgCl2, 0.1% BSA, 2 millimolar DTT) were dispensed in 384 microtiter plate. 20 nanoliters of test compound or positive and negative control (2.12 millimolar Y-27632 and DMSO, respectively) were then added to the appropriate wells. Each compound dilution was

assayed in triplicate, for a nominal total of 30 data points per dose response curve. The enzymatic reaction was initiated by dispensing 5 microliters of 8 nanomolar Rhok2 solution in assay buffer (50 millimolar HEPES pH 7.3, 10 millimolar MgCl2, 0.1% BSA, 2 millimolar DTT). After 1 hours of incubation at 25 degrees Celsius, 10 microliters of Kinase Glo reagent (Promega Corporation, Madison, WI) was added to each well. Plates were incubated for 10 minutes and luminescence was read on Perkin-Elmer Viewlux for 60 seconds.

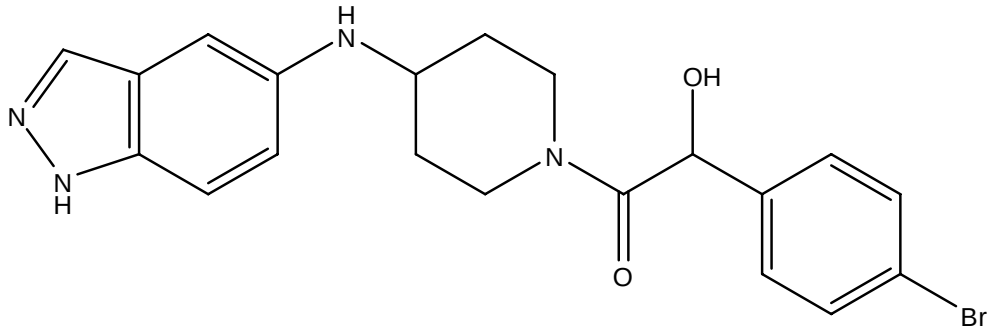
Each compound was tested in triplicate. The percent inhibition for each well has been calculated as follows:
%inhibition = (test_compound - median_negative_control)/(median_positive_control - median_negative_control)*100
where the positive control is Y-27632 (13 micromolar) and negative control is DMSO only.

For each compound, percentage inhibitions were plotted against compound concentration. A four parameter equation describing a sigmoidal dose-response curve was then fitted with adjustable baseline using Assay Explorer software (MDL Information Systems). The reported IC50 values were generated from fitted curves by solving for X-intercept at the 50% inhibition level of Y-intercept.In cases where the highest concentration tested (i.e. 20 micromolar) did not result in greater than 50% inhibition, the IC50 was determined manually as greater than 60 micromolar.

Compounds with IC50 values of greater than 10 micromolar were considered inactive, compounds with IC50 equal or less than 10 micromolar are considered active.

The activity score was calculated based on pIC50 values for compounds for which an exact IC50 value was calculated and based on the observed pIC50 range, specifically the maximum lower limit of the pIC50 value as calculated from the lowest concentration for which greater than 50% inhibition is observed. This results in a conservative estimate of the activity score for compounds for which no exact IC50 value is given while maintaining a reasonable rank order of all compounds tested.

Probe compound:

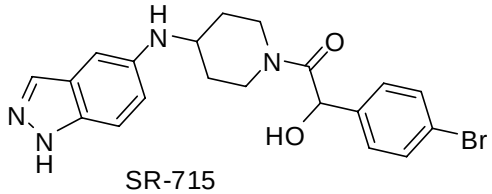


1-(4-(1H-indazol-5-ylamino)piperidin-1-yl)-2-(4-bromophenyl)-2-hydroxyethanone with one equivalent of trifluoro acetic acid.

Internally known as SR-715, this compound is submitted to PubChem and is registered as SID 26657388 and CID 16681731. ATP competition is determined to be the biological mode of action for this probe.

Chemistry:

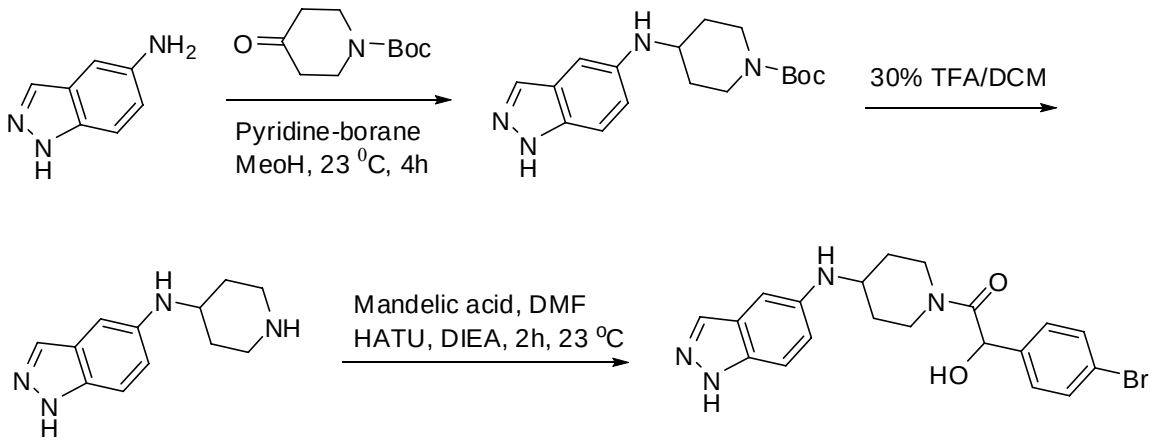
Three primary structural classes have been used as ROCK inhibitors: The isoquinoline scaffold, the 4-aminopyridine scaffold, and the indazole scaffold. Among these three, the indazoles contains perhaps the most potent ROCK inhibitors based on data from published papers and patent applications, but this scaffold is the least well characterized both *in vitro* and *in vivo*. Thus, several small focused libraries around 5-aminoindazole were prepared in Scripps Florida in order to further explore this scaffold as potential novel ROCK inhibitors. After in-house biological assays, SR-715 was identified as a good candidate from the 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-hydroxy(or 2-amino) sub-library.



SR-715

In these structures, the indazole head binds to the hinge of the ROCK ATP pocket. The aminopiperidine ring just functions as a spacer or a mimic of the sugar ring in ATP. In SR-715, a mandelic acid amide was used to function both as a H-bonding donor, and as a group to bind to the hydrophobic pocket in the protein.

The synthesis of SR-715 is shown in the scheme below:



Results:

Three primary structural classes have emerged as the most well-studied ROCK inhibitor series. The isoquinonline series is represented by fasudil[1-4] (IC50=150-550 nM) and H-1152P[5-7] (IC50=6-12nM). The 4-aminopyridine series is represented by Y-

27632[6,8,9] (IC50=140-260Nm). The indazole series contains perhaps the most potent ROCK inhibitors, but is the least characterized both in vitro and in vivo.

A series of 13 1-(4-(1H-indazol-5-yl)piperazin-1-yl)-2-hydroxy(or 2-amino) analogs [series 1] and another series of 10 (1-(4-(1H-indazol-5-yl amino)piperadin-1-yl)-2-hydroxy(or 2-amino) analogs [series 2] were measured for IC50 for ROCK-II and CYP3A4 inhibition.

In general, 2-amino analogs in both series were more potent for ROCK-II. Para substituted phenol group significantly improves the potency.

SR-715 (SID 26657388), Para-substituted bromo-phenyl or 2-hydroxy analog of series 2 is the most potent ROCK-II inhibitor of its series with an IC50 of 80nM. 2-amino para-bromo-phenyl analog, SR-1459 (SID 26522133) improves the potency to 13nM but it also increased the CYP3A4 inhibition to 7-fold compared to SR-715.

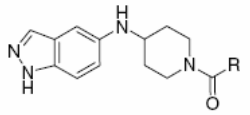
SR-715 show a 43 fold potency for CYP3A4 and SR-1459 shows a 38 fold potency but SR-1459's absolute potency of 490nM for CYP3A4 as opposed to SR-715's 3.5uM makes it less than desirable for further development unless further SAR can be developed that maintains the ROCK potency while eliminating the CYP3A4 activity.

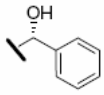
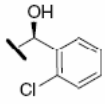
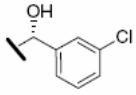
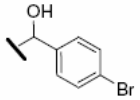
For details see PubChem AID =791 and reference number 10.

Appendices:

Structure Activity Relationship:

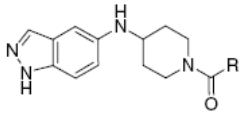
Table 3. IC_{50} values for ROCK II and CYP3A4 inhibition for 1-(4-(1H-indazol-5-yl amino)piperidin-1-yl)-2-hydroxy analogs

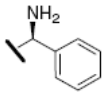
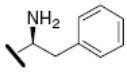
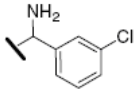
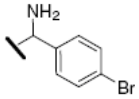


SID	R	ROCK II inhibition IC_{50}[a] (μM)	3A4 inhibition IC_{50}[a] (μM)	Ratio (3A4/ROCKII)
26522112		10.0	1.0	0.1
26522113		0.77 ± 0.006	1.0	1.3
26522114		1.7 ± 0.2	4.0	2.4
26522115		0.35 ± 0.002	2.0	5.7
26657388		0.08 ± 0.01	3.5	43.8

[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



SID	R	ROCK II inhibition IC_{50}[a] (μM)	3A4 inhibition IC_{50}[a] (μM)	Ratio (3A4/ROCKII)
26522129		6.0 ± 2.2	>10.0	>1.7
26522130		0.83 ± 0.1	0.45	0.54
26522131		0.47 ± 0.07	1.0	2.1
26522132		0.15 ± 0.04	0.17	1.1
26522133		0.013 ± 0.001	0.49	37.7

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

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