Supporting Information

Discovery of Subsituted 4-(Pyrazol-4-yl)-phenylbenzodioxane-2-carboxamides as Potent and Highly Selective Rho Kinase (ROCK-II) Inhibitors

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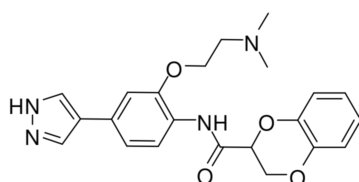
(1) Synthetic Procedures and Characterization:

Analytical HPLC data were generated by injecting 5 μL of very dilute sample solution in methanol or acetonitrile to a reverse phase HPLC system run over 14 min (5-95% acetonitrile / water with 0.1% TFA in each solvent). The products were detected by UV in the detection range of 215-310 nm. HRMS (electrospray ionization) experiments were performed with a Thermo Finnigan orbitrap mass analyzer. Data were acquired in the positive ion mode at a resolving power of 100,000 at m/z 400. Calibration was performed with an external calibration mixture immediately prior to analysis. Proton Nuclear magnetic Resonance (^{1}H NMR) spectra were recorded at 400 MHz (Brucker), chemical shifts are reported in ppm downfield (δ) from Me_4Si.



Chroman-3-carboxylic acid [2-(2-dimethylamino-ethoxy)-4-(1H-pyrazol-4-yl)-phenyl]-amide (5) (SR3677).

Step A. 2-(5-bromo-2-nitrophenoxy)-*N,N*-dimethylethanamine (**7**).

To a solution of 2-(dimethylamino)ethanol (0.610 mL, 6.06 mmol) in THF (25 mL) at 0 °C was added NaH (60% oil dispersion, 0.360 g, 9 mmol). After stirring for 15 min, 4-bromo-2-fluoro-1-nitrobenzene (**6**) (1.32 g, 6.00 mmol) was added and the resulting mixture was allowed to warm to room temperature and stirred for 7 h. The solvent was removed by rotary evaporation and the residue was treated with diethyl ether (50 mL) and an aqueous 0.5 N HCl solution (100 mL). The layers were separated and the aqueous layer was basified with an aqueous saturated $NaHCO_3$

solution and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, filtered, and concentrated to give the title compound **7** (1.59 g, 92%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.74 (d, *J* = 8.8 Hz, 1 H), 7.25 (d, *J* = 2.0 Hz, 1 H), 7.17 (dd, *J* = 8.8, 2.0 Hz, 1 H), 4.19 (t, *J* = 5.6 Hz, 2 H), 2.80 (t, *J* = 5.6 Hz, 2 H), 2.35 (s, 6 H). Single peak at 254 nm and 215 nm in analytical HPLC.

Step B. 4-bromo-2-(2-(dimethylamino)ethoxy)aniline (**8**).

To a solution of 2-(5-bromo-2-nitrophenoxy)-N,N-dimethylethanamine (Step A, 1.59 g, 5.50 mmol) in ethanol (50 mL) was added stannous chloride (6.2 g, 27.5 mmol) and the mixture was heated to 70 °C for 2 h. The reaction was mixture was diluted with ice water and concentrated by rotary evaporation. An aqueous Na_2CO_3 solution was added and the aqueous phase was extracted with ethyl acetate (3 × 60 mL). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, filtered, and concentrated to dryness to afford the title compound **8** (1.30 g, 91%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.90 (m, 2 H), 6.57 (d, *J* = 8.8 Hz, 1 H), 4.07 (t, *J* = 5.6 Hz, 2 H), 3.92 (br s, 2 H), 2.74 (t, *J* = 5.6 Hz, 2 H), 2.33 (s, 6 H). Single peak at 254 nm and 215 nm in analytical HPLC.

Step C. N-(4-bromo-2-(2-(dimethylamino)ethoxy)phenyl)-2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamide **9**.

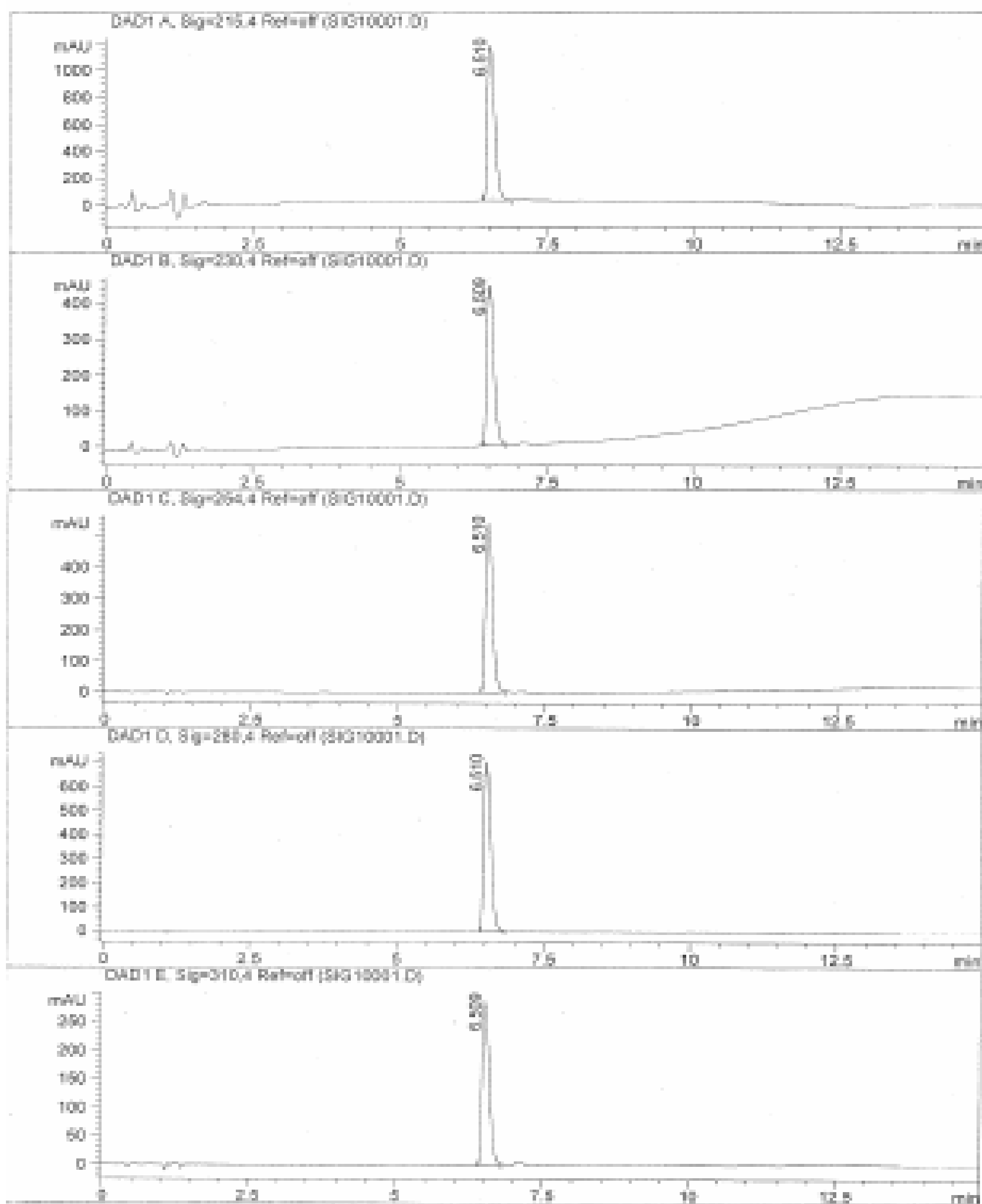
To a solution of amine **8** (0.1 g, 0.4 mmol), benzodioxane-2-carboxylic acid (1.1 equiv), and DIEA (3 equiv) in DMF was added HATU (0.18 g, 0.5 mmol) and the solution was gently stirred at 23 °C for 2h. After the solvent was removed by rotary

evaporation, the residue was suspended in ethyl acetate, washed with brine (2 x), saturated $NaHCO_3$ (2 x), brine (2 x), dried over Na_2SO_4, and evaporated to give the crude bromide **9** (0.13 g, 0.3 mmol, 75%), which was used directly in the next step without further purification.

Step D. chroman-3-carboxylic acid [2-(2-dimethylamino-ethoxy)-4-(1H-pyrazol-4-yl)-phenyl]-amide.

Pd[P(Ph)$_3$]$_4$ (15%) was added to a degassed (using argon) solution of **9** (0.13 g, 0.3 mmol), 1*H*-4-pyrazoleboronic acid pinacol ester (2 equiv), and K_2CO_3 (5 equiv) in dioxane/H_2O (4:1 by volume). After the solution was sealed in a high-pressure reactor, the suspension was stirred at 90-100 ℃ for 4 h, at which the Suzuki coupling was complete based on LC-MS analysis. After removing the solvent by rotary evaporation, the resulting residue was purified by preparative reverse phase HPLC to give compound **5** (**SR3677)** as a white solid (0.066 g, 35%). ^{1}H NMR (DMSO-d$_6$, 400 MHz), δ: 9.73 (b, 1H), 9.23 (s, 1H), 8.07 (s, 2H), 7.88 (d, *J* = 8.4 Hz, 1H), 7.34 (d, *J* = 1.6 Hz, 1H), 7.25 (dd, *J* = 1.6 Hz, *J* = 4.4 Hz, 1H), 7.09 (m, 1H), 6.93 (m, 3H), 5.09 (dd, *J* = 2.8 Hz, *J* = 5.6 Hz, 1H), 4.46 (dd, *J* = 2.8 Hz, *J* = 10.6 Hz, 1H), 4.37 (dd, *J* = 6.0 Hz, *J* = 11.6 Hz, 1H), 3.55 (m, 2H), 2.92 (m, 2H), 2.89 (s, 6H). Single peak in analytical HPLC trace; HRMS, MH^+ calcd for $C_{22}H_{24}N_4O_4$: 409.1876, obtained: 409.1860.

The analytical HPLC trace of SR3677 (**5**):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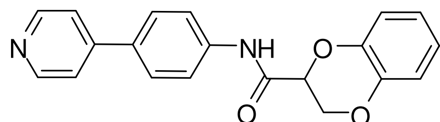
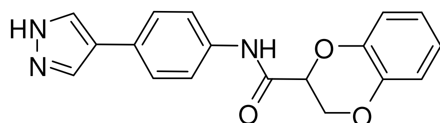


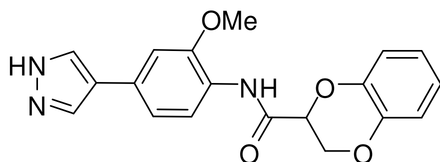
N-(4-(pyridin-4-yl)phenyl)-2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamide (2).

Similar procedures as in the synthesis of SR3677 were used to prepare this titled compound. ^{1}H NMR (DMSO, 400 MHz) δ 4.35-4.42 (dd, *J* = 5.6, 12.0 Hz, 1H), 4.47 (dd, *J* = 2.8, 12.0 Hz, 1H), 5.04 (dd, *J* = 3.2, 5.6 Hz, 1H), 6.84-6.93 (m, 3H), 7.04-7.07 (m, 1H), 7.86 (d, *J* = 8.8 Hz, 2H), 7.99 (d, *J* = 8.8 Hz, 2H), 8.12 (d, *J* = 6.8 Hz, 2H), 8.80 (d, *J* = 6.8 Hz, 2H), 10.4 (s, 1H); Single peak in analytical HPLC; HRMS, MH^+: calcd for $C_{20}H_{16}N_2O_3$: 333.1239, obtained: 333.1225.



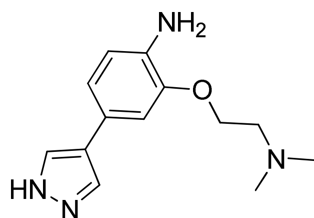
N-(4-(1H-pyrazol-4-yl)phenyl)-2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamide (3).

Similar procedures as in the synthesis of SR3677 were used to prepare this titled compound. ^{1}H NMR (DMSO, 400 MHz) δ 4.30-4.40 (dd, *J* = 5.6, 12.0 Hz, 1H), 4.47 (dd, *J* = 2.8, 12.0 Hz, 1H), 4.96 (dd, *J* = 2.4, 5.6 Hz, 1H), 6.83-6.92 (m, 3H), 7.02-7.06 (m, 1H), 7.56 (d, *J* = 8.8 Hz, 2H), 7.62 (dd, *J* = 2.0, 6.8 Hz, 2H), 8.01 (m, 2H), 10.10 (s, 1H), 13.0 (b, 1H); Single peak in analytical HPLC; MH^+: calcd for $C_{18}H_{15}N_3O_3$: 322.1192, obtained: 322.1180.



N-(2-methoxy-4-(1H-pyrazol-4-yl)phenyl)-2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamide (4).

Similar procedures as in the synthesis of SR3677 were used to prepare this titled compound. ^{1}H NMR (DMSO, 400 MHz) δ 3.88 (s, 3H), 4.41 (d, J = 4.0 Hz, 2H), 5.12 (t, J = 4.0 Hz, 1H), 6.86-6.95 (m, 3H), 7.06 (m, 1H), 7.52-7,65 (m, 2H), 8.01 (d, J = 8.4 Hz, 1H), 8.09 (b, 2H), 9.15 (s, 1H); Single peak in analytical HPLC; HRMS, MH^+: calcd for $C_{19}H_{17}N_3O_4$: 352.1297, obtained: 352.1288.



2-(2-(dimethylamino)ethoxy)-4-(1H-pyrazol-4-yl)aniline (10).

Similar procedures as in the synthesis of SR3677 were used to prepare this titled compound. ^{1}H NMR (DMSO, 400 MHz) δ 7.99 (s, 2H), 7.25 (s, 1H), 7.15-7.13 (m, 1H), 6.97-6.95 (m, 1H), 4.40 (t, J=4.8 Hz, 2H), 3.56 (t, J=4.8 Hz, 2H), 2.91 (s, 3H); Single peak in analytical HPLC; HRMS, MH+ calcd for $C_{13}H_{18}N_4O$:247.1559, obtained: 247.1546.

The analytic HPLC trace of compound 10

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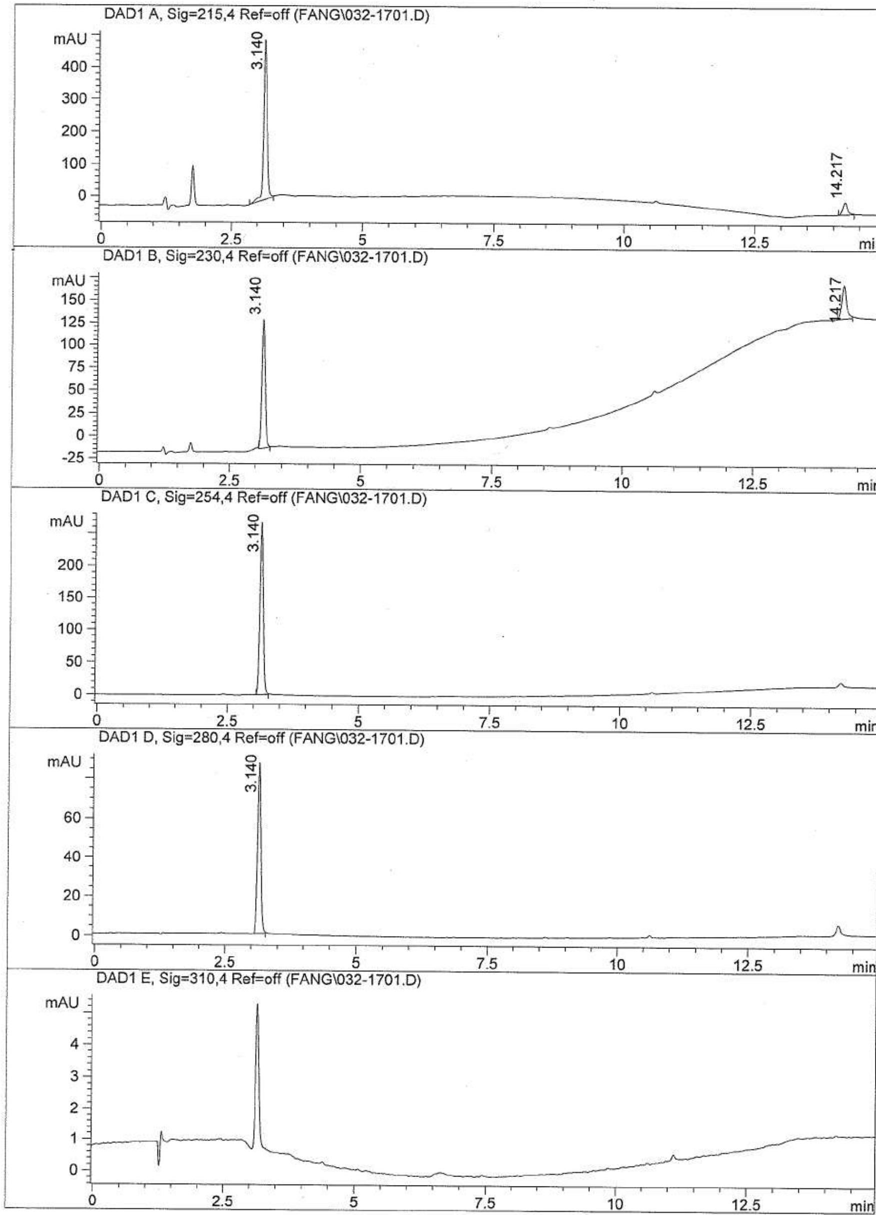

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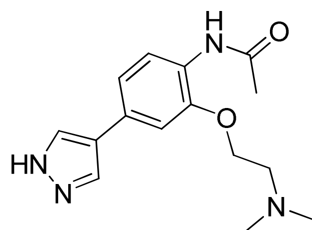

N-(2-(2-(dimethylamino)ethoxy)-4-(1H-pyrazol-4-yl)phenyl)acetamide (11).

Similar procedures as in the synthesis of SR3677 were used to prepare this titled compound. ^{1}H NMR (DMSO, 400 MHz) δ 9.82 (br, 1H), 9.07-9.06 (m, 1H), 8.04 (s, 2H), 7.87-7.84 (m, 1H), 7.31-7.30 (m, 1H), 7.21-7.19 (m, 1H), 4.43 (t, *J*=4.8 Hz, 2H), 3.60-3.59 (m, 2H), 2.93 (d, *J*=4.8 Hz, 6H), 2.10 (s, 3H); Single peak in analytical HPLC; HRMS, MH+: calcd for $C_{15}H_{20}N_4O_2$: 289.1665, obtained: 289.1651.

The analytical HPLC trace of compound 11:

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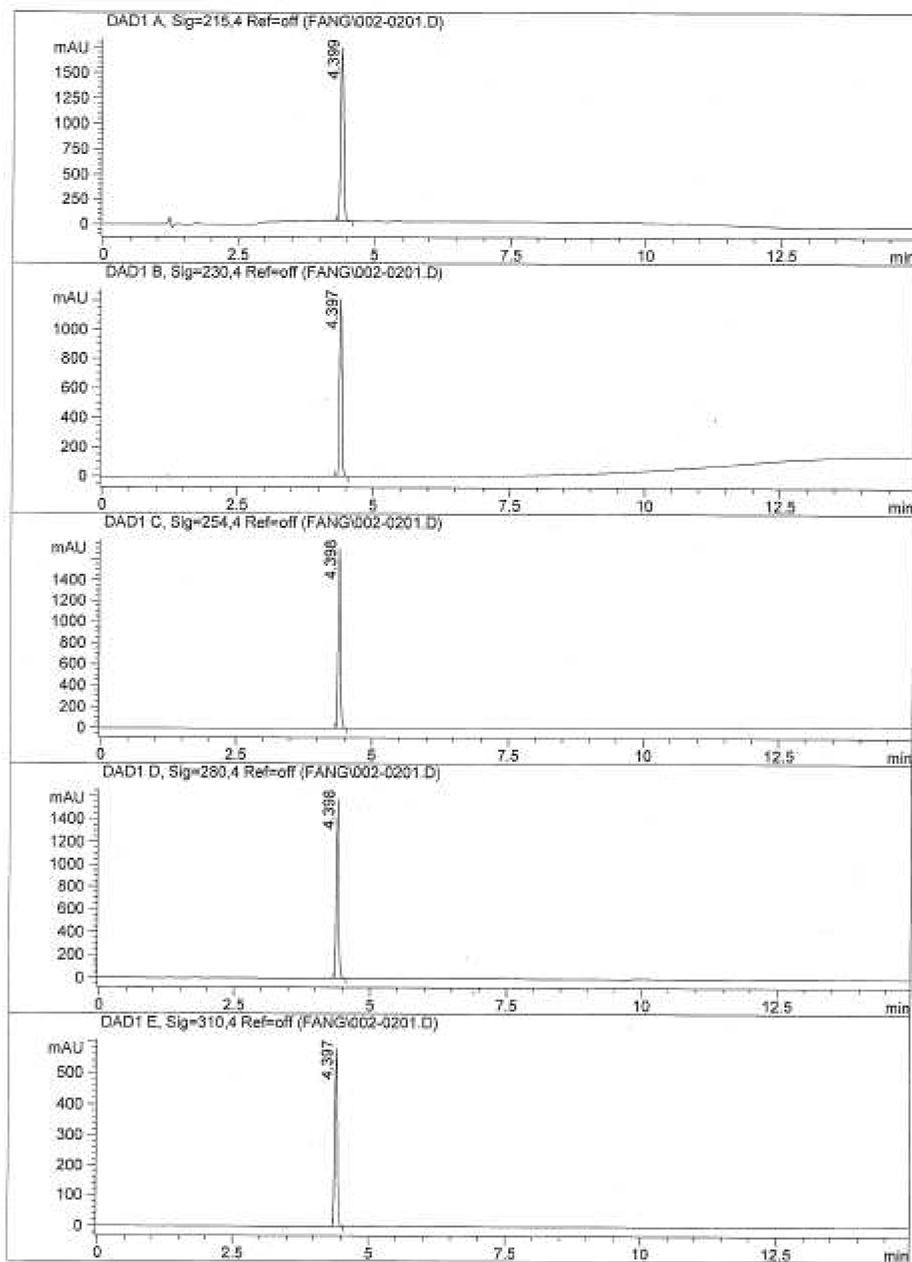

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(2) ROCK-II modeling and docking

After comparing the available experimental co-crystal and apo-crystal structures for Rho kinase with emphasis on the flexibility of the P loop we built a model of human ROCK-II based on the template of chain A of the human ROCK-I co-crystal structure with Y27632 (2ETR) using the automated STRUCTFAST method (85 % identity based on 395 aligned residues; ROCK-II sequence 22-416); the only sequence difference between ROCK-II an ROCK-I around 10Å of the Y27632 ligand are ROCK-I THR365 vs. ROCK-II Ser381 and ROCK-I Leu371 vs. ROCK2 ILE387.

Into the single chain ROCK-II model, which does not contain any water molecules, the original co-crystal ligand Y27632 was cloned followed by optimization of the hydrogen bonds. This complex was then minimized to 0.1 RMSG using the OPLS force field with the GB/SA solvent model around 8A of the ligand and a 6A shell with an increased force constant. The energy minimized structure was used to generate a docking grid of 18A length along the ligand Y27632.

Compound **5** (SR3677), which has only one stable tautomer, was protonated at pH 7 at the dimethylaminoethoxy moiety and for both enantiomers two 3D low energy ring conformations with equatorial and axial substitution at the dihydrobenzodioxine moiety respectively were generated using LigPrep from Schroedinger (Figure S1). The two different ring starting conformations are required to sample the conformational space because Glide's conformation generator or a force field minimization would not transform one into the other conformation. The prepared ligand structures were flexibly docked into the ROCK-II binding site using Glide from Schroedinger with both the SP and the XP scoring functions. Interestingly, different docking results were obtained

depending on the ligand starting conformation with much better docking scores and energies for the axial (SR3677-(*S*)_ax) compared to the equatorial (SR3677-(*S*)_eq) starting ring conformation. The differences in docking results (in terms of score and energy) were larger for the XP method. The best poses obtained by the SP and XP method starting from the axial ring conformation are almost identical. Starting from the equatorial conformation different poses are obtained depending on the scoring function; using the XP method the dihydrobenzodioxine moiety orients closer towards the P-loop (same pose derived from the axial starting conformation), but with a distorted (non-planar) amide bond (this is because the XP method better accounts for hydrophobic interaction, which are important in this case, but the equatorial conformation does not fit – without distortion – into this angled orientation); with the SP method a more linear pose is obtained with no interaction in the P-loop. Although the docking poses, scores, and energies of the (*R*) and (*S*) isomers do not vary much, in case of the axial starting conformation the (S) isomer is preferred slightly and in case of the equatorial conformation the (*R*) isomer. Overall better and more consistent results are obtained from the axial ring conformation (SR3677-(*S*)_ax) and the best pose (of the (*S*) isomer) is shown in figure 1 and a 2D representation is shown in figure S2 below.

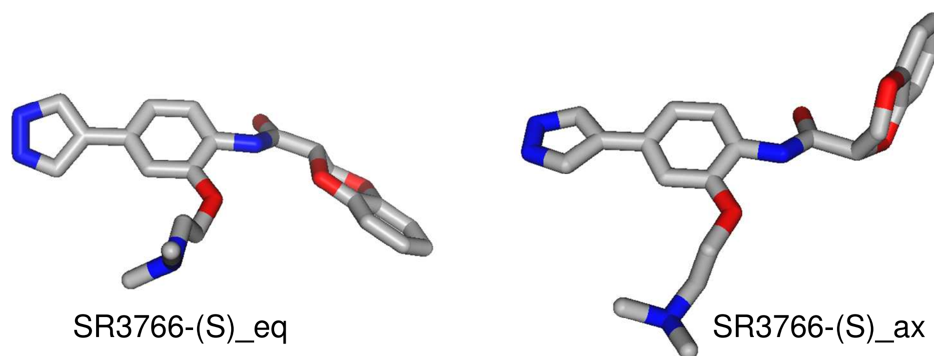

Figure S1. Starting ring conformations of **5** (SR3677) that were used for flexible docking into the ROCK-II model (depicted with Marvin Space).

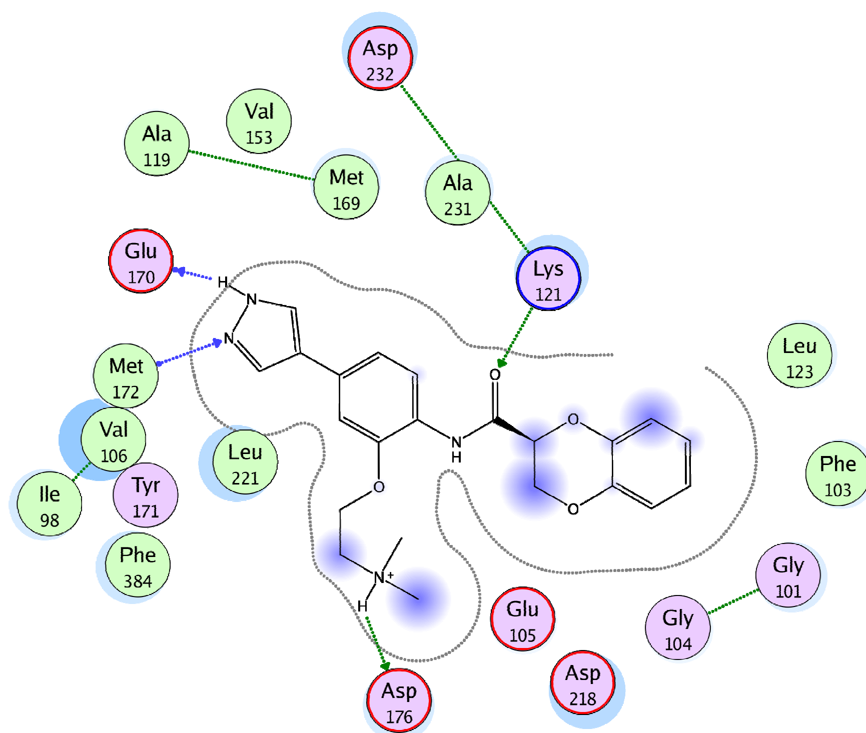

Figure S2. 2D representation of the binding mode of **5** (SR3677) in ROCK-II (residues around 5A of the ligand are shown, prepared with MOE).

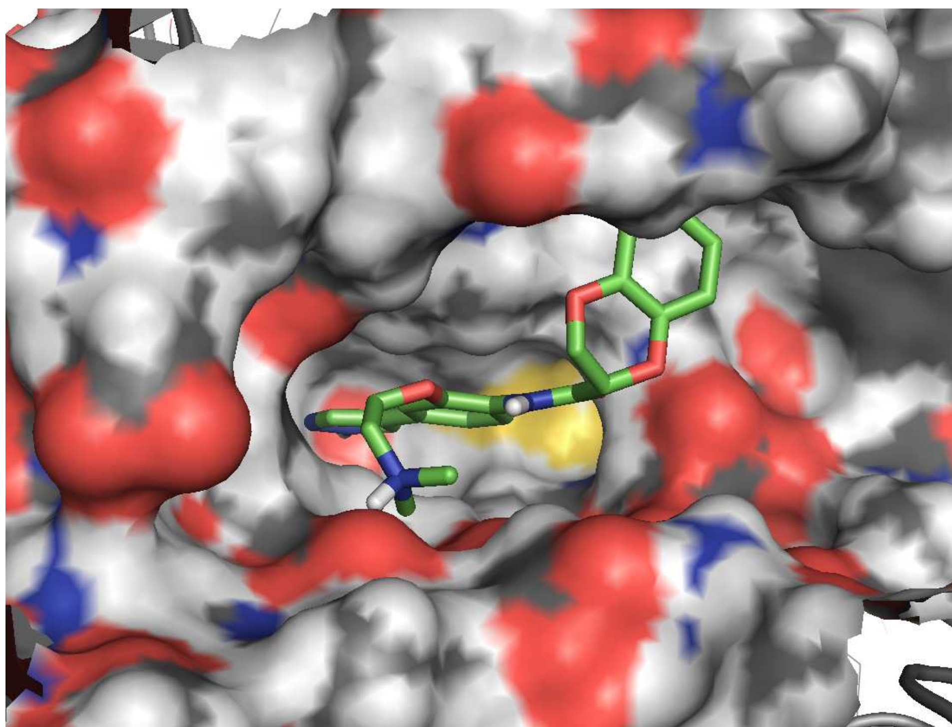

Figure S3. Surface representation of the binding pose of **5** (SR3677) in ROCK-II; note the hydrophobic pocket around the dihydrobenzodioxine moiety (figure prepared with PyMOL)

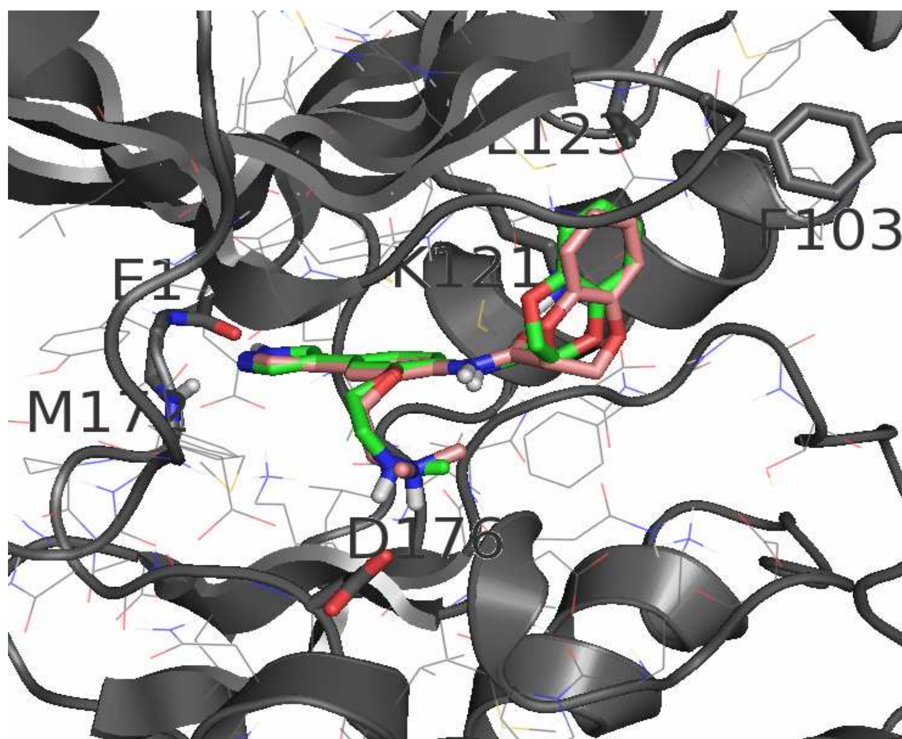

Figure S4. The docking of both the “S” (green) and the “R” (pink) enantiomers of **5** (SR3677) to ROCK-II (figure prepared with PyMOL).

(3) Pharmacokinetics

Pharmacokinetics studies were conducted in Sprague Dawley rats. The compound was formulated in a generic formulation at 1 mg/mL (e.g. 10:10:80, DMSO: tween 80: water, v:v:v) and dosed at 1 mg/kg intravenous into the femoral vein or 2 mg/kg by oral gavage. Blood was obtained at t = 5 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 6 hr, and 8 hr. Blood was collected into EDTA containing tubes and plasma was generated by standard centrifugation methods. All procedures and handling were according to standard operating procedures approved by the IACUC at Scripps Florida.

In order to assess in vivo pharmacokinetic parameters an LC-MS/MS bioanalytical method was developed where 25 µL of plasma was treated with 125 µL of acetonitrile containing an internal standard in a Millipore Multiscreen Solvinter 0.45 micron low binding PTFE hydrophilic filter plate (#MSRLN0450) and allowed to shake at room temperature for five minutes. The plate was then centrifuged for 5 minutes at 4000 rpm in a tabletop centrifuge and the filtrate was collected in a polypropylene capture plate. The filtrate (10 µL) is injected using an Agilent 1200 HPLC equipped with a Thermo Betasil C18 HPLC column 5 µm (50 x 2.1 mm) # 70105-052130. Mobile Phase A was water with 0.1% formic acid. Mobile Phase B was acetonitrile with 0.1% formic acid. Flow rate was 375 µL/min using a gradient of 90% A/10% B from 0 – 0.5 min, ramped to 5%A/95%B at 2 min, held at 5%A/95%B until 3.0 min, ramped to 90% A/10% B at 4 min, and held at 90% A/10% B until 7 min.

An API Sciex 4000 equipped with a turbo ion spray source was used for all analytical measurements. MRM methods were developed in positive ion mode. Peak areas of the analyte ion were measured against the peak areas of the internal standard. Data was fit using WinNonLin using an IV bolus model.

(4) Cytochrome P450 inhibition

Cytochrome P450 inhibition for four major isoforms are evaluated using a cocktail inhibition assay where the metabolism of specific marker substrates (CYP1A2 phenacetin demethylation to acetaminophen; CYP2C9, tolbutamide hydroxylation to hydroxytolbutamide; CYP2D6, bufuralol hydroxylation to 4′-Hydroxybufuralol; and

CYP3A4, midazolam hydroxylation to 1′-Hydroxymidazolam) in the presence or absence of 10 µM probe compound is evaluated. The concentration of each marker substrate is approximately its K_m value. Conditions were similar to those described by Tesino and Patonay[1] except 2C19 was not evaluated as we found that stock solutions of the 2C19 probe substrate, omeprazole, had poor stability. Specific inhibitors for each isoform are included in each run to validate the system.

(5) Biological Testing

(5a) Screening

All experiments were performed in Greiner FIA black 384-well low volume plates. All active enzymes except ROCK-II 1-543, which was cloned and purified as described earlier (Schröter, T., et al., *Comparison of Miniaturized Time-Resolved Fluorescence Resonance Energy Transfer and Enzyme-Coupled Luciferase High-Throughput Screening Assays to Discover Inhibitors of Rho-Kinase II (ROCK-II).* J. Biomol. Screen, 2008. **13**: p. 40-53.), were purchased from Upstate (now part of Milipore). Test compounds were dispensed in a 90% DMSO/10% water mixture using a 384 head offline Pintool system (GNF Systems)

ROCK-I/II assay

Assays were performed using the STK2 kinase system from Cisbio. 5 µl mixture of a 1 µM STK2 substrate and ATP (ROCK-I: 4 µM; ROCK-II: 20 µM) in STK-buffer was added to the wells using a BioRAPTR FRD™ Workstation (Aurora Discovery). 20nl of test compounds was dispensed. Reaction was started by addition of 5 µl of 2.5 nM

ROCK-I (Upstate #14-601) or 0.5 nM ROCK-II in STK-buffer. After 4 h at RT the reaction was stopped by addition of 10 µl of 1x antibody and 62.5 nM Sa-XL in detection buffer. After 1 h at RT the plates were read on the Viewlux in HTRF mode.

PKA assay

5 µl mixture of a 60 µM kemptide and 20 µM ATP in Kinase buffer (50 mM Hepes pH 7.3, 10 mM MgCl2, 0.1 % BSA, 2 mM DTT) was added to the wells using a BioRAPTR FRD™ Workstation (Aurora Discovery). 20 nl of test compounds was dispensed. Reaction was started by addition of 5 µl of 0.5 nM PKA (Upstate #14-440) in Kinase buffer (5 µl of kinase buffer for high wells). After 70 min at RT the reaction was stopped by addition of 10 µl Kinase-Glo reagent and plate was read after 10 min incubation time at RT on the Viewlux in luminescence mode.

MRCK assay

K_m for ATP and S6-peptide (LCB-AKRRRLSSLRA-NH_2) were determined by titration various concentration of ATP versus a constant concentration of peptide and vice versa using radioactive filter binding assays. S6-peptide, MRCK and ATP were mixed and reaction was started by addition of ATP and 1 uCi of ^{33}P-ATP/data point. After indicated time points aliquots were removed and reaction was stopped by 1 volume of 100 mM EDTA and 15 mM Ppi. Reaction was transferred to Millipore HTS PH plates and incubated for 5 min at RT, washed 5 times with 100 mM phosphoric acid (1.15 %). 50 ul/well scintillation cocktail was added and incorporation of radioactive phosphate was measured. For determination of IC_{50}s the determined biochemical parameters were used in a Kinase-Glo assay. As a final condition 5 µl mixture of a 40 µM S6-peptide (LCB-AKRRRLSSLRA-NH_2) and 10 µM ATP in Kinase buffer (50 mM

Hepes pH 7.3, 10 mM MgCl2, 0.1 % BSA, 2 mM DTT) was added to the wells using a BioRAPTR FRD™ Workstation (Aurora Discovery). 20 nl of test compounds was dispensed. Reaction was started by addition of 5 µl of 12 nM MRCK (Upstate #14-691) in Kinase buffer (5 µl of kinase buffer for high wells). After 75 min at RT the reaction was stopped by addition of 10 µl Kinase-Glo reagent and plate was read after 10 min incubation time at RT on the Viewlux in luminescence mode.

AKT1 assay

A 6 µl mixture of a 1 µM S6-peptide (LCB-AKRRRLSSLRA-NH_2) and 80 µM ATP in Kinase buffer (50 mM Hepes pH 7.3, 10 mM MgCl2, 0.1 % BSA, 2 mM DTT) was added to the wells using a BioRAPTR FRD™ Workstation (Aurora Discovery). 20 nl of test compounds was dispensed. Reaction was started by addition of 6 µl of 0.5 nM AKT1 (Upstate #14-276) in Kinase buffer. After 55 min at RT the reaction was stopped with a 8 µl mixture of 187.5 nM SreptavidinXL665 (Cisbio) and 2.5 ng of custom $Eu3^{+}$ Cryptate labeled (Cisbio, Cryptate/Antibody 4.17) phospho S6 peptide antibody (Cell Signaling # 2211) in Quench buffer (31.25 mM Hepes pH 7.5, 312.5 mM KF and 187.5 mM EDTA). After 1 h incubation at RT the plate was measured on the Viewlux in HTRF mode.

JNK assays:

Enzyme inhibition studies were performed in 384-well polystyrene HTRF plates (Grainier). For JNK3 incubations were performed for 15 min at ambient temperature (~22 °C) with, 0.2 μM biotinylated Flag-ATF2, 1 μM ATP, 0.3 nM activated JNK3α1 (with a control in the absence of kinase to determine the basal signal) For p38 incubations were performed for 30 min at ambient temperature (~22 °C) with, 0.4 μM

biotinylated Flag-ATF2, 10 μM ATP, 0.125 nM activated p38 (with a control in the absence of kinase to determine the basal signal). The reactions were carried out in 10μl volumes containing the final buffer concentrations; 50 mM Hepes pH 7.0, 2.5 mM MgCl2, 0.1 mg/ml bovine serum albumin, 1 mM DL-dithiothreitol, 0.01% Triton X-100 and 5% DMSO. A 10-point titration of all compounds was carried out in 3-fold dilutions from 2 μM – 10 pM. After the allotted time the kinase reaction was terminated by the addition 10μl of quenching solution (50 mM Hepes, pH 7.0, with 14 mM EDTA, 0.01% Triton X-100, 200 mM KF). The detection reagents, streptavidin-xl-APC (400nM) and europium cryptate-labeled rabbit polyclonal anti-phospho-ATF2 (0.43 ng/well) were purchased from CisBio. The HTRF signal was detected using a viewlux plate reader (PerkinElmer) 1 hr post-quenching. IC_{50} values were determined by fitting the data to the equation for a four-parameter logistic.

Target modulation using the LI-COR Odyssey system

A7r5 cells were plated at 5000 cells/well in a 96-well Packard View Plate (Perkin Elmer) in DMEM +10 % FBS. After allowing attachment overnight, cells were serum starved for 4 h and treated with inhibitor in 0.25% DMSO final concentration for 1 h at 37 °C. Cells were then treated with 10 µM LPA for 10 min. Following treatment, cells were immediately fixed with 4% paraformaldehyde for 30 minutes. After a brief wash in 0.1 M glycine, cells were permiablized in 0.2% Triton X for 10 minutes. Cells were then washed once in PBS and blocked in LI-COR blocking buffer (LI-COR Biosciences) for 1 h at 25 °C. Cells were probed for either phosphorylated myosin light chain 20 using 55 ng/mL primary rabbit antibody, total myosin light chain using 525 ng/mL primary rabbit

antibody or for α-tubulin using 1 μg/mL primary mouse antibody, and incubated overnight at 4 ℃. Following three washes, cells were probed with goat-anti-rabbit or goat-anti-mouse IR800 antibody (2 μg/mL in LI-COR block + 0.025% Tween-20) for 1 h at 25 ℃. Nuclei were stained with TO-PRO-3 iodide (642/661) (1:4000) for 20 minutes, washed twice in PBS/0.05% Tween-20 and read with an Odyssey Infrared Imaging System (LI-COR Biosciences).

(5b) Results from kinase panel screening for 3677 at 3 μM (Ambit screen):*

kinase	Percent Control	Conc. (nM)		kinase	Percent Control	Conc. (nM)
AAK1	No hit	3000		CDC2L1	No hit	3000
ABL1	No hit	3000		CDC2L2	No hit	3000
ABL1(E255K)	No hit	3000		CDK11	No hit	3000
ABL1(F317I)	No hit	3000		CDK2	No hit	3000
ABL1(F317L)	No hit	3000		CDK3	No hit	3000
ABL1(H396P)	No hit	3000		CDK5	No hit	3000
ABL1(M351T)	No hit	3000		CDK7	No hit	3000
ABL1(Q252H)	No hit	3000		CDK8	No hit	3000
ABL1(T315I)	No hit	3000		CDK9	No hit	3000
ABL1(Y253F)	No hit	3000		CDKL2	No hit	3000
ABL2	No hit	3000		CHEK1	No hit	3000
ACVR1	No hit	3000		CHEK2	No hit	3000
ACVR1B	No hit	3000		CIT	No hit	3000
ACVR2A	No hit	3000		CLK1	5.1	3000
ACVR2B	No hit	3000		CLK2	30	3000

ACVRL1	No hit	3000		CLK3	No hit	3000
ADCK3	No hit	3000		CLK4	10	3000
ADCK4	No hit	3000		CSF1R	No hit	3000
AKT1	No hit	3000		CSK	No hit	3000
AKT2	No hit	3000		CSNK1A1L	No hit	3000
AKT3	23	3000		CSNK1D	No hit	3000
ALK	No hit	3000		CSNK1E	No hit	3000
AMPK-alpha1	No hit	3000		CSNK1G1	No hit	3000
AMPK-alpha2	No hit	3000		CSNK1G2	No hit	3000
ANKK1	No hit	3000		CSNK1G3	No hit	3000
ARK5	No hit	3000		CSNK2A1	No hit	3000
AURKA	No hit	3000		CSNK2A2	No hit	3000
AURKB	No hit	3000		DAPK1	No hit	3000
AURKC	No hit	3000		DAPK2	No hit	3000
AXL	No hit	3000		DAPK3	No hit	3000
BIKE	No hit	3000		DCAMKL1	No hit	3000
BLK	No hit	3000		DCAMKL2	No hit	3000
BMPR1A	No hit	3000		DCAMKL3	No hit	3000
BMPR1B	No hit	3000		DDR1	No hit	3000
BMPR2	No hit	3000		DDR2	No hit	3000
BMX	No hit	3000		DLK	No hit	3000
BRAF	No hit	3000		DMPK	No hit	3000
BRAF(V600E)	No hit	3000		DMPK2	No hit	3000
BRSK1	No hit	3000		DRAK1	No hit	3000

BRSK2	No hit	3000		DRAK2	No hit	3000
BTK	No hit	3000		DYRK1B	No hit	3000
CAMK1	No hit	3000		EGFR	No hit	3000
CAMK1D	No hit	3000		EGFR(E746-A750del)	No hit	3000
CAMK1G	No hit	3000		EGFR(G719C)	No hit	3000
CAMK2A	No hit	3000		EGFR(G719S)	No hit	3000
CAMK2B	No hit	3000		EGFR(L747-E749del, A750P)	No hit	3000
CAMK2D	No hit	3000		EGFR(L747-S752del, P753S)	No hit	3000
CAMK2G	No hit	3000		EGFR(L747-T751del,Sins)	No hit	3000
CAMK4	No hit	3000		EGFR(L858R)	No hit	3000
CAMKK1	No hit	3000		EGFR(L861Q)	No hit	3000
CAMKK2	No hit	3000		EGFR(S752-I759del)	No hit	3000
EPHA1	No hit	3000		JAK1(Kin.Dom.1)	No hit	3000
EPHA2	No hit	3000		JAK1(Kin.Dom.2)	No hit	3000
EPHA3	No hit	3000		JAK2(Kin.Dom.2)	No hit	3000
EPHA4	No hit	3000		JAK3(Kin.Dom.2)	No hit	3000
EPHA5	No hit	3000		JNK1	No hit	3000
EPHA6	No hit	3000		JNK2	No hit	3000
EPHA7	No hit	3000		JNK3	No hit	3000
EPHA8	No hit	3000		KIT	No hit	3000
EPHB1	No hit	3000		KIT(D816V)	No hit	3000

EPHB2	No hit	3000	KIT(V559D)	No hit	3000
EPHB3	No hit	3000	KIT(V559D,T670I)	No hit	3000
EPHB4	No hit	3000	KIT(V559D,V654A)	No hit	3000
ERBB2	No hit	3000	LATS1	No hit	3000
ERBB4	No hit	3000	LATS2	12	3000
ERK1	No hit	3000	LCK	No hit	3000
ERK2	No hit	3000	LIMK1	No hit	3000
ERK3	No hit	3000	LIMK2	No hit	3000
ERK4	No hit	3000	LKB1	No hit	3000
ERK5	No hit	3000	LOK	No hit	3000
ERK8	No hit	3000	LTK	No hit	3000
FER	No hit	3000	LYN	No hit	3000
FES	No hit	3000	MAP3K3	No hit	3000
FGFR1	No hit	3000	MAP3K4	No hit	3000
FGFR2	No hit	3000	MAP3K5	No hit	3000
FGFR3	No hit	3000	MAP4K1	No hit	3000
FGFR3(G697C)	No hit	3000	MAP4K2	No hit	3000
FGFR4	No hit	3000	MAP4K3	No hit	3000
FGR	No hit	3000	MAP4K4	No hit	3000
FLT1	No hit	3000	MAP4K5	No hit	3000
FLT3	No hit	3000	MAPKAPK2	No hit	3000
FLT3(D835H)	No hit	3000	MAPKAPK5	No hit	3000
FLT3(D835Y)	No hit	3000	MARK1	No hit	3000
FLT3(ITD)	No hit	3000	MARK2	No hit	3000

FLT3(K663Q)	No hit	3000		MARK3	No hit	3000
FLT3(N841I)	No hit	3000		MARK4	No hit	3000
FLT4	No hit	3000		MEK1	No hit	3000
FRK	No hit	3000		MEK2	No hit	3000
FYN	No hit	3000		MEK3	No hit	3000
GAK	No hit	3000		MEK4	No hit	3000
GCN2(Kin.Dom.2,S808G)	No hit	3000		MEK6	No hit	3000
GSK3A	No hit	3000		MELK	No hit	3000
GSK3B	No hit	3000		MERTK	No hit	3000
HCK	No hit	3000		MET	No hit	3000
HIPK1	No hit	3000		MINK	No hit	3000
IGF1R	No hit	3000		MKNK1	No hit	3000
IKK-alpha	No hit	3000		MKNK2	No hit	3000
IKK-beta	No hit	3000		MLCK	No hit	3000
IKK-epsilon	No hit	3000		MLK1	No hit	3000
INSR	No hit	3000		MLK2	No hit	3000
INSRR	No hit	3000		MLK3	No hit	3000
IRAK3	No hit	3000		MRCKA	No hit	3000
ITK	No hit	3000		MRCKB	No hit	3000
MST1	No hit	3000		PKN1	No hit	3000
MST1R	No hit	3000		PKN2	No hit	3000
MST2	No hit	3000		PLK1	No hit	3000
MST3	No hit	3000		PLK3	No hit	3000
MST4	No hit	3000		PLK4	No hit	3000

MUSK	No hit	3000		PRKCD	No hit	3000
MYLK	No hit	3000		PRKCE	No hit	3000
MYLK2	No hit	3000		PRKCH	No hit	3000
MYO3A	No hit	3000		PRKCQ	No hit	3000
MYO3B	No hit	3000		PRKD1	No hit	3000
NDR2	No hit	3000		PRKD2	No hit	3000
NEK1	No hit	3000		PRKD3	No hit	3000
NEK2	No hit	3000		PRKG1	No hit	3000
NEK5	No hit	3000		PRKG2	No hit	3000
NEK6	No hit	3000		PRKR	No hit	3000
NEK7	No hit	3000		PRKX	No hit	3000
NEK9	No hit	3000		PTK2	No hit	3000
NLK	No hit	3000		PTK2B	No hit	3000
p38-alpha	No hit	3000		PTK6	No hit	3000
p38-beta	No hit	3000		RAF1	No hit	3000
p38-delta	No hit	3000		RET	No hit	3000
p38-gamma	No hit	3000		RET(M918T)	No hit	3000
PAK1	No hit	3000		RET(V804L)	No hit	3000
PAK2	No hit	3000		RET(V804M)	No hit	3000
PAK3	No hit	3000		RIOK1	No hit	3000
PAK4	No hit	3000		RIOK2	No hit	3000
PAK6	No hit	3000		RIOK3	No hit	3000
PAK7/PAK5	No hit	3000		RIPK1	No hit	3000
PCTK1	No hit	3000		RIPK2	No hit	3000

PCTK2	No hit	3000		RIPK4	No hit	3000
PCTK3	No hit	3000		ROCK2	0.4	3000
PDGFRA	No hit	3000		ROS1	No hit	3000
PDGFRB	No hit	3000		RPS6KA1(Kin.Dom.1)	No hit	3000
PDPK1	No hit	3000		RPS6KA1(Kin.Dom.2)	No hit	3000
PFTAIRE2	No hit	3000		RPS6KA2(Kin.Dom.1)	No hit	3000
PFTK1	No hit	3000		RPS6KA2(Kin.Dom.2)	No hit	3000
PHKG1	No hit	3000		RPS6KA3(Kin.Dom.1)	No hit	3000
PHKG2	No hit	3000		RPS6KA4(Kin.Dom.1)	No hit	3000
PIK3C2B	No hit	3000		RPS6KA4(Kin.Dom.2)	No hit	3000
PIK3CA	No hit	3000		RPS6KA5(Kin.Dom.1)	No hit	3000
PIK3CA(E545K)	No hit	3000		RPS6KA5(Kin.Dom.2)	No hit	3000
PIK3CB	No hit	3000		RPS6KA6(Kin.Dom.1)	No hit	3000
PIK3CD	No hit	3000		RPS6KA6(Kin.Dom.2)	No hit	3000
PIK3CG	No hit	3000		SgK085	No hit	3000
PIM1	No hit	3000		SgK110	No hit	3000
PIM2	No hit	3000		SLK	No hit	3000
PIM3	No hit	3000		SNARK	No hit	3000
PIP5K1A	No hit	3000		SNF1LK	No hit	3000
PIP5K2B	No hit	3000		SNF1LK2	No hit	3000
PKAC-alpha	No hit	3000		SRC	No hit	3000
PKAC-beta	No hit	3000		SRMS	No hit	3000
PKMYT1	No hit	3000		SRPK1	No hit	3000
SRPK2	No hit	3000		TRKC	No hit	3000

SRPK3	No hit	3000		TSSK1	No hit	3000
STK16	No hit	3000		TTK	No hit	3000
STK33	No hit	3000		TXK	No hit	3000
STK35	No hit	3000		TYK2(Kin.Dom.1)	No hit	3000
STK36	No hit	3000		TYK2(Kin.Dom.2)	No hit	3000
SYK	No hit	3000		TYRO3	No hit	3000
TAK1	No hit	3000		ULK1	No hit	3000
TAOK1	No hit	3000		ULK2	No hit	3000
TAOK3	No hit	3000		ULK3	No hit	3000
TEC	No hit	3000		VEGFR2	No hit	3000
TESK1	No hit	3000		WEE1	No hit	3000
TGFBR1	No hit	3000		WEE2	No hit	3000
TGFBR2	No hit	3000		YANK2	No hit	3000
TIE1	No hit	3000		YANK3	No hit	3000
TIE2	No hit	3000		YES	No hit	3000
TLK1	No hit	3000		YSK1	No hit	3000
TLK2	No hit	3000		ZAK	No hit	3000
TNIK	No hit	3000		ZAP70	No hit	3000
TNK1	No hit	3000		TRKA	No hit	3000
TNK2	No hit	3000		TRKB	No hit	3000
TNNI3K	No hit	3000				

- The scores shown are percentage of enzyme activity relative to that seen in a DMSO control experiment. Assays were scored “no hit” if enzyme activity was statistically indistinct from that observed upon exposure only to a DMSO control.

(5c) Results from counter screening of SR3677 against a panel of 70 non-kinase enzymes and receptors:

EXPERIMENTAL RESULTS - BIOCHEMICAL ASSAYS

Cat. #	TARGET	BATCH*	SPP.	n=	CONC.	%	↑% INHIBITION (-100, -50, 0, 50, 100)	IC_{50}	K_i	n_H	R
107300	Peptidase, Angiotensin Converting Enzyme	208157	rabbit	2	3 μM	1					
200510	Adenosine A_1	208162	hum	2	3 μM	-4					
200610	Adenosine A_{2A}	208163	hum	2	3 μM	5					
200720	Adenosine A_3	208165	hum	2	3 μM	-4					
♦ 203100	Adrenergic α_{1A}	208136	rat	2	3 μM	53					
♦ 203200	Adrenergic α_{1B}	208137	rat	2	3 μM	68					
203400	Adrenergic α_{1D}	208139	hum	2	3 μM	43					
♦ 203620	Adrenergic α_{2A}	208140	hum	2	3 μM	76					
204010	Adrenergic β_1	208169	hum	2	3 μM	43					
204110	Adrenergic β_2	208170	hum	2	3 μM	19					
285010	Androgen (Testosterone) AR	207809	rat	2	3 μM	10					
210020	Angiotensin AT_1	208052	hum	2	3 μM	-1					
210110	Angiotensin AT_2	208054	hum	2	3 μM	-1					
212510	Bradykinin B_1	208111	hum	2	3 μM	-3					
212610	Bradykinin B_2	208113	hum	2	3 μM	-18					
214510	Calcium Channel L-Type, Benzothiazepine	208032	rat	2	3 μM	47					
214600	Calcium Channel L-Type, Dihydropyridine	208128	rat	2	3 μM	24					
216000	Calcium Channel N-Type	208037	rat	2	3 μM	-6					
219500	Dopamine D_1	208175	hum	2	3 μM	17					
219700	Dopamine D_{2S}	208177	hum	2	3 μM	6					
219800	Dopamine D_3	208179	hum	2	3 μM	15					
219900	Dopamine $D_{4.2}$	208181	hum	2	3 μM	-2					
224010	Endothelin ET_A	208073	hum	2	3 μM	1					
224110	Endothelin ET_B	208075	hum	2	3 μM	-1					
225510	Epidermal Growth Factor (EGF)	208077	hum	2	3 μM	-1					
226010	Estrogen ERα	208081	hum	2	3 μM	-2					
226300	G Protein-Coupled Receptor GPR103	208027	hum	2	3 μM	25					
226600	$GABA_A$, Flunitrazepam, Central	208186	rat	2	3 μM	-18					
226500	$GABA_A$, Muscimol, Central	208141	rat	2	3 μM	5					
228610	$GABA_{B1A}$	208090	hum	2	3 μM	4					

EXPERIMENTAL RESULTS - BIOCHEMICAL ASSAYS

Cat. #	TARGET	BATCH*	SPP.	n=	CONC.	%	† % INHIBITION (-100, -50, 0, 50, 100)	IC_{50}	K_i	n_H	R
232700	Glutamate, Kainate	208189	rat	2	3 μM	-11					
232810	Glutamate, NMDA, Agonism	208190	rat	2	3 μM	-4					
232910	Glutamate, NMDA, Glycine	208191	rat	2	3 μM	-10					
233000	Glutamate, NMDA, Phencyclidine	208192	rat	2	3 μM	-2					
239610	Histamine H_1	208195	hum	2	3 μM	7					
239710	Histamine H_2	208196	hum	2	3 μM	1					
239810	Histamine H_3	207911	hum	2	3 μM	-2					
241000	Imidazoline I_2, Central	208129	rat	2	3 μM	14					
243510	Interleukin IL-1	207950	mouse	2	3 μM	-2					
250460	Leukotriene, Cysteinyl $CysLT_1$	207922	hum	2	3 μM	0					
251600	Melatonin MT_1	207927	hum	2	3 μM	7					
252610	Muscarinic M_1	208208	hum	2	3 μM	6					
252710	Muscarinic M_2	208209	hum	2	3 μM	4					
252810	Muscarinic M_3	208210	hum	2	3 μM	3					
257010	Neuropeptide Y Y_1	208219	hum	2	3 μM	-8					
257110	Neuropeptide Y Y_2	208221	hum	2	3 μM	0					
258590	Nicotinic Acetylcholine	208028	hum	2	3 μM	12					
258700	Nicotinic Acetylcholine α1, Bungarotoxin	208029	hum	2	3 μM	7					
260110	Opiate δ (OP1, DOP)	208229	hum	2	3 μM	-9					
260210	Opiate κ (OP2, KOP)	208230	hum	2	3 μM	-7					
260410	Opiate μ (OP3, MOP)	208231	hum	2	3 μM	5					
264500	Phorbol Ester	208038	mouse	2	3 μM	10					
265010	Platelet Activating Factor (PAF)	208217	hum	2	3 μM	-4					
265600	Potassium Channel [K_{ATP}]	208224	ham	2	3 μM	2					
265900	Potassium Channel hERG	208134	hum	2	3 μM	31					
268410	Prostanoid EP_4	208215	hum	2	3 μM	5					
268700	Purinergic P_{2X}	207821	rabbit	2	3 μM	4					
268810	Purinergic P_{2Y}	207823	rat	2	3 μM	0					
270000	Rolipram	208135	rat	2	3 μM	-1					
271110	Serotonin (5-Hydroxytryptamine) $5\text{-}HT_{1A}$	207915	hum	2	3 μM	6					

EXPERIMENTAL RESULTS - BIOCHEMICAL ASSAYS

Cat. #	TARGET	BATCH*	SPP.	n=	CONC.	†% INHIBITION (%)	IC_{50}	K_i	n_H	R
271910	Serotonin (5-Hydroxytryptamine) 5-HT_7	208130	hum	2	3 μM	2				
278110	Sigma σ_1	208142	hum	2	3 μM	-18				
278200	Sigma σ_2	208144	rat	2	3 μM	-4				
279510	Sodium Channel, Site 2	208133	rat	2	3 μM	22				
255510	Tachykinin NK_1	208104	hum	2	3 μM	-6				
285900	Thyroid Hormone	208282	rat	2	3 μM	-1				
220320	Transporter, Dopamine (DAT)	208023	hum	2	3 μM	37				
226400	Transporter, GABA	208095	rat	2	3 μM	17				
204410	Transporter, Norepinephrine (NET)	208021	hum	2	3 μM	16				
274030	Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	208026	hum	2	3 μM	3				

[1] Testino, S. A., Jr;.Patonay, G. High-throughput inhibition screening of major human cytochrome P450 enzymes using an in vitro cocktail and liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2003**, *30*, 1459-1467.