Supporting Information

Hit to Lead Account of the Discovery of Bisbenzamide and Related Ureidobenzamide Inhibitors of Rho Kinase

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1. **ATP competition using IMAP assay**
2. **Comparison of IMAP and luciferase data for dimethylaminomethylphenyl-substituted analogs and selected comparators**
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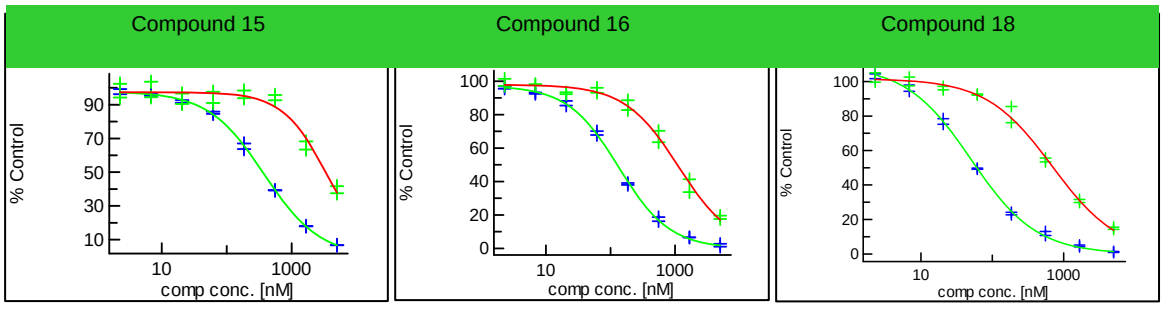
1. ATP competition for compounds **15, 16,** and **18** (IMAP assay):



Green line: 10 μM ATP; Red line: 200 μM ATP

2. Comparison of IMAP and luciferase results

A comparison of results from these two assay formats for analogs demonstrating weak inhibition of the luciferase enzyme used to quantify residual ATP in the luciferase assay is reported in Table 1. Three standards using alternative RHS fragments are included. This comparison shows that while the IC_{50}'s were approximately 2–4-fold higher using the IMAP technology, the relative potencies were consistent with the exception of **49** which demonstrated a smaller shift. The reduction in potency in the IMAP assay as compared to the luciferase assay is likely due in part to the varying concentrations of ATP (750 nM – luciferase assay; 100 μM – IMAP assay).

Table 1. Comparison of hROCK2 IC_{50} (nM) data from luciferase and IMAP assay.

Entry	luciferase	IMAP
16	100	320
17	53	140
21	69	230
30	>7000	>7000
31	1300	>6500
32	>6000	>6400
33	266	660
34	92	230
35	160	350
36	670	1300
37	1700	>6500
43	14	48
44	65	160
45	70	210

46	10	45
47	8	14
48	74	180
49	240	290

3. IMAP dose–response assay method

The activity of ROCK2 (1-543) kinase was measured utilizing IMAP FP Screening Express Kit (Catalogue no. R8127) and FAM S6 substrate peptide (Catalogue no. R7184) from Molecular Devices (Sunnyvale, CA). The assay was performed in 96-well half-area, black, non-binding surface microtiter plates (Corning, catalog no. 3686) in assay buffer consisting of 10 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 0.05% NaN_3, 0.1% phosphate-free BSA, and 1 mM DTT. Test compounds received as 5 mg/mL DMSO stocks were serially diluted 1:3 in DMSO for the 10-point concentration response. The DMSO dilutions were further diluted in the assay buffer, and 10 µL of this dilution was added to the assay plate, for a final starting assay concentration of 3 µg/mL in 1% DMSO. A 10 µL aliquot of 3 nM ROCK2 (29.0 µM stock) diluted in kinase reaction buffer was added to each assay well for a final concentration of 1 nM kinase. A 10 µL aliquot of a mixture of 600 nM FAM S6 peptide and 300 µM ATP diluted in kinase reaction buffer was added to each well for a final concentration of 200 nM peptide and 100 µM ATP. The kinase reaction mixture was incubated for 60 min at room temperature followed by addition of 60 µL IMAP progressive binding reagent diluted 400-fold in 1X Binding buffer A. After 30 min of incubation at room temperature, the plates were read for fluorescence polarization (FP) on an Analyst plate reader (Molecular Devices) using Ex to 485 nm, Em 530 nm, and FL 505 dichroic mirror. The FP signals were converted to

POC values and the IC_{50} for the concentration-responsive compounds was determined as described above.

4. Selectivity data for **18** and **50**:

18:

AMPK, ANKRD3, Aurora B, CAMK1, CDK2, CHK1, CHK2, CK1, CK2, CLK1, CRAC, CSK, DAPK1, DDR1, DDR2, DMPK, DYRK1A, EF2K, EGFR ERK8, GSG2, GSK3β, ICE, IGF1R, IKKE, JAK3, JNK1α1, JNK3α1, LRRK2, MAPK2, MAPKAP-K1A, MAPKAP-K1b, MAPKAP-K2, MAPKAP-K3, MARK3, MKK1, MNK1, MNK2, MSK1, MST2, MTOR, NEK2α, NEK6, NEK7, NIK, p38α, PBK, PDK1, PKA, PKBβ, PKBDPH, PKCa, PKD1, PLK1, PRAK, PRK2, RSK2, S6K1, SAPK2α, SGK, SMMLCK, SRC, SRPK1

All > 50 POC @ 3-6 μM

ITK, PLK, MAP3K11, PIM2, LYN, PTK2, MST4, VEGFR1, PRKCD

All > 10 μM (IC_{50})

PRKG1 5600 nM (IC_{50})

50

AMPK, Aurora B, CAMK1, CDK2, CHK1, CHK2, CK1, CK2, CSK, DYRK1A, EF2K, ERK8, GSK3β, JNK1α1, JNK3α1, MAPK2, MAPKAP-K1A, MAPKAP-K1b, MAPKAP-K2, MAPKAP-K3, MARK3, MKK1, MNK1, MNK2, MSK1, MST2, NEK2α, NEK6, NEK7, PBK, PDK1, PIM2, PKA, PKBβ, PKBDPH, PKD1, PLK1, PRAK, S6K1, SAPK2α, SGK, SMMLCK, SRC, SRPK1

All > 50 POC @ 3 μM

PRK2 33POC @ 3 μM; PKCα 50POC @ 3 μM; PRKG1 3000 nM (IC_{50})

5. Profiling assay methods:

High Throughput Solubility

A Beckman BioMek FX workstation (Beckman Coulter, Fullerton, CA) was used as the automation platform. Test compound kinetic solubility was measured using a *u*Sol Evolution Solubility Analyzer (pION, Woburn, MA). For solubility experiments,

duplicate dosing solutions of test compounds were prepared from 10 mM DMSO stock solutions in system solutions at pH 4.5 and 7.4 and were added to 96-well assay plates. After a specified time (16-19 hr), a sample of the test solution is vacuum filtered and the filtrate is transferred to a UV sample plate. Spectra are collected from 250-498 nm using a Spectramax Plus UV spectrophotometer (Molecular Devices, Sunnyvale, CA) and the solubility values (mcg/ml) were determined with pION software.

Fluorescent Cytochrome P450 Inhibition Assay

A Tecan Genesis 150 workstation, programmed with Gemini software (Tecan US, Durham, NC) was used as the automation platform and was integrated with a Tecan Genios Pro multifunctional plate reader.

This assay measured the IC_{50} of test compounds to inhibit isoforms of the human hepatic xenobiotic metabolizing enzymes CYP1A2, 2C9, 2C19, 2D6, and 3A4. Eight concentrations (0.01-30 μM in half-log intervals) of each test compound were tested in 25-200 mM K_3PO_4, 1.3-8.2 mM NADP+, 0.41-3.3 mM glucose-6-phosphate, 0.41-3.3 mM $MgCl_2$, and 0.4 Units/mL glucose-6-phosphate dehydrogenase. Test compounds, which were previously dissolved in acetonitrile, were separately diluted in assay buffer. Duplicate 100 μL aliquots of diluted test compound were dispensed into Falcon Microtest 96-well assay plates (BD Biosciences, Franklin Lakes, NJ), and the plates were preincubated for at least 10 min at 37 ^{o}C. A 100 μL volume of human recombinant CYP isoforms (Supersomes, BD Gentest, Woburn, MA; 2.5-15.0 pmol/mL) and substrate (CYP1A2: 7-ethoxy-3-cyanocoumarin, 5 μM; CYP2C9: 7-methoxy-4-trifluoromethylcoumarin, 75 μM); CYP2C19: 7-ethoxy-3-cyanocoumarin, 25 μM;

CYP2D6: 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin, 1.5 μM; CYP3A4BQ: 7-benzyloxyquinoline, 40 μM; CYP3A4BFC: 7-benzyloxy-4-trifluoromethylcoumarin, 50 μM) were then added to each test well. The reaction was incubated for 15-45 min at 37 ^{o}C. Standard inhibition curves using assay-specific reference inhibitors (BD Gentest, Woburn, MA) were run with each assay plate. The reaction was terminated with the addition of 75 μL 80% acetonitrile/20% 0.5 M Tris base. The assay plate was read with the fluorescent plate reader at product-specific excitation and emission wavelengths. The IC_{50} values were calculated using XL Fit.

LCMS/MS Cytochrome P450 lnhibition Assay

A Caliper SciClone ALH3000 workstation, programmed with Maestro software (Caliper Life Science, Hopkinton, MA) was used as the automation platform.

This assay measured the IC_{50} of test compounds to inhibit isoforms of the human hepatic xenobiotic metabolizing enzymes CYP1A2, 2C9, 2C19, 2D6, and 3A4. Two concentrations (1 and 10 μM) of each test compound were tested in 100 mM K_3PO_4 and 2 mM NADPH. Test compounds, which were previously dissolved in 0.1% DMSO, were separately diluted in assay buffer. Duplicate 100 μL aliquots of diluted test compound were dispensed into 96-well assay plates, and the plates were preincubated for at least 10 min at 37 ^{o}C. Human recombinant CYP isoforms (BD Gentest, Woburn, MA; 0.5 mg/mL protein) and substrate (4-60 μM) were then added to each test well. The reaction was incubated for 8-30 min at 37 ^{o}C. Standard inhibition curves using assay-specific reference inhibitors (Sigma-Aldrich, St. Louis, MO) were run with each assay plate. The reaction was terminated with the addition of equal volumes of acetonitrile and

an analytical internal standard. After centrifugation, the supernatants are analyzed by LC-MS/MS (Applied Biosystems/MDS Sciex, Toronto, ON) for acetaminophen (CYP1A2), 4'-hydroxydiclofenac (CYP2C9), 4'-hydroxy-(S)-mepheytoin (CYP2C19), dextrophan (CYP2D6), and 6-beta-hydroxy-testosterone (CYP3A4). High throughput IC_{50} values and slopes were calculated by fitting the percent of control data into a 2-parameter Hill equation using XLFit.

Parallel Artificial Membrane Permeability Assay (PAMPA)

A Tecan Genesis CPW 150 workstation (Tecan US, Durham, NC) was used as the automation platform. The workstation was programmed with Gemini software. Test compound permeability was measured using a filter-immobilized artificial membrane coated with a lipid solution, which is mounted on a PAMPA Evolution Permeability Analyzer (pION, Woburn, MA). For permeability experiments, dosing solutions of test compounds were prepared from 10 mM DMSO stock solutions in a system solution at pH 7.4 and were added to the donor side of the 96-well assay plates, with a chemical sink buffer in the acceptor side. After a specified time (3-18 hr), the ratio of the test concentration in the acceptor and donor wells represents the passive permeability. Analysis was done by uv spectrometry, and the apparent permeability coefficients (Pe x 10^{-6} cm/s) were determined with XLFit

Microsomal stability (Human Liver Microsomes)

Human liver microsomes were diluted in assay buffer (50 mM potassium phosphate buffer, pH 7.4 and 2.5 mM NADPH) to a final concentration of 1 mg protein/mL. A volume of 25 μL compound **1** (1-10 μM) and 50 μL microsome suspension were added

to 825 μL assay buffer. The preparation was incubated for 5 min at 37 ^{o}C. The reaction was started by the addition of 100 μL NADPH. Volumes of 80 μL were removed from the incubation mix at 0, 3, 6, 10, 15, 20, 40, and 60 min after the start of the reaction and added to 160 μL acetonitrile. The samples were shaken for 20 sec and then centrifuged. A 200 μL volume of the supernatant was transferred to 0.25 mm glass fiber filter plates and centrifuged. Injection volumes of 10 μL were added to Zorbax SB C8 HPLC columns with formic acid in water/acetonitrile at a flow rate of 1.5 mL/min. Percent loss of parent compound was calculated from the area under each time point to determine the half-life.

Caco-2 permeability

A dosing solution of **1** in Hank's Balanced Salt Solution (pH 7.4) at a concentration of 10 μM was stirred overnight at ambient temperature, filtered, and added to the donor side (apical or basolateral) of the Caco-2 monolayers, and Hank's Balanced Salt Solution (pH 7.4) was added to the receiver side of the monolayers. At each time point (30, 60, 90, and 120 min) samples were collected from the receiver side and analyzed by LC/MS/MS.