Supporting Information

Discovery of an orally efficacious imidazo[5,1-*f*][1,2,4]triazine dual inhibitor of IGF-1R and IR

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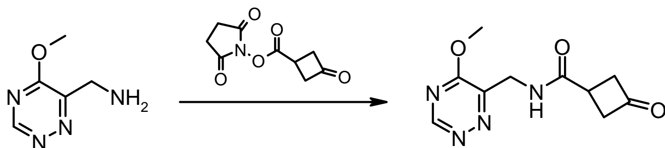
Experimental

General Synthetic and Analytical Chemistry Methods:

Unless otherwise noted, all materials/reagents were obtained from commercial suppliers and used without further purification. Reactions were monitored by thin layer chromatography (TLC) on silica gel 60 F_{254} (0.2 mm) precoated aluminum foil and visualized using UV light. Flash chromatography was performed with silica gel (400-230 mesh). Preparatory TLC was performed on Whatman LK6F silica gel 60 Å size 20 × 20 cm plates with a thickness of 1000 μm. Mass-directed HPLC purification of compounds was performed on a Waters system composed of the following: 2767 Sample Manager, 2525 Binary Gradient Module, 600 Controller, 2487 Dual λ Absorbance Detector, Micromass ZQ for mass ionization, Phenomenex Luna C18(2) 5 μM 100 Å, 150 × 21.2

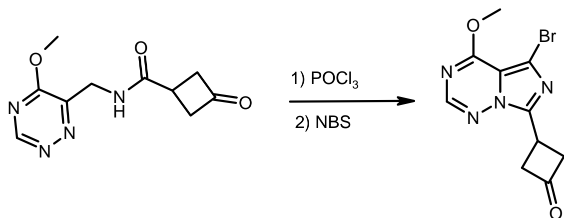
mm column with mobile phases of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in HPLC water (B). The general method is a flow rate of 30 mL/min, run time of 13 min, and a gradient profile of 0.00 min 2% A, 2.10 min 10% A, 8.00 min 50% A, 12.0 min 99% A, 12.8 min 2% A. LC-MS data was collected on either ZQ2 or ZQ3. ZQ2 is an Agilent 1100 HPLC equipped with a Gilson Auto injector and Waters Micromass ZQ for ionization. ZQ3 is an Agilent 1100 HPLC equipped with an HP Series 1100 auto injector and Waters Micromass ZQ for ionization. Both systems use the Xterra MS C18, 5 μM particle size, 50 × 4.6 mm with a mobile phase of acetonitrile (A) and 0.01% formic acid in HPLC water (B). The general method is a flow rate of 1.3 mL/min, run time of 5 min, and a gradient profile of 0.00 min 5% A, 3.00 min 90% A, 3.50 min 90% A, 4.00 min 5% A, 5.00 min 5% A. High resolution mass spectra (HRMS) were obtained by UPLC-TOF-MS on a LCT PREMIER XE mass spectrometer (Waters), using positive electrospray ionization mode and a scan range of m/z = 150–850 and were accurate to ±5 ppm. The UPLC system used a Waters BEH C18, 1.7 μM, 50 × 2.1 mm column heated to 50 ºC conditioned with a mobile phase of acetonitrile (A) and 10% acetonitrile in 10 mM aq. ammonium acetate (B) and a gradient profile of 0.0 min 0% A, 0.5 min 0% A, 2.3 min 90% A, 2.4 min 90% A, 2.5 min 0% A, 3.0 min 0% A. The flow rate was 0.8 mL/min. Samples were approximately 5 or 10 μg/mL of each compound in mobile phase A, with an internal standard for mass calibration. ^{1}H NMR (400 MHz) and ^{13}C NMR (100.6 MHz) spectra were recorded on Bruker or Varian instruments at ambient temperature with TMS or the residual solvent peak as the internal standard. The line positions or multiples are given in ppm (δ) and the coupling constants (J) are given as absolute values in Hertz (Hz). All melting points were determined with a Mel-Temp II apparatus and are uncorrected. All the final compounds have HPLC purity greater than 97%.

***N*-[(5-Methoxy-1,2,4-triazin-6-yl)methyl]-3-oxocyclobutanecarboxamide**



A suspension of 1-(5-methoxy-1,2,4-triazin-6-yl)methanamine (1.00 g, 7.13 mmol) and 1-{[(3-oxocyclobutyl)carbonyl]oxy}pyrrolidine-2,5-dione (1.81 g, 8.56 mmol) in THF (15 mL) was charged with a 10% (w/w) aq. $NaHCO_3$ solution (10 mL) at 0 ºC. The resulting mixture was slowly warmed to rt and stirred at rt for 3.5 h. The solvent was removed under reduced pressure and the mixture was extracted with ethyl acetate (30 mL x 3). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated under reduce pressure to give a crude residue, which was purified by a silica gel chromatography (eluent: 2% methanol in methylene chloride) to give the desired product as a white solid (Yield: 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.13-3.31 (m, 3H), 3.47-3.58 (m, 2H), 4.12 (s, 3H), 4.75 (d, J = 4.0 Hz, 2H), 7.11 (br. s., 1H), 9.08 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.4, 173.7, 161.1, 156.0, 148.9, 54.9, 51.8, 39.0, 28.7; MS (ES^+): *m/z* 237.10 (MH^+); HPLC: t_R = 2.21 min (OpenLynx, polar_5min); HRMS (ESI): Calcd for $C_{10}H_{13}N_4O_3[MH^+]$: 237.0988, Found: 237.0986; mp = 127 ºC.

3-(5-Bromo-4-methoxy-imidazo[5,1-f][1,2,4]triazin-7-yl)-cyclobutanone

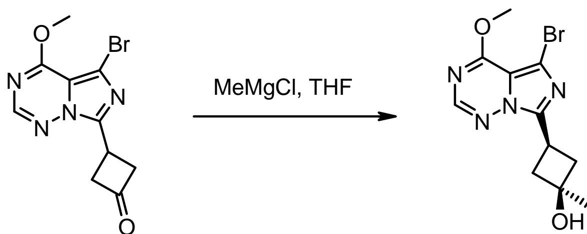


A solution of 3-oxo-cyclobutanecarboxylic acid (5-methoxy-[1,2,4]triazin-6-ylmethyl)-amide (2.40 g, 8.33 mmol) in anhydrous MeCN (100 mL) and anhydrous DMF (20 mL) was charged with $POCl_3$ (3.10 mL, 33.3 mmol) dropwise over 10 min period at 0 ºC. The resulting mixture was allowed to warm to rt and stirred at rt overnight. The reaction mixture was concentrated under reduced pressure to give a residue which was poured over ice. Solid $NaHCO_3$ was added to adjust pH to ~8 and the mixture was extracted with DCM (100 mL x 3). The combined organic layers were washed with brine, dried

over Na_2SO_4, and concentrated under reduced pressure to give a crude product (LC-MS purity >90%), which was used for next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.51-3.62 (m, 3H), 3.68-3.78 (m, 2H), 4.18 (s, 3H), 7.71 (s, 1H), 8.05 (s, 1H); MS (ES^+): *m/z* 219.07 (MH^+); HPLC: t_R = 2.41 min (OpenLynx, polar_5min).

A solution of the material prepared above in anhydrous DMF (60 mL) was charged with NBS (1.48 g, 8.33 mmol) in 3 portions at 0 ºC. The resulting mixture was stirred at 0 °C for an additional 2 h. The reaction was quenched with 10% aq. $Na_2S_2O_3$ solution (20 mL) and the resulting mixture was extracted with ethyl acetate (200 mL x 3). The combined organic layers were washed with brine (20 mL x 2), dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give a crude residue, which was purified by a silica gel chromatography (eluent: methylene chloride) to give the desired product as a white solid (Yield: 55% over two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.45-3.60 (m, 3H), 3.66-3.77 (m, 2H), 4.19 (s, 3H), 8.01 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 205.5, 161.9, 148.6, 145.1, 113.1, 107.0, 54.9, 52.1, 19.9; MS (ES^+): *m/z* 296.98, 299.02 (MH^+); HPLC: t_R = 3.18 min (OpenLynx, polar_5min); HRMS (ESI): Calcd for $C_{10}H_{10}N_4O_2{}^{79}Br$ [MH^+]: 296.9987, Found: 296.9980 mp = 174 ºC.

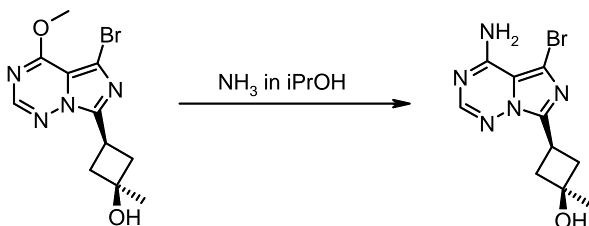
cis-3-(5-Bromo-4-methoxyimidazo[5,1-_f_][1,2,4]triazin-7-yl)-1-methylcyclobutanol



A solution of 3-(5-bromo-4-methoxyimidazo[5,1-*f*][1,2,4]triazin-7-yl)cyclobutanone (3.0 g, 10.1 mmol) in anhydrous THF (75 mL) was charged with a solution of methylmagnesium chloride in THF (3.0 M, 6.75 mL, 20.3 mmol) dropwise at -78 ºC over a 15 min period under N_2. The resulting mixture was stirred at –78 ºC for an additional 2 h then warmed to –20 °C for 30 min. The mixture was cooled back to –78 °C, quenched with sat. NH_4Cl (60 mL) at -78 ºC and then warmed to rt. The inorganic solids were removed by filtration, washed with EtOAc (100 mL x 2) and the combined filtrate was washed with brine (200 mL), dried over Na_2SO_4, filtered, and concentrated *in vacuo* to

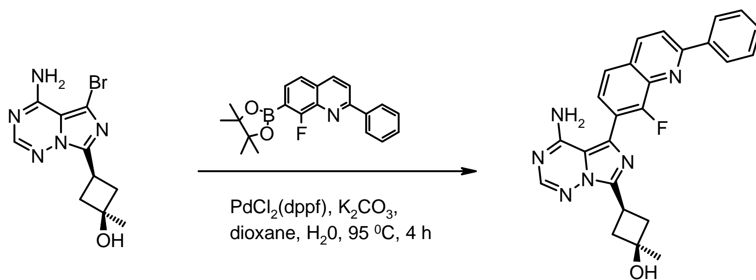
give the desired product as a white solid (Yield: 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.50 (s, 3H), 2.60-2.64 (m, 4H), 3.68 (m, 1H), 4.18 (s, 3H), 7.98 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.1, 148.6, 146.7, 112.6, 107.3, 67.8, 55.0, 42.9, 27.0, 21.2; MS (ES^+): *m/z* 312.94, 314.96 [MH^+]; HPLC: t_R = 2.68 min. (OpenLynx, polar_5min); HRMS (ESI): Calcd for $C_{11}H_{14}N_4O_2{}^{79}Br$ [MH^+]: 313.0301, Found: 313.0305; mp = 164 ºC.

cis-3-(4-Amino-5-bromoimidazo[5,1-_f_][1,2,4]triazin-7-yl)-1-methylcyclobutanol



A solution of 3-(5-bromo-4-methoxyimidazo[5,1-*f*][1,2,4]triazin-7-yl)-1-methyl cyclobutanol (2.0 g, 6.40 mmol) in 2 N NH_3 in *i*-PrOH (24.0 mL, 48.0 mmol) was heated at 50 ºC in a Par bomb apparatus for 16 h. The reaction mixture was cooled to –10 °C, the resulting solid was collected by filtration, washed with cold *i*-PrOH (5 mL x 2) and dried in a high vacuum oven overnight to give desired product as a white solid (Yield: 84%). ^{1}H NMR (400 MHz, CD_3OD) δ 1.48 (s, 3H), 2.41-2.62 (m, 4H), 3.61 (m, 1H), 7.79 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 154.8, 149.7, 145.4, 111.4, 105.5, 67.4, 42.6, 26.6, 20.6; MS (ES^+): *m/z* 297.99, 299.95 [MH^+]; HPLC: t_R = 2.56 min. (OpenLynx, polar_5min); HRMS (ESI): Calcd for $C_{10}H_{13}N_5O^{79}Br$ [MH^+]: 298.0303, Found: 298.0306; mp = 235 ºC (decomp.).

cis-3-[4-Amino-5-(8-fluoro-2-phenylquinolin-7-yl)imidazo[5,1-_f_][1,2,4]triazin-7-yl]-1-methylcyclobutanol



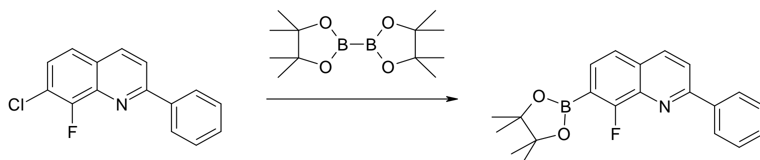
A mixture of *cis*-3-(4-amino-5-bromo-imidazo[5,1-*f*][1,2,4]triazin-7-yl)-1-methyl-cyclobutanol (1.50 g, 5.30 mmol), 8-fluoro-2-phenyl-7-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-quinoline (3.50 g, 10.0 mmol), potassium carbonate (2.10 g, 15.2 mmol) and 1,1'-bis(diphenylphosphino)ferrocenepalladium(II) dichloride (0.41 g, 0.50 mmol) in degassed dioxane/water (4:1, v/v, 72 mL) was heated at 95 ºC under N_2 for 4 h. The reaction mixture was evaporated to dryness and the residue obtained was heated with water (25 mL x 2) and decanted. The residual was purified by silica gel chromatography using a gradient (100% CH_2Cl_2 → 3% MeOH in CH_2Cl_2) to give the desired product as a yellow solid (Yield: 65%). ^{1}H NMR (400 MHz, CD_3OD) δ 1.49 (s, 3H), 2.52-2.59 (m, 2H), 2.60-2.68 (m, 2H), 3.71 (m, 1H), 7.48-7.58 (m, 3H), 7.73 (dd, J = 8.4, 6.6 Hz, 1H), 7.87-7.91 (m, 2H), 8.17 (d, J = 8.8 Hz, 1H), 8.23-8.27 (m, 2H), 8.49 (dd, J = 8.8, 1.1 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 156.2, 155.7, 154.3, 149.5, 145.9, 138.3, 138.2, 137.1, 129.9, 128.9, 128.7, 128.6, 127.3, 126.8, 123.3, 120.6, 119.7, 112.15, 67.8, 42.9, 26.9, 21.1; MS (ES^+): *m/z* 441.01 [MH^+]; HPLC: t_R = 2.92 min. (OpenLynx, polar_5min); HRMS (ESI): Calcd for $C_{25}H_{21}FN_6O$[MH^+]: 441.1839, Found: 441.1846; mp = 160 ºC.

cis-3-[4-Amino-5-(8-fluoro-2-phenylquinolin-7-yl)imidazo[5,1-_f_][1,2,4]triazin-7-yl]-1-methylcyclobutanol hydrochloride salt

A mixture of *cis*-3-[4-amino-5-(8-fluoro-2-phenylquinolin-7-yl)imidazo[5,1-*f*][1,2,4]triazin-7-yl]-1-methylcyclobutanol (8.6 g, 19.5 mmol) in absolute ethanol (800 mL) was charged with a solution of 2M HCl in ether (39.0 mL, 78.0 mmol) dropwise over 10 min period. The resulting solution was stirred at rt overnight and the precipitate was collected by filtration, washed with dry EtOH and diethyl ether, and dried in a vacuum oven at 45–50 ˚C to give the desired product (7.7 g). ^{1}H NMR (400 MHz,

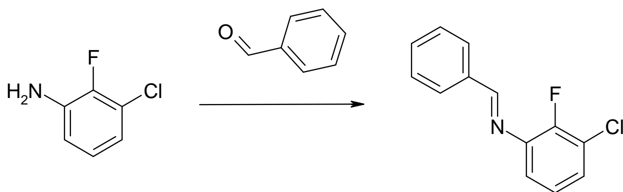
DMSO-d_6) δ 10.40 (br. s., 1H), 9.99 (br. s., 3H), 9.18 (br. s., 1H), 8.66 (d, J = 8.1 Hz, 1H), 8.31-8.43 (m, 4H), 8.02 (d, J = 8.3 Hz, 1H), 7.81 (dd, J = 8.3, 6.6 Hz, 1H), 7.48-7.68 (m, 3H), 3.60 (quin, J = 9.0 Hz, 1H), 2.52-2.71 (m, 2H), 2.35-2.52 (m, 2H), 1.41 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.7, 154.9, 150.2, 149.9, 139.5, 138.1, 138.1, 137.4, 133.9, 130.2, 129.4, 129.1, 127.8, 127.4, 124.0, 120.4, 118.3, 110.4, 67.9, 42.9, 26.9, 21.1; Anal. Calcd for $C_{25}H_{21}FN_6O·1.8HCl·0.4H_2O$: C, 58.50; H, 4.63; N, 16.37. Found: C, 58.30; H, 4.59; N, 16.32; mp = 201 ºC.

8-Fluoro-2-phenyl-7-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-quinoline



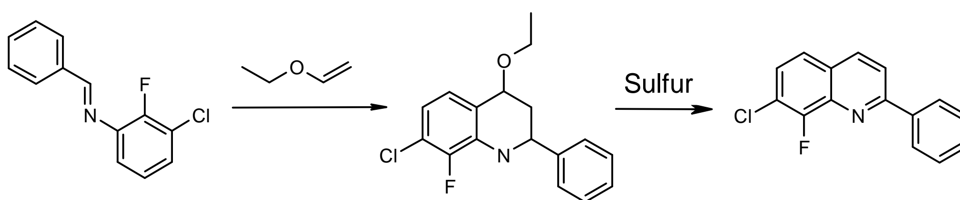
A suspension of 7-chloro-8-fluoro-2-phenylquinoline (0.80 g, 3.10 mmol), bis(pinacolato)diboron (0.91 g, 3.60 mmol), 1,3-bis(2,6-diisopropylphenyl)imidazol-2-ylidene chloride (0.13 g, 0.32 mmol), $Pd(OAc)_2$ (0.035 g, 0.16 mol) and AcOK (0.76 g, 7.76 mol) in anhydrous THF (20 mL) was heated at 72 °C under N_2 overnight. The reaction mixture was allowed to cool to rt and was diluted with ethyl acetate (50 mL). The mixture was filtered through a pad of celite and the filtrate was concentrated under reduced pressure. A white solid precipitated and was collected by filtration and rinsed multiple times with hexanes and further dried under high vacuum to give the desired product (82%). ^{1}H NMR (400 MHz, CD_3OD) δ 1.42 (s, 12H), 7.48-7.58 (m, 3H), 7.67-7.78 (m, 2H), 8.12 (d, J = 8.8 Hz, 1H), 8.21 (dd, J = 8.2, 1.4 Hz, 2H), 8.41 (dd, J = 8.7, 1.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.2, 156.4, 138.2, 137.3, 137.0, 130.8, 130.3, 129.9, 128.9, 127.3, 123.0, 120.7, 84.0, 24.7; MS (ES^+): *m/z* 349.14, 350.21, 351.28 [MH^+]; HPLC: t_R = 4.31 min. (OpenLynx, polar_5min.); HRMS (ESI): Calcd for $C_{15}H_{11}BFNO_2$ [MH^+] (boronic acid): 268.0945, Found: 268.0941; mp = 153 ºC.

(3-Chloro-2-fluoro-phenyl)-[1-phenyl-meth-(E)-ylidene]-amine



A solution of 3-chloro-2-fluoroaniline (300 g, 1.99 mol) and benzaldehyde (257 g, 2.40 mol) in PhMe (1.5 L) was heated to reflux using a Dean-Stark trap at 150 °C for 16 h. The reaction mixture was concentrated to dryness on the high vacuum pump to give the desired crude product, which was used for the next step without further purification. ^{1}H NMR (400 MHz, CD_3CN) δ 7.12-7.23 (m, 2H) 7.29-7.36 (m, 1H) 7.50-7.61 (m, 3H) 7.92-7.97 (m, 2H) 8.58 (s, 1H).

7-Chloro-8-fluoro-2-phenylquinoline



To a solution of (3-chloro-2-fluoro-phenyl)-[1-phenyl-meth-(*E*)-ylidene]-amine (155.7 g, 0.66 mol) in TFE (250 mL) was added ethyl vinyl ether (72.8 g, 1.00 mol) in TFE (250 mL) via an addition-funnel over a period of 15 min. The resulting mixture was stirred at rt for 48 h, then charged with EtOH (390 mL) and powder sulfur (53.4 g, 1.66 mol) and heated at 85 °C for 1 h. The sulfur was filtered off and the product was recrystallized from EtOH at 0 °C to yield the desired product (55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20-8.28 (m, 3H), 7.97 (d, J = 8.6 Hz, 1H), 7.47-7.62 (m, 5H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.2, 152.8, 137.9, 137.8, 137.5, 130.2, 128.9, 127.4, 127.3, 127.2, 124.5, 120.1, 119.1; HRMS (ESI): Calcd for $C_{15}H_9ClFN$ [MH^+]: 258.0486, Found: 258.0475; mp = 108 ºC.

Cell lines. 3T3/huIGF-1R fibrosarcoma cells are derived from NIH 3T3 cells stably overexpressing full-length human IGF-1R (also designated LISN). GEO human colorectal cancer cells were maintained in McCoy's 5A medium supplemented with 10%

FCS and 1% L-glutamine or in serum-free conditioned culture system. MCF7, NCI-H292, Colo-205, HT29, H358, H1703, BxPC3, A673, SW620, DU4475, HepG2 and Hepa-1 cells were cultured in the appropriate media according to the American Type Culture Collection (ATCC) recommendations. Specifically, HepG2 cells were maintained in MEM supplemented with 10% FCS, 1% NEAA and 1% L-glutamine. Hepa-1 cells were maintained in MEM-alpha supplemented with 10% FCS and 1% L-glutamine.

Antibodies. The following antibodies were used for immunoprecipitation or as the capture antibody in ELISA assays: human IGF-1R (Ab-1, Calbiochem, EMD, CA) and IR (Ab-2, Lab Vision Corp., CA) for capture; human IGF-1Rβ (sc-713, Santa Cruz Biotechnology) for immunoprecipitation. The following antibodies were used for immunoblotting analysis: human IGF-1Rβ (sc-713, Santa Cruz Biotechnology), antiphosphotyrosine (X1021, Exalpha Biologicals, MA), antiphosphotyrosine-horseradish peroxidase (HRP) conjugate (mouse anti-Phosphotyrosine-HRP, Invitrogen-ZYMED), $pAKT^{473}$ (9271, Cell Signaling Technology), p-p70S6K (9205, Cell Signaling Technology), and GAPDH (9482, Abcam).

Protein kinase biochemical assays. Protein kinase assays were either performed in-house by ELISA-based assay methods (IGF-1R, IR, EGFR and KDR) or at Upstate Inc. (Charlottesville, VA) by a radiometric method (KinaseProfiler service) with ATP at 100 μmol/L concentration. In-house ELISA assays used poly(Glu:Tyr) (Sigma, St. Louis, MO) as the substrate bound to the surface of 96-well assay plates, and phosphorylation

was detected using an antiphosphotyrosine antibody conjugated to HRP. The bound antibody was quantified using ABTS as the peroxidase substrate by measuring absorbance at 405/490 nm. All assays used purified recombinant kinase catalytic domains. Recombinant enzymes of human IGF-1R or EGFR were expressed as an NH_2-terminal glutathione *S*-transferase fusion protein in insect cells and were purified in-house. The human IR protein was purchased from Calbiochem (cat# 407697). IC_{50} values were determined from the sigmoidal dose-response plot of percent inhibition versus log_{10} compound concentration (Xlfit 3.0, IDBS). A minimum of three measurements, performed in duplicate, were carried out with in-house assays unless otherwise indicated.

Compound **9b** was profiled versus a panel of 167 kinases using the ProfilerPro™ Kinase Selectivity Assay Kit following the instructions provided by the manufacturer (Caliper Life Sciences). Compound **9b** at 1 μM conc. yielded significant inhibition of IGF-1R (97%) and IR (82%). The non-receptor protein tyrosine FER kinase was inhibited 76%. All others were less than 50% inhibited. The modest inhibition of FER observed at 1 μM did not translate into a significant IC_{50} when **9b** was profiled in Invitrogen selectivity panel (FER IC_{50} > 10 μM).

Protein kinase inhibition in intact cells. Quantitative 96-well ELISA assays were developed to study the cellular effects of IGF-1R/IR inhibitors. Cells were placed into 96-well plates in media containing low serum (0.5% FCS) at 37 ºC for 2 h (3T3/huIGF-1R) or overnight (HepG2 and Hepa-1), and then were treated with various concentrations of compound for 2 h before lysis (the final DMSO concentration in the assay was 0.4%),

and the appropriate ligand was added for the final 15 min of the compound treatment period (100 ng/mL IGF-I, R&D systems or 10 ng/mL insulin, Roche). Lysates were prepared in TGH buffer (1% Triton-100, 10% glycerol, 50 mmol/L HEPES, pH 7.4) supplemented with 150 mmol/L NaCl, 1.5 mmol/L MgCl, 1 mmol/L EDTA and fresh protease and phosphatase inhibitors (10 μg/mL leupeptin, 25 μg/mL aprotinin, and 200 μmol/L Na_3VO_4). ELISA assays of the target protein phosphorylation were done by transferring lysates into a second 96-well plate that was precoated with the appropriate capture antibody. The target proteins were then probed with an antiphosphotyrosine antibody-HRP conjugate using a chemiluminescent HRP substrate (Pierce) for detection by luminometry. In experiments to evaluate the effect of plasma protein binding on potency, whole plasma from mouse or human was incorporated into the quantitative 96-well assays at a concentration of 90%. In these experiments, plasma was added to the cell culture medium prior to compound addition.

For immunoblotting analysis, lysates were cleared of insoluble material by centrifugation at 15,000 × *g* for 5 min at 4 ºC and the resultant supernatant was subjected to immunoprecipitation with anti-IGF-1R antibody (sc-713) coupled to Protein G-sepharose beads (Sigma, St. Louis, MO), followed by SDS-PAGE and immunoblotting with antiphosphotyrosine antibody-HRP conjugate and chemiluminescent detection. Alternatively, for highly abundant protein targets (IGF-1R, pErk1/2, pAkt, and p-p70S6K), lysates were analyzed directly by SDS-PAGE and immunoblotting.

Cell proliferation assay. For assays of cell proliferation, cells were seeded into 96-well plates in appropriate media containing 10% FCS and incubated for 3 d in the presence of

compound at various concentrations. Inhibition of cell growth was determined by luminescent quantitation of intracellular ATP content using CellTiterGlo (Promega, Madison, WI). Data are presented as fraction of maximal proliferation, calculated by dividing the cellular density in the presence of varying concentrations of compound by the cellular density of control cells, treated with vehicle (DMSO) only.

Metabolic stability assay. Compound was mixed with human and mouse microsomes, and the reaction was initiated by the addition of NADPH for 0 (pre-NADPH addition), 5, 10, 20 or 40 min. The reactions were terminated with chilled methanol. After centrifugation to precipitate protein, supernatants were analyzed by LC-MS/MS. Various pharmacokinetic parameters were calculated, including half-life ($t_{½}$), intrinsic clearance (Cl_{int}), and scaled hepatic clearance (Cl_h), dependant upon the species. An 'Extraction Ratio' (ER) was calculated according to the following formula: $ER = Cl_h/\text{hepatic blood flow}$.

Cytochrome P450 3A4 assay. Cytochrome P450 3A4 activity was measured using P450-Glo™ CYP3A4 Screening Systems (Promega) according to the instructions provided by the manufacturer.

Protein-binding assay. Pooled plasma samples (approximately 2 ml) from various species (Bioreclamation, Inc., Hicksville, NY 11802) were spiked with compound solutions in DMSO to give final nominal concentrations of 1 and 10 µM (and final DMSO concentration of 0.1% v/v). The samples were gently vortex-mixed and

incubated at room temperature for 30 min. Replicate aliquots (20 µl) were transferred to micro-centrifuge tubes for analysis of total (bound + free) concentrations. Replicate samples (250 µL) were transferred to polycarbonate tubes (Beckman P/N 343775), placed in an ultracentrifuge (Beckman Optima L-80 with 42.2Ti rotor) and spun at approximately 155,000 g for 4 h at 20 ºC to pellet the plasma proteins. Following centrifugation, aliquots (20 µl) of the clear supernatants were carefully transferred to microcentrifuge tubes for analysis of free (unbound) concentration. All aliquots were diluted 1 in 5 with blank mouse plasma and extracted by protein precipitation with 4 volumes of ice-cold methanol. Peak areas in the original plasma samples and the resulting supernatants were measured by HPLC-MS/MS. The HPLC conditions (Agilent 1100 series pump) consisted of a binary, linear gradient of formic acid (0.1% v/v) and methanol on a 4 µ Synergi Hydro-RP, 30 × 2.0 mm column (Phenomenex). The mass spectrometer was an Applied Biosystems model API 3000 operated in positive ion MRM mode, with source and collision cell conditions optimized for the parent-daughter ion transition of *m/z* 422.3 > 364.3. Corresponding mean concentrations of total drug and free drug were calculated for each species. Percentage binding was calculated according to the following equation: % bound = 100 – ([free] x 100) / [total].

Animals. Female CD-1 and athymic nude *nu*/*nu* CD-1 mice (6-8 wks, 25-29 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were allowed to acclimate for a minimum of one week prior to initiation of a study. Female Sprague Dawley rats with implanted jugular vein canulas were obtained from Hilltop Lab Animals (Scottdale, PA) and were allowed to acclimate for a minimum of one day prior to a study.

Throughout the studies, animals were allowed sterile rodent chow and water *ad libitum*, and immunocompromised animals were maintained under specific pathogen-free conditions. All rodent animal studies were conducted at OSI facilities with the approval of the Institutional Animal Care and Use Committee in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited vivarium and in accordance with the Institute of Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals, NIH, Bethesda, MD). Animals for pharmacokinetic studies on male beagle dogs were housed at MPI Research (Mattawan, MI).

Pharmacokinetic analysis. For pharmacokinetic analysis, the compound was formulated in saline adjusted to pH 2 with 0.01 mol/L hydrochloric acid for intravenous injection and in 25 mmol/L tartaric acid for oral administration. Female CD-1 mice (6-8 weeks old) received either a single intravenous dose or a single oral dose of compound at the indicated doses. For intravenous dosing, compound was delivered via tail vein injection at a dosing volume of 4 mL/kg. For oral dosing, compound was delivered via oral gavage in a dosing volume of 10 mL/kg. Subsequently, three animals were sacrificed at each designated time point (1, 4, 8, 16 and 24 h) and blood samples were collected in EDTA. After centrifugation at 1500 × *g* for 10 min, plasma samples were prepared by protein precipitation with methanol and analyzed by HPLC-MS/MS (PE Sciex API 3000 LC/MS/MS System, Applied Biosystems). Pharmacokinetic parameters for the plasma time-concentration profile, including C_{max}, AUC, elimination half-life ($t_{½}$), volume of distribution at steady state (V_{ss}), clearance (CL) and oral bioavailability, were calculated by non-compartmental analysis.

***In vivo pharmacodynamic analysis**:* To assess the ability of **9b** to inhibit phosphorylation of IGF-1R and IR in tumor tissue, animals with established tumors of 300 ± 50mm^3 size were dosed orally with **9b** in 40% Trappsol at 10 mg/kg dose. Tumor samples were collected, snap frozen in liquid nitrogen, and homogenized in a Precellys-24 homogenizer (MO BioLaboratories, CA) with tumor lysis buffer (1% Triton X-100, 10% glycerol, 50mM HEPES (ph 7.4), 150mM NaCl, 1.5mM $MgCl2$, 1mMEDTA supplemented with protease and phosphatase inhibitor cocktails (Sigma, MO), 10mM NaF and 1mM sodium orthovanadate). Homogenates were clarified by centrifugation (14,000g for 5 min at 4°C) and supernatants were analyzed by phospho-RTK array for phospho-IGF-1R and phospho-IR as per manufacturer's protocol (R&D Systems, Minneapolis, MN).

***In vivo antitumor efficacy studies**:* Female *nu/nu* CD-1 mice were used for xenograft studies. To assess anti-tumor efficacy, cells were implanted s.c. in the right flank. Tumors were allowed to establish to 200±50mm^3 before randomization into treatment groups. **9b** was administered as indicated. Tumor volumes were determined twice weekly from caliper measurements by V = (length x width^2)/2. Tumor growth inhibition (TGI) was determined by %TGI = $\{1 – [(T_t/T_0) / (C_t/C_0)] / 1 – [C_0/C_t]\}$ x 100, where T_t = tumor volume of treated animal x at time t, T_0 = tumor volume of treated animal x at time 0, C_t = median tumor volume of control group at time t, and C_0 = median tumor volume of control group at time 0. Mean %TGI was calculated for the entire dosing period for each group. Significant anti-tumor activity is defined as mean %TGI >50%.