Supporting Information

Discovery of novel insulin-like growth factor-1 receptorinhibitors with unique time dependent binding kinetics

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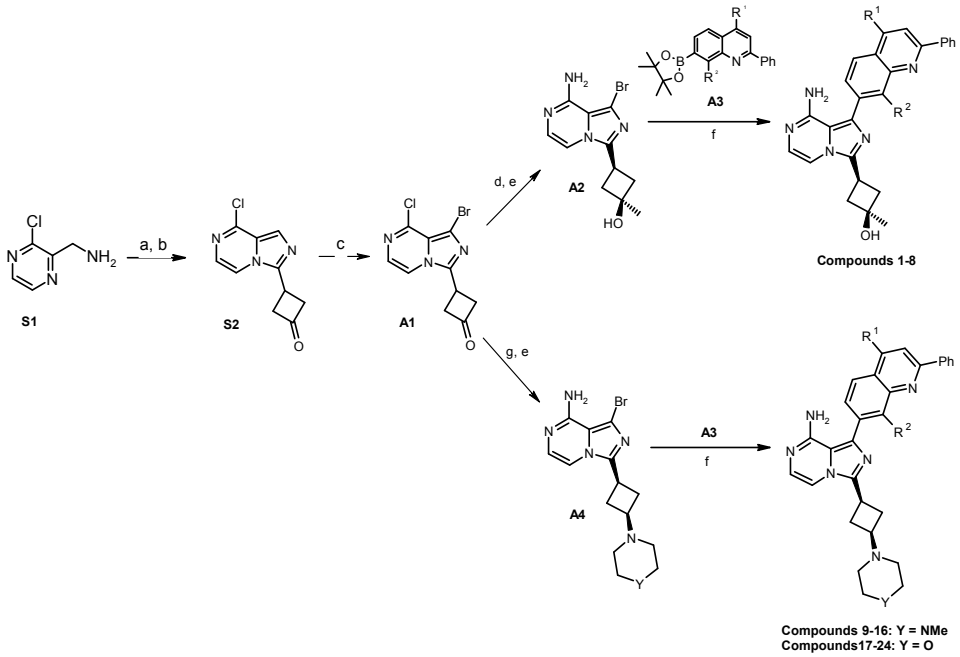
Experimental

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 LC-MS and/or thin layer chromatography (TLC). 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. TLC was performed 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) 5μM 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 of30 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

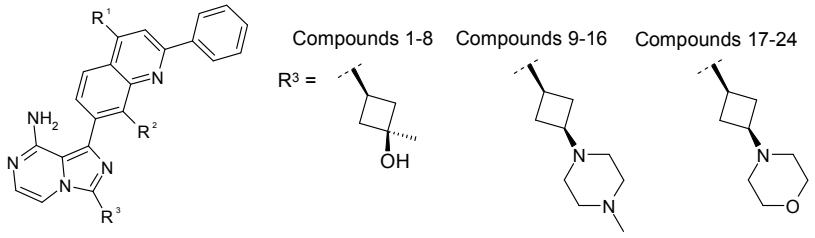
2% A. High resolution mass spectra (HRMS) were obtained by UPLC-TOF-MS on a LCT PREMIER XE mass spectrometer (Waters), using positive electrospray ionization mode and a scan range of *m/z* = 150–850 and were accurate to ±5 ppm. The UPLC system used a Waters BEH C18, 1.7 µM, 50 × 2.1 mm column heated 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%.

Scheme 1S: General synthesis of imidazo[1,5-*a*]pyrazine-derived IGF-1R slow off-rate inhibitors[a]



[a]Reagents and conditions: (a)3-oxo-cyclobutanecarboxylic acid 2,5-dioxo-pyrrolidin-1-yl ester, THF, rt; (b) $POCl_3$, DMF, rt, 75% for two steps; (c) NBS, DMF, rt, 0 ºC, 90%;(d) MeMgCl, THF, –78 °C, 72%; (e) 2N NH_3 in iPrOH, 90 °C, 84-95%; (f) $PdCl_2$(dppf), K_2CO_3, dioxane-H_2O (4:1, v:v), 95 °C, 62-75%; (g) $HN(CH_2CH_2)_2Y$, $NaBH(OAc)_3$, THF, rt, 70-75%.

Table 1S: Imidazo[1,5-*a*]pyrazine derived IGF-1R slow off-rate inhibitors



Cpd	R^1	R^2	Inhibitor-IGF-1R half life ($t_{1/2}$, h)	IGF-1R cell IC_{50} (nM)[a]
1	OMe	H	2.7	3
2	OEt	H	133	4
3	OiPr	H	4.2	10
4	OPh	H	5.6	23
5	OMe	F	10	4
6	OEt	F	995	5
7	OiPr	F	43	10
8	OPh	F	34	8
9	OMe	H	3.3	16
10	OEt	H	ND[b]	24
11	OiPr	H	3.3	29
12	OPh	H	14	195
13	OMe	F	8.3	9
14	OEt	F	743	9
15	OiPr	F	11	26
16	OPh	F	1.6	72
17	OMe	H	3.7	5
18	OEt	H	49	12
19	OiPr	H	5.4	25
20	OPh	H	13	100
21	OMe	F	9.9	8
22	OEt	F	897	12
23	OiPr	F	27	18
24	OPh	F	4.5	72

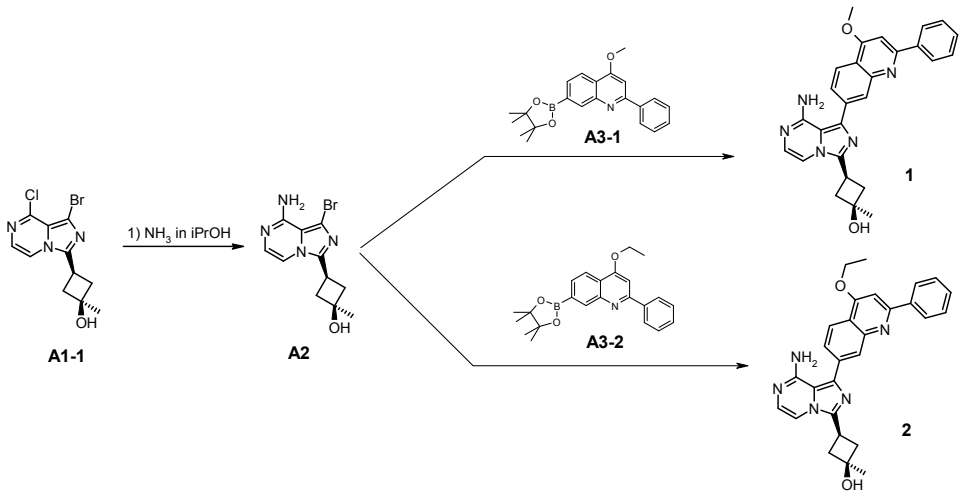
a. Cellular mechanistic assay in 3T3/huIGF-1R cell;
b. Not determined

Table 3S: Physicochemical and ADMET properties of compound **2**

Microsomal stability (ER)		CYP inhibition		Miscellaneous phys-chem and ADMET properties	
Species	Value	CYP isoform	IC_{50} (μM)		
Mouse	0.28	1A2	>20	MW	465
Rat	0.15	2C9	>20	AlogP/PSA	4.09/98.6Å
Dog	0.43	2C19	>20	LLE[a]	5.13
Monkey(rhesus)	0.75	2D6	>20	PAMPA (10^{-6}cm/s)	1120 (pH7.4) 280 (pH5.0)
Human	0.59	3A4	>20	Aq. solubility (μM)	2.0 (pH7.4) >106 (pH3.0)

a. LLE = $_{p}$IC50 – ALogP

Synthesis of compound 1 and 2:

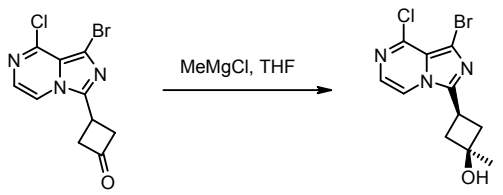


To a mixture of 3-(1-bromo-8-chloro-imidazo[1,5-*a*]pyrazin-3-yl)-1-methylcyclobutanol (**A1-1**, 15.17 g, 0.048mol) in 2-butanol (60 mL) in a Parr vessel was added aqueous ammonium hydroxide (85 mL). The vessel was sealed and the mixture was heated in an oil bath set at 90 °C for 16 h. Thereaction vessel was then cooled in an ice bath, the reaction mixture was transferred to a 500 mL flask. The bulk of solvent was removed under reduced pressure. Water (100 mL) was added to the residue, the solids were collected by filtration and washed with water to give desired product**A2** (yield: 90%). This material was used for next step without any further purifications.^{1}H NMR (400 MHz, CD_3OD) δ 1.48 (s, 3H), 2.31-2.66 (m, 4H), 3.33-3.48 (m, 1H), 6.93 (d, J = 5.0 Hz, 1H), 7.39 (d, J = 5.3 Hz, 1H).MS (ES^+): *m/z* 296.94/298.77 (80/100) [MH^+]; HPLC: t_R = 1.30 min (OpenLynx, polar_5min).

To a stirred mixture of 3-(8-amino-1-bromo-imidazo[1,5-*a*]pyrazin-3-yl)-1-methylcyclobutanol (**A2**, 3.92 g, 0.013 mol), potassium carbonate (5.46 g, 0.04 mol) in 1,2-dimethoxyethane (80 mL) and H_2O (20 mL) was added 4-methoxy-2-phenyl-7-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-quinoline (**A3-1**, 5.43 g, 0.015 mol) followed by 1,1'bis-(dipenylphosphino)ferrocene) palladium dichloride (0.54 g, 0.00066 mol) under an atmosphere of nitrogen. The resulting mixture was then heated and stirred at 90°C for 4 h. The reaction was then cooled to room temperature, the bulk of solvent was removed under reduced pressure to give a crude residue. It was then purified by a silica gel flash chromatography (eluent: 1→10% methanol in methylene chloride) to give the desired product **1**(yield: 75%).^{1}H NMR (400 MHz, DMSO) δ 1.41 (s, 3H), 2.44-2.49 (m, 4H), 3.39 - 3.56 (m, 1H), 4.20 (s, 3H), 6.20 (br. s., 2H), 7.09 (d, *J*= 4.8 Hz, 1H), 7.47 - 7.63 (m, 5H), 7.87 (dd, *J*= 8.6, 1.8 Hz, 1H), 8.18 (d, *J*= 1.3 Hz, 1H), 8.23 (d, *J*= 8.6 Hz, 1H), 8.32 (dd, *J*= 8.3, 1.3 Hz, 2H);^{13}C NMR (100 MHz, DMSO) 21.5, 27.2, 42.8, 56.6, 68.2, 98.8, 106.9, 114.3, 119.2, 122.1, 127.3, 127.7, 128.0, 128.9, 129.0, 130.0, 133.5, 136.7, 139.4, 144.2, 148.7, 151.8, 158.7, 162.8. MS (ES^+): *m/z* 452.40[MH^+]; HPLC: t_R = 0.61 min (Analytical_2min); HRMS (ESI): Calcd for $C_{27}H_{25}N_5O_2$[MH^+]: 452.2087, Found: 452.2099.

To a stirred mixture of 3-(8-amino-1-bromo-imidazo[1,5-*a*]pyrazin-3-yl)-1-methylcyclobutanol (**A2,** 13.07 g, 0.044 mol), potassium carbonate (18.21 g, 0.13 mol) in 1,2-dimethoxyethane (240 mL) and H_2O (60 mL) was added 4-ethoxy-2-phenyl-7-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-quinoline (**A3-2**, 18.75 g, 0.050 mol) followed by 1,1'bis-(dipenylphosphino)ferrocene) palladium dichloride (1.80 g, 0.0022 mol) under an atmosphere of nitrogen. The resulting mixture was then heated and stirred at 90°C for 4 h. The reaction was then cooled to room temperature, the bulk of solvent was removed under reduced pressure to give a crude residue. It was then purified by a silica gel flash chromatography (eluent: 1→10% methanol in methylene chloride) to give the desired product **2**(yield: 71%).^{1}H NMR (400 MHz, DMSO) δ 1.41 (s, 3H), 1.55 (t, *J* = 6.9 Hz, 3H), 2.40 - 2.47 (m, 4H), 3.49 (t, *J* = 8.7 Hz, 1H), 4.50 (q, *J* = 6.9 Hz, 2H), 6.18 (br. s., 2H), 7.08 (d, *J* = 4.8 Hz, 1H), 7.43 - 7.65 (m, 5H), 7.86 (dd, *J* = 8.6, 1.5 Hz, 1H), 8.17 (d, *J* = 1.5 Hz, 1H), 8.23 (d, *J* = 8.6 Hz, 1H), 8.31 (dd, *J* = 8.3, 1.3 Hz, 2H);^{13}C NMR (100 MHz, $CDCl_3$) δ 14.5, 22.5, 27.2, 43.2, 64.2, 70.6, 98.9, 106.4, 114.3, 119.9, 122.6, 126.8, 127.6, 128.0, 128.7, 129.3, 134.8, 136.2, 140.2, 144.5, 149.2, 151.6, 159.5, 162.0;MS (ES^+): *m/z* 466.42 [MH^+]; HPLC: t_R = 0.67 min (Analytical_2min); HRMS (ESI): Calcd for $C_{28}H_{27}N_5O_2$[MH^+]: 466.2243, Found: 466.2246.

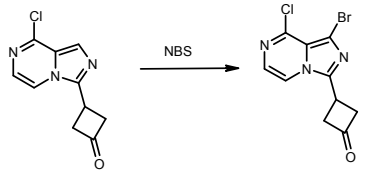
Synthesis of compound A1-1:



A1 **A1-1**

To a stirred solution of 3-(1-bromo-8-chloro-imidazo[1,5-*a*]pyrazin-3-yl)-cyclobutanone (**A1**, 14.40 g, 0.048 mol) in anhydrous THF (400 mL) was added a solution of methylmagnesium chloride in THF (3.0 M, 32.40 mL) via a syringe over 10 minutes at -78 °Cunder an atmosphere of nitrogen. The resulting solution was stirred at -78 °Cfor 2h. The reaction was then quenched with saturated aqueous NH_4Cl (50 mL) at -78 °C. The mixture was allowed to warm to room temperature, and the bulk of solvent was removed under reduced pressure. To the residue was added ethyl acetate (450 mL) followed by brine (150 mL). The aqueous layer was separated, the organic layer was washed with brine (150 mL), dried over anhydrous sodium sulfate, and concentrated to give a crude product which was then recrystallized in diisopropyl ether to give the desired product as desired product**A1-1**(yield: 72%).^{1}H NMR (400 MHz, CD_3OD) δ 1.49 (s, 3H), 2.45-2.63 (m, 4H), 3.40-3.55 (m, 1H), 7.28 (d, *J* = 5.0 Hz, 1H), 8.00 (d, *J* = 5.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$)δ 21.8, 26.9, 43.1, 69.4, 110.9, 113.4, 120.6, 127.3, 144.4, 145.7.MS (ES^+): *m/z*316.01/317.99/319.83 (70/100/30) [MH^+]; HPLC: t_R = 0.94 min (Analytical_2min); HRMS (ESI): Calcd for $C_{11}H_{11}{}^{79}Br\,{}^{37}ClN_3O$ [MH^+]: 317.9832, Found: 317.9841.

Synthesis of compound A1:

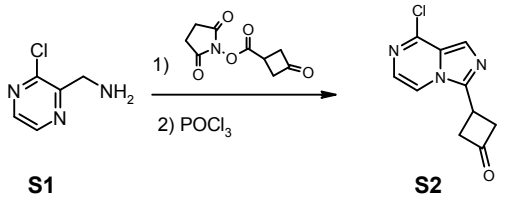


S2 **A1**

To a stirred solution of 3-(8-chloroimidazo[1,5-*a*]pyrazin-3-yl)cyclobutanone (**S2**, 14.37 g, 0.065 mol) in DMF (150 mL) under an atmosphere of nitrogen was slowly added a solution of N-bromosuccinimide (12.46 g, 0.070 mol) in DMF (100 mL) at0 ºC. The resulting mixture was stirred at same temparature for 15 min. Water (400 mL) was then added and the resulting solidswere collected by filtration and washed with water to give the title compound**A1**(yield:90%).^{1}HNMR(400 MHz, $CDCl_3$) δ 3.55-3.65 (m, 2H), 3.70-3.79 (m, 2H), 3.79-3.89 (m, 1H), 7.38 (d, *J* = 5.0 Hz, 1H), 7.55 (d, *J* = 4.8 Hz, 1H).^{13}CNMR (100 MHz,

$CDCl_3$)δ 19.8, 51.9, 108.6, 115.5, 120.2, 126.6, 142.9, 143.4, 204.5;MS (ES^+): *m/z*299.91/301.92/303.82 (80/100/30)[MH^+]; HPLC: t_R = 1.02 min (Analytical_2min);HRMS (ESI): Calcd for$C_{10}H_7{}^{79}Br{}^{37}ClN_3O$[$MH^+$]: 301.9519, Found: 301.9508.

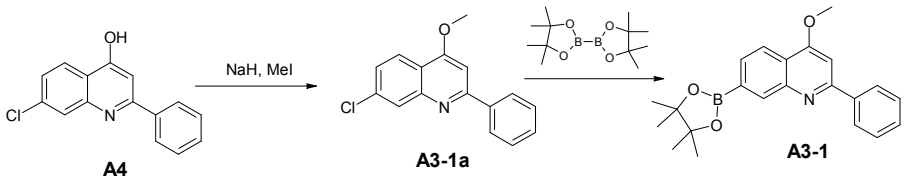
Synthesis of compound S2:



S1 **S2**

To a stirred solution of 3-oxo-cyclobutanecarboxylic acid 2,5-dioxo-pyrrolidin-1-yl ester (19.62 g, 0.093 mol) in THF (300 mL) were added C-(3-chloro-pyrazin-2-yl)-methylamine hydrochloride salt (**S1**, 15.40 g, 0.085 mol) at room temparature, followed by addition of a solution of $NaHCO_3$ (7.56 g, 0.090 mol) in water (50 mL). The resulting mixture was stirred at room temparature for 90 min, and the layers were allowed to separate. The aqueous layer was extracted with ethyl acetate (300 mL x 2). The combined organic layers were washed with brine (350 mL), dried over anhydrous sodium sulfate, and concentrated in vacuo to provide the title compound, which was used for next step without any further purifications. This solid was then suspended in ethyl acetate (300 mL), to the mixture was added dimethylformamide (20 mL) and the solution was cooled to 10 °C. Phosphorus oxychloride (15.90 mL, 0.17 mol) was then added over 15 minutes and the reaction was allowed to stir at room temparature for 45 min. The reaction solution was then poured slowly into a 20% aqueous Na_2CO_3 solution at 10°C. The resulting mixture was extrated with ethyl acetate (300 mL x 3). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to give a crude residue. It was then purified by a silica gel flash chromatography (eluent: 1→10% ethyl acetate in heptane) to give the desired product **S2**(yield: 75% for 2 steps). ^1H NMR (400 MHz, $CDCl_3$) δ 3.57–3.69 (m , 2H), 3.72–3.82 (m , 2H), 3.85–3.96 (m , 1H), 7.41 (d, *J* = 4.8 Hz, 1H), 7.60 (d, *J* = 4.8 Hz, 1H), 7.86 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.7, 52.9, 112.8, 124.9, 125.1, 127.4, 143.1, 146.3, 203.3. MS (ES^+): *m/z*222.00/223.93(100/70) [MH^+]; HPLC: t_R = 0.76 min (Analytical_2min); HRMS (ESI): Calcd for $C_{10}H_8{}^{35}ClN_3O$ [MH^+]: 222.0434, Found: 222.0443.

Synthesis of compound A3-1:

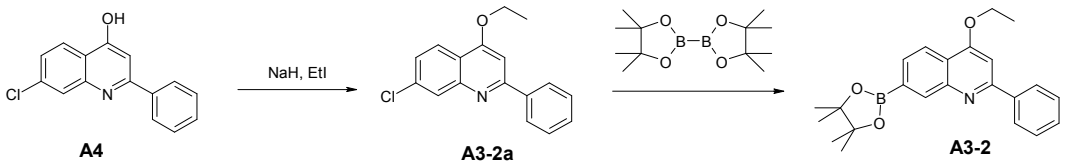


A4 **A3-1a** **A3-1**

To a stirred solution of 4-hydroxy-7-chloro-2-phenylquinoline (**A4**, 7.65 g, 0.030 mol) in dry DMF (200 mL) was added NaH (60% in mineral oil, 1.32 g, 0.033 mmol) in portions at 0 °C. The resulting mixture was stirred for 10 min at room temperature, followed by addition of methyl iodide (2.25 mL, 0.036 mol) in drop-wise at 40°C and stirring was continued for an additional 1 h. The reaction mixture was then poured into ice-water and extracted with methylene chloride (200 mL x 3). The organic layers were combined, washed with water, dried over Na_2SO_4 and concentrated to give a crude residue. The residue was further purified by a silica gel flash chromatography (eluent: 10% ethyl acetate in hexane) to give desired product **A3-1a**(yield: 88%).^{1}H NMR (400 MHz, DMSO) δ 4.13 (s, 3H), 7.43 - 7.56 (m, 5H), 7.99 (d, *J*= 2.0 Hz, 1H),8.08 (d, *J*= 8.8 Hz, 1H), 8.21 - 8.27 (m, 2H);^{13}C NMR (100 MHz, $CDCl_3$) δ 55.7; 98.1, 118.8, 123.1, 126.2, 127.5, 128.1, 128.8, 129.6, 135.9, 139.9, 149.7, 159.9, 162.8.MS (ES^+): *m/z*270.20/272.13 (100/70)[MH^+]; HPLC: t_R = 1.34 min (Analytical_2min); HRMS (ESI): Calcd for $C_{16}H_{12}{}^{35}ClNO[MH^+]$: 270.0686, Found: 270.0695.

To a stirred mixture of bis(pinacoloto)diborane (16.00 g, 0.063 mol), potassium acetate (14.70 g, 0.15 mol) and 7-chloro-4-methoxy-2-phenylquinoline (**A3-1a**, 14.20 g, 0.053 mol) in anhydrous dioxane (400 mL) was added tris(debenzyledeneacetone)dipalladium (0) (2.40 g, 0.0027 mmol) and tricyclohexylphosphine (1.50 g, 0.0054 mol) under an atmosphere of nitrogen. The resulting mixture was then heated at 90 °C for 2 h. The reaction mixture was cooled to room temperature, filtered through a short silica gel pad to remove inorganic material, and the silica gel pad was washed with methylene chloride (50 mL). The filtrate was concentrated to give a crude residuewhich was purified by recrystallization from isopropyl ether to give the desired product**A3-1** (yield: 85%).^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.36 (s, 12H), 4.18 (s, 3H), 7.46 - 7.63 (m, 4H), 7.75 (dd, *J*= 8.3, 1.0 Hz, 1H), 8.13 (d, *J*= 8.3 Hz, 1H), 8.25 - 8.36 (m, 3H);^{13}C NMR (100 MHz, $CDCl_3$) δ 24.9, 55.6, 84.0, 98.3, 120.6, 122.0, 127.5, 128.7, 129.2, 130.0, 130.8, 137.1, 140.2, 148.4, 158.4, 162.6.MS (ES^+): *m/z* 280.09[MH^+] (corresponding boronic acid); HPLC: t_R = 0.64 min (Analytical_2min); HRMS (ESI): Calcd for $C_{22}H_{24}BNO_3[MH^+]$: 362.1927, Found: 362.1922.

Synthesis of compound A3-2:

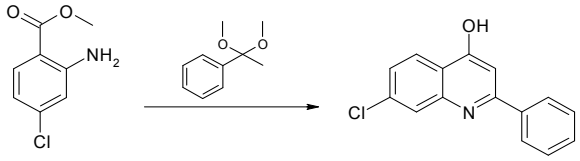


To a stirred solution of 4-hydroxy-7-chloro-2-phenylquinoline (**A4**, 12.75 g, 0.050 mol) in dry DMF (350 mL) was added NaH (60% in mineral oil, 2.20 g, 0.055 mmol) in portions at 0 °C. The resulting mixture was stirred for 10 min at room temperature, followed by addition of ethyl iodide (9.36 g, 0.060 mol) in drop-wise at 40°C and stirring was continued for an additional 1 h. The reaction mixture was then poured into ice-water and extracted with methylene chloride (200 mL x 3). The organic layers were combined, washed with water, dried over Na_2SO_4 and concentrated to give a crude residue. The residue was further purified by a silica gel flash chromatography (eluent: 10% ethyl acetate in hexane) to give desired product**A3-2a** (yield: 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.60 (t, *J* = 6.9 Hz, 3H), 4.31 (q, *J* = 6.9 Hz, 2H), 7.12 (s, 1H), 7.41 (dd, *J* = 8.8, 2.0 Hz, 1H), 7.44 - 7.57 (m, 3H), 8.00 - 8.18 (m, 4H);^{13}C NMR (100 MHz, $CDCl_3$)δ 14.6, 64.9, 99.5, 118.9, 124.0, 126.3, 127.8, 127.8, 129.0, 130.1, 135.0, 139.1, 149.4, 159.4, 162.0;MS (ES^+): *m/z*284.21/286.16 (100/80)[MH^+]; HPLC: t_R = 1.45 min (OpenLynx, polar_5min); HRMS (ESI): Calcd for $C_{17}H_{14}{}^{35}ClNO[MH^+]$: 284.0842, Found: 284.0831.

To a stirred mixture of bis(pinacoloto)diborane (10.67 g, 0.042 mol), potassium acetate (9.80 g, 0.10 mol) and 7-chloro-4-ethoxy-2-phenylquinoline (**A3-2a**, 10.00 g, 0.035 mol) inanhydrous dioxane (300 mL) was added tris(debenzyledeneacetone)dipalladium (0) (1.60 g, 0.0018 mmol) and tricyclohexylphosphine (1.00 g, 0.0036 mol) under an atmosphere of nitrogen. The resulting mixture was then heated at 90 °C for 2 h. The reaction mixture was cooled to room temperature, filtered through a short silica gel pad to remove inorganic material, and the silica gel pad was washed with methylene chloride (50 mL). The filtrate was concentrated to give a crude residuewhich was purified by recrystallization from isopropyl ether to give the desired product**A3-2** (yield: 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 12H), 1.62 (t, *J* = 6.9 Hz, 3H), 4.36 (q, *J* = 6.9 Hz, 2H), 7.19 (s, 1H), 7.41-7.62 (m, 3H), 7.84 (d, *J* = 8.0 Hz, 1H), 8.09-8.17 (m, 2H), 8.19 (d, *J* = 8.3 Hz, 1H), 8.64 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.8, 25.2, 64.3, 84.3, 99.1, 121.1, 122.5, 127.7, 128.9, 129.4, 130.1, 130.8, 137.5, 140.5, 148.8, 158.7, 162.2;MS (ES^+): *m/z* 294.22[MH^+] (corresponding boronic acid); HPLC: t_R = 0.74 min

(OpenLynx, polar_2min); HRMS (ESI): Calcd for $C_{23}H_{26}BNO_3[MH^+]$: 376.2084, Found: 376.2083.

Synthesis of compound A4:



A5 **A4**

To a stirred mixture of methyl 4-chloroanthranilate (**A5**, 27.84 g, 0.15 mol) in diphenyl ether (200 mL) was added acetophenone dimethyl acetal (24.90 g, 0.15mol)under an atmosphere of nitrogen. The resulting mixtue was heated to 120°C for 30 minutes, then to 200°C for 30 minutes and finally to the 250°C for 10 h. The reaction mixture was cooled to room temperature, hexane (100 mL) was added and the mixture was stirred at room temperature for 30 minutes. The solids formed were filtered, washed with hexane and dried in vacuum oven to give desired product**A4**(yield: 70%). ^{1}H NMR (400 MHz, DMSO) δ 6.37 (s, 1H), 7.29-7.43 (m, 1H), 7.50-7.67 (m, 3H), 7.76–7.89 (m, 3H), 8.09 (d, J = 8.6 Hz, 1H), 11.75 (s, 1H);MS (ES^+): *m/z*256.12/258.13 (100/50)[MH^+]; HPLC: t_R = 0.95 min (Analytical_2min); HRMS (ESI): Calcd for $C_{15}H_{10}{}^{35}ClNO$ [MH^+]:256.0529, Found:256.0535.

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.

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;. 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).

Biochemical IC_{50} determination:

The Omnia Assay (Invitrogen) was optimized for the GST-tagged catalytic domain of IGF-1R (Invitrogen PV3250). In this assay system, Omnia Y Peptide 12 (Invitrogen KPZ3121C) functions as a substrate for IGF-1R. Phosphorylation of this SOX-containing peptide by IGF1R results in an increase in fluorescence at 485 nm upon excitation at 360 nm.

Assays are carried out in 384-well OptiPlates (Perkin Elmer 6007290) in a total volume of 20 μL containing IGF-1R (0.25 nM), Omnia Y Peptide 12 (10 μM), ATP (100 μM), and test compound (variable) in assay buffer (50 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 0.003% Brij, 0.004% Tween20, 1 mM DTT, 1 mM EGTA, 0.0016% BSA) with 1% DMSO.

IC_{50}s for test compounds were determined using an 11-point three-fold serial dilution with a final assay concentration ranging from 0.056 nM to 3.30 μM for initial assays or from 0.019 nM to 1.11 μM for assays incorporating a 24-hour IGF-1R-compound preincubation step. All compound concentrations were assayed in duplicate. Initial compound dilutions were prepared at 100X concentration in 100% DMSO from a 10 mM stock solution. Compounds were further diluted 25-fold into assay buffer resulting in a 4X concentrated solution.

For assays without a preincubation step, 5 μL of the 4X concentrated compound solution (or 4% DMSO for positive controls) was added to the assay plate followed by 5 μL of a solution containing peptide (40 μM) and ATP (400 μM) in assay buffer. The reactions were initiated by the addition of 10 μL of IGF-1R (0.5 nM) in assay buffer, or assay buffer alone for negative controls.

For assays incorporating a preincubation step, 5 μL of the 4X concentrated compound solution (or 4% DMSO for positive controls) was added to the assay plate followed by 10 μL of a solution containing IGF-1R (0.5 nM) and ATP (200 μM). The assay plate was covered and incubated at 24 hours at room temperature prior to the addition of 5 μL of peptide (40 μM) to initiate the reaction.

The increase in fluorescence due to peptide phosphorylation was monitored continuously as a function of time using a Spectramax M5 plate reader (Molecular Devices) equipped with SoftMax Pro 5.2 software.

Assays were monitored for 1.5 h for initial screening or for 10 h to assess reaction linearity subsequent to 24-hour incubation. Following background subtraction, compound IC_{50}s were determined by plotting either the assay signal at the 60 minute time point (no preincubation) or the slope from the linear portion of the reaction progress curves (24-hr preincubation) as a function of log inhibitor concentration and fitting to a four-parameter sigmoidal dose response model using GraphPad Prism.

Measurement of IGF-1R compound dissociation rates:

To measure compound dissociation rates, IGF-1R (50 nM) was incubated for 1.5 h at room temperature with excess compound at 20-fold over the previously determined IC_{50} value in assay buffer (50 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 0.003% Brij, 0.004% Tween20, 1 mM DTT, 1 mM EGTA, 0.0016% BSA) with 1% DMSO. Compound-saturated IGF-1R was then diluted 100-fold and immediately diluted 2-fold further into an Omnia assay mixture containing peptide Y12 (20 μM) and ATP (1 mM) in assay buffer with 1% DMSO for a total assay volume of 20 μL. Final assay mixtures were plated in triplicate and contained IGF-1R (0.25 nM), Y12 peptide (10 μM) and ATP (500 μM) with compound present 10-fold below the previously determined IC_{50} value.

Following rapid dilution, the increase in fluorescence due to peptide phosphorylation was monitored continuously for 40 to 100 h using a Spectramax M5 plate reader (Molecular Devices) equipped with SoftMax Pro 5.2 software.

Resulting curves were imported into GraphPad Prism for analysis. Data points falling beyond the linear range subsequent to activity regain, presumably due to either substrate depletion or decay of enzymatic stability, were excluded from analysis prior to curve fitting. Data were fit to the following equation to determine the compound dissociation rate:

$$y = b + v_s x + \left(\frac{v_i - v_s}{k_{obs}} \right) \left(1 - \exp^{-k_{obs} x} \right)$$

Where, v_i is initial velocity, v_s is final velocity, x is time in minutes, k_{obs} is the observed rate constant for the transition between $v_i$and v_s, and b is invoked to correct for nonzero initial fluorescence values observed in many cases. To obtain meaningful results, the following

constraints were applied during curve fitting: $v_i = 0$, $k_{obs} < 1$. For compounds with extremely slow off-rates, where v_s was not well-defined by the reversibility curve, it was sometimes necessary to constrain v_s to fitted values obtained for a control compound obtained within the same data set to obtain an estimate for k_{obs}. Averaged dissociation rates from triplicate measurements were then converted to $t_{½}$ values according to the relationship $t_{½} = \ln 2/k_{obs}$.

***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), 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 4ºC 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.

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/hepatic blood flow.

Cytochrome P450 3A4 assay:

Cytochrome P450 1A2, 2C9, 2C19, 2D6 and 3A4 activity was measured using P450-Glo™ Screening Systems (Promega) according to the instructions provided by the manufacturer.

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).

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 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. Compounds were 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%.