Supporting Information

Potent and Selective MEK 1,2 Inhibitors. Part 1: 4-(4-Bromo-2-fluorophenylamino)-1-methyl-pyridin-2(1H)-ones

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Chemistry Experimental Section

The reactions set forth below were done generally under a positive pressure of nitrogen or argon or with a drying tube (unless otherwise stated) in anhydrous solvents, and the reaction flasks were typically fitted with rubber septa for the introduction of substrates and reagents via syringe or cannula. Glassware was oven dried and/or heat dried. All reagents and solvents were used without further purification unless otherwise stated. Reactions were monitored by either analytical TLC or analytical HPLC. Analytical TLC was performed using glass plates pre-coated with silica gel (Manufacturer: EMD, Silica Gel 60 F_{254}, 250 μm). Analytical HPLC was performed on YMC ODS-AQ 3 μm, 120Å, 3.0 x 50 mm column using a 0.01% HFBA/1% IPA/water/acetonitrile gradient and UV detection at 220 and 254 nm. Flash column chromatography was performed on a Biotage system (Manufacturer: Dyax Corporation) having pre-packed silica gel columns

(Manufacturer: Biotage, part no. FPK0-1107-15046, FPK0-1107-17026, FPK0-1107-17046, or FK0-1107-1804C) or on Isolute SPE columns (Manufacturer: International Sorbent Technology, part no. 450-0500-E or 450-1000-F). Mass spectra were recorded on Thermo Finnigan LCQ Duo Flow Injection APCI (±). Combustion analyses were performed by Schwarzkopf Microanalytical Laboratory, Inc. (Woodside, NY). All analyzed compounds are within ±0.4% of the theoretical value unless otherwise indicated. ^{1}H-NMR spectra were recorded on a Varian Mercury (400 MHz) NMR spectrometer. ^{19}F-NMR spectra were recorded on a Varian Mercury (376 MHz) NMR spectrometer. Chemical shifts are expressed in parts per million (ppm, δ scale) using tetramethylsilane as the reference standard. When peak multiplicities are reported, the following abbreviations are used: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet), dt (doublet of triplet), br (broad). Coupling constants are reported in Hertz (Hz).

4-(4-bromo-2-fluorophenylamino)-N-(cyclopropylmethoxy)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (6).

Step A: Preparation of ethyl 4-hydroxy-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylate: To diethyl 1,3-acetonedicarboxylate (50.0 mL, 276 mmol) was added triethyl orthoformate (45.8 mL, 276 mmol) followed by acetic anhydride (52.1 mL, 551 mmol). The resulting mixture was stirred for 1 h at 135 ^{o}C. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. To the resulting residue at 0 ^{o}C was added methylamine (53 mL, 690 mmol, 40% in water) followed by water (200 mL) with stirring. The reaction mixture was stirred at room temperature for 16 h. The reaction mixture was diluted with EtOAc (300 mL). The organic layer was separated. The aqueous phase was neutralized with 10% HCl solution to produce the desired product as a white precipitate that was filtered and washed with water. The filtrate was extracted with EtOAc. The combined organic extracts dried over $MgSO_4$ and concentrated under reduced pressure to give a white solid that was rinsed with Et_2O and combined with the first crop to yield 29 g (54%) of the desired product. MS APCI (+) *m/z* 198 (M+) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 8.48 (s, 1H), 5.82 (s, 1H), 4.40 (q, *J* = 7.2 Hz, 2H), 3.59 (s, 3H), 1.38 (t, *J* = 7.6 Hz, 3H).

Step B: Preparation of ethyl 4-chloro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylate: To a suspension of ethyl 4-hydroxy-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylate (10.0 g, 50.7 mmol) and $POCl_3$ (27.8 mL, 304 mmol) was added triethylamine (7.07 mL, 50.7 mmol). After stirring for 16 h, the reaction mixture was concentrated under reduced pressure to remove $POCl_3$. The resulting residue was poured onto ice, carefully neutralized with sat'd aq K_2CO_3 solution, and extracted with EtOAc. The combined organic extracts were dried over $MgSO_4$ and concentrated under reduced pressure to yield 7.3 g (67%) of the desired product that was used directly without further purification. MS APCI (+) *m/z* 216, 218 (M+, Cl pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 8.49 (s, 1H), 6.62 (s, 1H), 4.32 (q, *J* = 7.2 Hz, 2H), 3.59 (s, 3H), 1.36 (t, *J* = 7.6 Hz, 3H).

Step C: Preparation of 4-chloro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylic acid: To a solution of ethyl 4-chloro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylate (925 mg, 4.29 mmol) in a 4:1 mixture of THF-MeOH (20 mL) was added 1 N aq LiOH (8.6 mL, 8.6 mmol) at room temperature. After stirring for 30 minutes, the reaction mixture was acidified to pH 1 with 10% aq HCl and extracted with EtOAc. The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure to give 0.732 g (91%) of the desired product that was used directly without further purification. MS APCI (-) *m/z* 186, 188 (M-, Cl pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 8.53 (s, 1H), 6.62 (s, 1H), 3.58 (s, 3H).

Step D: Preparation of 4-(4-bromo-2-fluorophenylamino)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylic acid: To a solution of i-Pr_2NH (0.39 mL, 2.80 mmol) in THF (4 mL) at 0 ^{o}C was added n-BuLi (1.10 mL, 2.80 mmol, 2.5 M solution in hexanes). After stirring for 15 min, the resulting mixture was cooled to -78 ^{o}C. 4-Bromo-2-fluoroaniline (0.38 g, 2.0 mmol) was added. After vigorous stirring for 10 minutes, a mixture of the 4-chloro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylic acid (0.15 g, 0.80 mmol) in THF (5 mL) was added. After stirring for 30 min at –78 ^{o}C, the dry-ice bath was removed. The reaction mixture was slowly warmed to room temperature and stirred for 17 h. The reaction mixture was treated with 10% aq HCl (15 mL), extracted with EtOAc, dried over $MgSO_4$, and concentrated under reduced pressure to give the crude material that was triturated with CH_2Cl_2 to afford 0.21 g (77%) of the desired product. MS APCI (-) *m/z* 339, 341 (M-, Br pattern) detected; ^{1}H NMR (400 MHz,

DMSO-d_6) δ 9.61 (s, 1H), 8.53 (s, 1H), 7.69 (dd, *J* = 10.8, 2.0 Hz, 1H), 7.46 (m, 2H), 5.47 (s, 1H), 3.41 (s, 3H); ^{19}F NMR (376 MHz, DMSO-d_6) δ –121.4.

Step E: Preparation of 4-(4-bromo-2-fluorophenylamino)-N-(cyclopropylmethoxy)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (**6**): A mixture of 4-(4-bromo-2-fluorophenylamino)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylic acid (78 mg, 0.23 mmol), EDCI (130 mg, 0.69 mmol), and HOBt (93 mg, 0.69 mmol) in DMF (5 ml) was stirred for 30 minutes at room temperature. O-Cyclopropylmethylhydroxylamine (60 mg, 0.69 mmol) was added followed by Et_3N (0.096 mL, 0.69 mmol). After stirring for 1 h, the reaction mixture was diluted with EtOAc and washed with sat'd aq NH_4Cl, sat'd aq $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to yield 83 mg (89%) of the desired product. MS APCI (+) *m/z* 410, 412 (M+, Br pattern) detected; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.57 (s, 1H), 9.42 (s, 1H), 8.10 (s, 1H), 7.67 (d, *J* = 10.4 Hz, 1H), 7.43 (m, 2H), 3.70 (d, *J* = 7.2 Hz, 2H), 3.35 (s, 3H), 1.11 (m, 1H), 0.54 (m, 2H), 0.27 (m, 2H); ^{19}F NMR (376 MHz, DMSO-d_6) δ –121.8.

4-(4-Bromo-2-fluorophenylamino)-5-chloro-N-(cyclopropylmethoxy)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (7).

Preparation of **7** is as described for **6** with the addition of the following chlorination step after Step B.

Preparation of ethyl 4,5-dichloro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylate: A mixture of ethyl 4-chloro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylate (1.00 g, 4.64 mmol) and NCS (0.68 g, 5.10 mmol) in DMF (30 mL) was stirred for 1 h at room temperature. The reaction mixture was diluted with EtOAc and washed with 0.1 N aq HCl. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give 1.10 g (95%) of the desired product that was used directly without further purification. MS APCI (+) *m/z* 250, 252 (M+, Cl pattern) detected; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (s, 1H), 4.36 (q, *J* = 7.2 Hz, 2H), 3.67 (s, 3H), 1.39 (t, *J* = 7.0 Hz, 3H).

4-(4-Bromo-2-fluorophenylamino)-5-chloro-N-(cyclopropylmethoxy)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (**7**): MS APCI (+) *m/z* 444, 446 (M+, Cl, Br pattern) detected; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.52 (s, 1H), 8.78 (s, 1H), 7.98 (s, 1H), 7.52 (dd, *J* = 10.4, 2.0 Hz, 1H), 7.29 (d, *J* = 8.4 Hz, 1H), 6.93 (dd, *J* = 8.4, 8.4 Hz, 1H), 3.47

(s, 3H), 3.40 (d, *J* = 7.2 Hz, 2H), 1.01 (m, 1H), 0.50 (m, 2H), 0.20 (m, 2H); ^{19}F NMR (376 MHz, DMSO-d_6) δ –122.4.

4-(4-Bromo-2-fluorophenylamino)-N-(cyclopropylmethoxy)-5-fluoro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (8).

Preparation of **8** is as described for **6** with the addition of the following fluorination step after Step B.

Preparation of 4-chloro-5-fluoro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylic acid: A mixture of ethyl 4-chloro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylate (1.00 g, 4.64 mmol) and Selectfluor [1-(chloromethyl)-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane-bis(tetrafluoroborate)] (3.29 g, 9.27 mmol) in MeCN (50 mL) was stirred at 85 ^{o}C for 1 h. The reaction was cooled to room temperature and concentrated under reduced pressure. The residue was dissolved in water and extracted with EtOAc. The combined organic extracts were dried over $MgSO_4$ and concentrated under reduced pressure to give the crude material that was purified by silica gel flash column chromatography (40% EtOAc in hexanes) to give the partially purified ethyl ester. To a solution of the partially purified ethyl ester in a 4:1 mixture of THF:MeOH (10 mL) was added 1 N aq LiOH (9.30 mL, 9.30 mmol) at room temperature. After stirring for 1 h, the reaction mixture was acidified to pH 1 with 10% aq HCl and extracted with EtOAc. The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure to give the crude material that was triturated with diethyl ether to yield 0.25 g (26% for two steps) of 4-chloro-5-fluoro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylic acid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.48 (s, 1H), 3.56 (s, 3H); ^{19}F NMR (376 MHz, DMSO-d_6) δ –133.9.

4-(4-Bromo-2-fluorophenylamino)-N-(cyclopropylmethoxy)-5-fluoro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (**8**): MS APCI (+) *m/z* 428, 430 (M+, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.85 (s, 1H), 7.36 (dd, *J* = 10.8, 2.0 Hz, 1H), 7.28 (d, *J* = 8.4 Hz, 1H), 7.01 (dt, *J* = 8.8, 4.4 Hz, 1H), 3.69 (d, 2H), 3.57 (s, 3H), 0.89 (m, 1H), 0.58 (m, 2H), 0.30 (m, 2H); 19F NMR (376 MHz, CD_3OD) δ –125.8, –152.1.

4-(4-Bromo-2-fluorophenylamino)-N-(cyclopropylmethoxy)-1,5-dimethyl-6-oxo-1,6-dihydropyridine-3-carboxamide (9).

Preparation of ethyl 4-hydroxy-1,5-dimethyl-6-oxo-1,6-dihydropyridine-3-carboxylate:

To diethyl 2-methyl-3-oxopentanedioate (Caliskan et al *Aust. J. Chem.* **1999**, *52*, 1013-1020) (5.00 g, 23.1 mmol) was added triethyl orthoformate (3.85 mL, 23.1 mmol) and acetic anhydride (4.37 mL, 46.2 mmol) at room temperature. The resulting mixture was stirred for 1 h at 135 ^{o}C. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. To the resulting residue 0 ^{o}C was added methylamine (5.00 mL, 57.8 mmol, 40% in water) followed by water (20 mL). The reaction mixture was warmed to room temperature and stirred for 16 h. The reaction mixture was extracted with EtOAc. The aqueous layer was acidified to pH 2 with 10% aq HCl. The acidified aqueous layer was extracted with EtOAc. The combined organic extracts were dried over $MgSO_4$ and concentrated under reduced pressure to give the crude material that was purified by trituration with diethyl ether to yield 4.88 g (55%) of the desired product. MS APCI (+) *m/z* 212 (M+) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 8.39 (s, 1H), 4.40 (q, *J* = 7.2 Hz, 2H), 3.57 (s, 3H), 1.97 (s, 3H), 1.39 (t, *J* = 7.2 Hz, 3H).

Compound **9** was prepared as described in Steps B – E for compound **6**.

4-(4-Bromo-2-fluorophenylamino)-N-(cyclopropylmethoxy)-1,5-dimethyl-6-oxo-1,6-dihydropyridine-3-carboxamide (**9**): MS APCI (+) *m/z* 424, 426 (M+, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.88 (s, 1H), 7.33 (dd, *J* = 10.4, 1.2 Hz, 1H), 7.20 (d, *J* = 8.8 Hz, 1H), 6.65 (dd, *J* = 8.4, 8.4 Hz, 1H), 3.59 (d, *J* = 6.8 Hz, 2H), 3.57 (s, 3H), 1.83 (s, 3H), 1.10 (m, 1H), 0.54 (m, 2H), 0.25 (m, 2H); ^{19}F NMR (376 MHz, CD_3OD) δ –128.2.

4-(4-Bromo-2-fluorophenylamino)-N-(2-hydroxyethoxy)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (10).

Step A: Preparation of 4-(4-bromo-2-fluorophenylamino)-1-methyl-6-oxo-N-(2-(vinyloxy)ethoxy)-1,6-dihydropyridine-3-carboxamide: A mixture of 4-(4-bromo-2-fluorophenylamino)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylic acid (120 mg, 0.35 mmol), EDCI (100 mg, 0.53 mmol), and HOBt (71 mg, 0.53 mmol) in DMF (5 mL) was stirred at room temperature for 3 h. O-(2-Vinyloxyethyl)hydroxylamine (0.071 mL, 0.70 mmol) was added followed by Et_3N (0.098 mL, 0.70 mmol). After stirring for 2 h, the reaction mixture was diluted with EtOAc and washed with sat'd aq NH_4Cl, sat'd aq

$NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give the crude material that was purified by silica gel flash column chromatography (3% MeOH in CH_2Cl_2) to afford 78 mg (52%) of the desired product. The desired product was used without further characterization except MS. MS APCI (+) *m/z* 426, 428 (M+, Br pattern) detected.

Step B: Preparation of 4-(4-bromo-2-fluorophenylamino)-N-(2-hydroxyethoxy)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (**10**): To a solution of 4-(4-bromo-2-fluorophenylamino)-1-methyl-6-oxo-N-(2-(vinyloxy)ethoxy)-1,6-dihydropyridine-3-carboxamide (77 mg, 0.18 mmol) in a 1:1 mixture of EtOH:THF (6 mL) was added 1 N aq HCl (0.36 mL, 0.36 mmol) at room temperature. After stirring for 1 h, the pH of the reaction mixture was adjusted to 5 to 7 with 2 N aq NaOH. The resulting mixture was diluted with EtOAc, washed with water, dried over $MgSO_4$, and concentrated under reduced pressure to give the crude material that purified by trituration with diethyl ether to yield 55 mg (76%) of the desired product. MS APCI (-) *m/z* 398, 400 (M-, Br pattern) detected; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.65 (s, 1H), 9.41 (s, 1H), 8.13 (s, 1H), 7.68 (d, *J* = 9.6 Hz, 1H), 7.43 (m, 2H), 5.53 (s, 1H), 4.74 (m, 1H), 3.91 (t, *J* = 4.8 Hz, 2H), 3.62 (m, 2H), 3.36 (s, 3H); ^{19}F NMR (376 MHz, DMSO-d_6) δ –121.8.

4-(4-Bromo-2-chlorophenylamino)-5-chloro-N-(2-hydroxyethoxy)-6-methoxynicotinamide (14).

Step A: Preparation of ethyl 5-chloro-4,6-dihydroxynicotinate: To a solution of ethyl 4,6-dihydroxynicotinate (20.0 g, 109 mmol, *J. Heterocyclic Chem.* **1983**, *20*, 1363) in DMF (400 mL) at room temperature was portionwise added NCS (17.5 g, 131 mmol) over 10 min. The resulting mixture was stirred for 3 days. After addition of sat'd aq $NaHCO_3$, the resulting mixture was stirred for 2 h at room temperature. The mixture was concentrated under reduced pressure to give the crude material that was partitioned between EtOAc and water. The precipitates were filtered off and dried under reduced pressure to afford 6.12 g (26%) of the desired product. MS APCI (-) *m/z* 216, 218 (M-; Cl pattern) detected. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.06 (s, 1H), 4.32 (q, *J* = 7.2 Hz, 2H), 1.30 (t, *J* = 7.2 Hz, 3H).

Step B: Preparation of ethyl 4,5,6-trichloronicotinate: To a suspension of ethyl 5-chloro-4,6-dihydroxynicotinate (6.12 g, 28.1 mmol) in $POCl_3$ (40 mL) was slowly added TEA

(4.00 mL, 28.7 mmol) at room temperature. The resulting mixture was stirred for 4 h at 60 ^{o}C. The reaction mixture was cooled to room temperature, poured onto ice, and neutralized by sat'd aq K_2CO_3 and NaOH pellets. The resulting mixture was extracted with EtOAc followed by Et_2O, dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give the crude material that was purified by silica gel flash column chromatography (EtOAc:hexanes = 1:5) to afford 6.04 g (84%) of the desired product. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (s, 1H), 4.45 (q, *J* = 6.8 Hz, 2H), 1.42 (t, *J* = 6.8 Hz, 3H).

Step C: Preparation of 4,5,6-trichloronicotinic acid: To a solution of ethyl 4,5,6-trichloronicotinate (7.76 g, 30.5 mmol) in a 4:1 mixture of THF-MeOH (150 mL) was added 1 N aq NaOH (61 mL, 61 mmol) at room temperature. The reaction mixture was stirred for 30 min, acidified to pH 1 with conc. HCl, and diluted with EtOAc. The mixture was washed with water and brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield 6.77 g (98%) of the crude product that was used directly without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.86 (s, 1H).

Step D: Preparation of 4-(4-bromo-2-chlorophenylamino)-5,6-dichloronicotinic acid: To a solution of 4-bromo-2-chloroaniline (11.4 g, 55.3 mmol) in a 1:3 mixture of THF-hexanes (92 mL) at –78 ^{o}C was added LiHMDS (70.7 mL, 70.7 mmol, 1 M solution in THF) over 7 min. The resulting mixture was stirred for 1 h at –78 ^{o}C. A solution of 4,5,6-trichloronicotinic acid (5.12 g, 22.6 mmol) in THF (15 mL) was added at –78 ^{o}C. The reaction mixture was slowly warmed to room temperature and stirred for 3 h. The reaction mixture was quenched with water and acidified to pH 1-2 with 2 N aq HCl. The resulting mixture was cooled to 10 ^{o}C with ice-water bath and stirred for 25 min. The precipitates were filtered and dried under reduced pressure to afford 8.64 g (99%) of the desired product. MS APCI (-) *m/z* 393, 395, 397 (M-; Cl, Br pattern) detected. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.90 (s, 1H), 8.68 (s, 1H), 7.79 (d, *J* = 2.0 Hz, 1H), 7.48 (dd, *J* = 2.0, 8.8 Hz, 1H), 7.04 (d, *J* = 8.8 Hz, 1H).

Step E: Preparation of methyl 4-(4-bromo-2-chlorophenylamino)-5,6-dichloronicotinate: To a suspension of 4-(4-bromo-2-chlorophenylamino)-5,6-dichloronicotinic acid (14.7 g, 37.0 mmol) in EtOH (300 mL) was slowly added trimethylsilyldiazomethane (60 mL,

120 mmol, 2.0 M solution in hexanes). After the addition was complete the resulting slurry was diluted with hexanes (300 mL) and the precipitates were filtered and washed with hexanes to give 10.06 g of the desired product. The organic washes were concentrated under reduced pressure to give the crude material that was purified by silica gel flash column chromatography (CH_2Cl_2) to afford an additional 3.83 g of the desired product. A total of 13.89 g (91%) of the desired product was isolated. MS APCI (+) *m/z* 409, 411, 413 (M+; Cl, Br pattern) detected. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.30 (s, 1H), 8.57 (s, 1H), 7.81 (d, *J* = 2.4 Hz, 1H), 7.49 (dd, *J* = 2.4, 8.4 Hz, 1H), 7.05 (d, *J* = 8.4 Hz, 1H), 3.68 (s, 3H).

Step F: Preparation of methyl 4-(4-bromo-2-chlorophenylamino)-5-chloro-6-methoxynicotinate: To a solution of methyl 4-(4-bromo-2-chlorophenylamino)-5,6-dichloronicotinate (343 mg, 0.84 mmol) in a 4:1 mixture of MeOH-THF (5 mL) was slowly added NaH (100 mg, 4.18 mmol) portionwise. The resulting mixture was stirred for 16 h at room temperature. The reaction mixture was diluted with EtOAc, washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give the crude material that was purified by silica gel flash column chromatography (100% CH_2Cl_2 to 10% MeOH in CH_2Cl_2) to afford 165 mg (49%) of the desired product. MS APCI (-) *m/z* 403, 405, 407 (M-, Cl, Br pattern) detected. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.45 (s, 1H), 8.69 (s, 1H), 7.55 (d, *J* = 2.0 Hz, 1H), 7.26 (dd, *J* = 2.0, 8.4 Hz, 1H), 6.68 (d, *J* = 8.4 Hz, 1H), 4.08 (s, 3H), 3.91 (s, 3H).

Step G: Preparation of 4-(4-bromo-2-chlorophenylamino)-5-chloro-6-methoxynicotinic acid: To a suspension of methyl 4-(4-bromo-2-chlorophenylamino)-5-chloro-6-methoxynicotinate (17 mg, 0.042 mmol) in a 1:1 mixture of THF-H_2O (4 mL) was added 1 N aq NaOH (0.21 mL, 0.21 mmol) at room temperature. After stirring for 3 h, the reaction mixture was concentrated under reduced pressure to half of the volume and acidified to pH 1 with 1 N aq HCl. The resulting suspension was extracted with EtOAc-THF, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the desired product quantitatively that was used directly without further purification. ^{1}H NMR (400 MHz, CD_3OD) δ 8.70 (s, 1H), 7.60 (d, *J* = 2.4 Hz, 1H), 7.35 (dd, *J* = 2.4, 8.8 Hz, 1H), 6.79 (d, *J* = 8.4 Hz, 1H), 4.03 (s, 3H).

Step H: Preparation of 4-(4-bromo-2-chlorophenylamino)-5-chloro-N-(2-hydroxyethoxy)-6-methoxynicotinamide (**14**): The title compound **14** was prepared in 47% yield by the procedures previously described for compound **10** using 4-(4-bromo-2-chlorophenylamino)-5-chloro-6-methoxynicotinic acid (15 mg, 0.038 mmol) and O-(2-vinyloxyethyl)hydroxylamine (5.9 mg, 0.057 mmol). MS APCI (-) *m/z* 448, 450, 452 (M-, Cl, Br pattern) detected. ^{1}H NMR (400 MHz, CD_3OD) δ 8.17 (s, 1H), 7.59 (d, *J* = 2 Hz, 1H), 7.33 (dd, *J* = 8.4, 2.0 Hz, 1H), 6.76 (d, *J* = 8.8 Hz, 1H), 4.03 (s, 3H), 3.86 (t, *J* = 4.8 Hz, 2H), 3.68 (t, *J* = 4.8 Hz, 2H).

4-(4-Bromo-2-chlorophenylamino)-5,6-dichloro-N-(2-hydroxyethoxy)nicotinamide (15).

The title compound **15** was prepared in 32 % yield by the procedures previously described for compound **10** using 4-(4-bromo-2-chlorophenylamino)-5,6-dichloronicotinic acid (35 mg, 0.088 mmol, prepared in step D for compound **14**) and O-(2-vinyloxyethyl)hydroxylamine (14 mg, 0.13 mmol). MS APCI (+) *m/z* 454, 456, 458 (M+, Cl, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 8.26 (s, 1H), 7.63 (s, 1H), 7.40 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.93 (d, *J* = 8.8 Hz, 1H), 3.82 (m, 2H), 3.70 (m, 2H).

4-(4-Bromo-2-fluorophenylamino)-5-fluoro-N-hydroxy-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (20).

4-(4-Bromo-2-fluorophenylamino)-5-fluoro-N-hydroxy-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide was prepared from 4-(4-bromo-2-fluorophenylamino)-N-tert-butoxy-5-fluoro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide by TFA mediated deprotection. 4-(4-Bromo-2-fluorophenylamino)-N-tert-butoxy-5-fluoro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (35 mg, 0.081 mmol) was treated with TFA (0.63 mL). After stirring for 4 days, the reaction mixture was concentrated under reduced pressure to give the crude material that was purified by silica gel flash column chromatography (10% MeOH in CH_2Cl_2) followed by sat'd aq $NaHCO_3$ wash of an ethyl acetate solution of the product to yield 10 mg (33%) of the desired product. MS APCI (+) *m/z* 356, 358 (M-OH, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.84 (s,

1H), 7.35 (d, *J* = 10.4 Hz, 1H), 7.28 (d, *J* = 8.8 Hz, 1H), 7.01 (m, 1H), 3.57 (s, 3H); ^{19}F NMR (376 MHz, CD_3OD) δ –126.0, –151.5.

The following compounds were prepared as described above.

4-(4-Bromo-2-fluorophenylamino)-5-chloro-N-(2-hydroxyethoxy)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (11).

MS APCI (+) *m/z* 434, 436 (M+, Cl, Br pattern) detected; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.60 (s, 1H), 8.82 (s, 1H), 8.03 (s, 1H), 7.52 (d, *J* = 10.4 Hz, 1H), 7.28 (d, *J* = 8.4 Hz, 1H), 6.92 (dd, *J* = 8.4, 8.4 Hz, 1H), 4.69 (m, 1H), 3.68 (m, 2H), 3.52 (m, 2H), 3.47 (s, 3H); ^{19}F NMR (376 MHz, DMSO-d_6) δ –122.7.

4-(4-Bromo-2-fluorophenylamino)-5-fluoro-N-(2-hydroxyethoxy)-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (12).

MS APCI (+) *m/z* 418, 420 (M+, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.90 (s, 1H), 7.36 (dd, *J* = 10.4, 2.0 Hz, 1H), 7.28 (d, *J* = 8.8 Hz, 1H), 7.01 (td, *J* = 8.8, 4.0 Hz, 1H), 3.98 (m, 2H), 3.75 (m, 2H), 3.58 (s, 3H); ^{19}F NMR (376 MHz, CD_3OD) δ –125.8, –152.1.

4-(4-Bromo-2-fluorophenylamino)-N-(2-hydroxyethoxy)-1,5-dimethyl-6-oxo-1,6-dihydropyridine-3-carboxamide (13).

MS APCI (+) *m/z* 414, 416 (M+, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.93 (s, 1H), 7.33 (d, *J* = 10.4 Hz, 1H), 7.20 (d, *J* = 8.4 Hz, 1H), 6.65 (dd, *J* = 8.8, 8.8 Hz, 1H), 3.88 (m, 2H), 3.68 (m, 2H), 3.57 (s, 3H), 1.83 (s, 3H); ^{19}F NMR (376 MHz, CD_3OD) δ –128.4; Anal. ($C_{16}H_{17}BrFN_3O_4$) C, H, N.

4-(4-Bromo-2-fluorophenylamino)-5-fluoro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (16).

MS (+) *m/z* 358, 360 (M+, Br pattern) detected; ^{1}H NMR (400 Hz, CD_3OD) δ 8.11 (s, 1H), 7.36 (dd, *J* = 10.4, 2.0 Hz, 1H), 7.28 (d, *J* = 8.8 Hz, 1H), 7.02 (m, 1H), 3.58 (s, 3H); ^{19}F NMR (376 MHz, CD_3OD) δ –125.8, –152.3; Anal. ($C_{13}H_{10}BrF_2N_3O_2$) H; C: calcd, 43.60; found, 42.98; N: calcd, 11.73; found, 11.04.

4-(4-Bromo-2-fluorophenylamino)-5-fluoro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxylic acid (17).

MS APCI (-) *m/z* 357, 359 (M-1, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 8.33 (s, 1H), 7.30 (d, *J* = 8.6 Hz, 1H), 7.26 (d, *J* = 8.7 Hz, 1H), 7.03 (td, *J* = 8.8, 4.0 Hz, 1H), 3.62 (s, 3H); ^{19}F NMR (376 MHz, CD_3OD) δ –125.2, –54.2.

4-(4-Bromo-2-fluorophenylamino)-5-fluoro-N,1-dimethyl-6-oxo-1,6-dihydropyridine-3-carboxamide (18).

MS APCI (+) *m/z* 372, 374 (M+, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.93 (s, 1H), 7.35 (d, *J* = 10.4 Hz, 1H), 7.27 (d, *J* = 8.4 Hz, 1H), 7.0 (td, *J* = 8.8, 4.4 Hz, 1H), 3.57 (s, 3H), 2.83 (s, 3H); ^{19}F NMR (376 MHz, CD_3OD) δ –126.0, –152.1.

4-(4-Bromo-2-fluorophenylamino)-N-(cyclopropylmethyl)-5-fluoro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (19).

MS APCI (+) *m/z* 412, 414 (M+, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.97 (s, 1H), 7.32 (dd, *J* = 10.4, 1.6 Hz, 1H), 7.25 (d, *J* = 8.8 Hz, 1H), 6.98 (dt, *J* = 8.8, 4.8 Hz, 1H), 3.60 (s, 3H), 3.15 (d, *J* = 7.2 Hz, 2H), 1.04 (m, 1H), 0.54 (m, 2H), 0.26 (m, 2H); ^{19}F NMR (376 MHz, CD_3OD) δ –125.5, –151.4.

4-(4-Bromo-2-fluorophenylamino)-5-fluoro-N-methoxy-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (21).

MS APCI (+) *m/z* 388, 390 (M+, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.87 (s, 1H), 7.35 (dd, *J* = 10.4, 2.0 Hz, 1H), 7.27 (d, *J* = 8.8 Hz, 1H), 7.01 (td, *J* = 8.8, 4.4 Hz, 1H), 3.75 (s, 3H), 3.58 (s, 3H); ^{19}F NMR (376 MHz, CD_3OD) δ –125.7, –151.8.

4-(4-Bromo-2-fluorophenylamino)-N-ethoxy-5-fluoro-1-methyl-6-oxo-1,6-dihydropyridine-3-carboxamide (22).

MS APCI (+) *m/z* 402, 404 (M+, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.87 (s, 1H), 7.35 (dd, *J* = 10.0, 1.6 Hz, 1H), 7.27 (d, *J* = 10.0 Hz, 1H), 7.01 (dt, *J* = 8.0, 4.0 Hz, 1H), 3.94 (q, *J* = 7.2 Hz, 2H), 3.58 (s, 3H), 1.27 (t, *J* = 6.8 Hz, 3H); ^{19}F NMR (376 MHz, CD_3OD) δ –125.9, –152.0; Anal. ($C_{15}H_{14}BrF_2N_3O_3$) C, H, N.

4-(4-Bromo-2-fluorophenylamino)-N-methoxy-1,5-dimethyl-6-oxo-1,6-dihydropyridine-3-carboxamide (23).

MS APCI (-) *m/z* 382, 384 (M-, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.90 (s, 1H), 7.33 (m, 1H), 7.20 (m, 1H), 6.66 (dd, *J* = 8.8, 8.8 Hz, 1H), 3.65 (s, 3H), 3.57 (s, 3H), 1.83 (s, 3H); ^{19}F NMR (376 MHz, CD_3OD) δ –128.27; Anal. ($C_{15}H_{15}BrFN_3O_3$) C, H, N.

4-(4-bromo-2-fluorophenylamino)-N-ethoxy-1,5-dimethyl-6-oxo-1,6-dihydropyridine-3-carboxamide (24).

MS APCI (-) *m/z* 396, 398 (M-, Br pattern) detected; ^{1}H NMR (400 MHz, CD_3OD) δ 7.90 (s, 1H), 7.33 (dd, *J* = 10.8, 2.4 Hz, 1H), 7.20 (dd, *J* = 8.8, 1.6 Hz 1H), 6.65 (dd, *J* = 8.8, 8.8 Hz, 1H), 3.83 (q, *J* = 6.8 Hz, 2H), 3.57 (s, 3H), 1.83 (s, 3H), 1.22 (t, *J* = 6.8 Hz, 3H); ^{19}F NMR (376 MHz, CD_3OD) δ –128.32; Anal. ($C_{16}H_{17}BrFN_3O_3$) C, H, N.

Elemental Analyses for Compounds for 13, 16, 22, 23, and 24

Compound	Formula	Calculated	Found
13	$C_{16}H_{17}BrFN_3O_4$	C 46.39	C 46.54
		H 4.14	H 3.80
		N 10.14	N 10.17
16	$C_{13}H_{10}BrF_2N_3O_2$	C 43.60	C 42.98
		H 2.81	H 3.19
		N 11.73	N 11.04
22	$C_{15}H_{14}BrF_2N_3O_3$	C 44.79	C 44.69
		H 3.51	H 3.26
		N 10.45	N 10.19
23	$C_{15}H_{15}BrFN_3O_3$	C 46.89	C 47.20
		H 3.94	H 3.63
		N 10.94	N 11.00
24	$C_{16}H_{17}BrFN_3O_3$	C 48.26	C 48.23
		H 4.30	H 3.98
		N 10.55	N 10.57

Biological Assays

MEK enzyme assay: N-terminal 6 His-tagged, constitutively active MEK-1 (2-393) is expressed in *E. coli* and protein is purified by conventional methods (Mansour S.J.; Matten W.T.; Hermann A.S.; Candia J.M.; Rong S.; Fukasawa K.; Vande Woude G.F.; Ahn N.G. *Science* **1994**, *265*, 966-970). The activity of MEK1 is assessed by measuring the incorporation of γ-^{33}P-phosphate from γ-^{33}P-ATP onto N-terminal His tagged ERK2, which is expressed in *E. coli* and is purified by conventional methods, in the presence of MEK-1. The assay is carried out in 96-well polypropylene plate. The incubation mixture (100 µL) comprises of 25 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 5 mM β-glycerolphosphate, 100 µM Na-orthovanadate, 5 mM DTT, 5 nM MEK1, and 1 µM ERK2. Inhibitors are suspended in DMSO, and all reactions, including controls are performed at a final concentration of 1% DMSO. Reactions are initiated by the addition of 10 µM ATP (with 0.5 µCi γ-^{33}P-ATP/well) and incubated at ambient temperature for 45 minutes. Equal volume of 25% TCA is added to stop the reaction and precipitate the proteins. Precipitated proteins are trapped onto glass fiber B filterplates, and excess labeled ATP washed off using a Tomtec MACH III harvestor. Plates are allowed to air-dry prior to adding 30 µL/well of Packard Microscint 20, and plates are counted using a Packard TopCount.

pERK measurement in Malme-3M cells: Malme-3M melanoma cells were plated in 96-wells and treated with various concentrations of test compounds for 1 hr at 37 °C. The cells were fixed, permeabilized, and incubated with an anti-phospho-ERK antibody and an anti-ERK1,2 antibody. Plates were washed and fluorescently-labeled secondary antibodies were added. Plates were analyzed on a LICOR fluorescence imager. The pERK signal is normalized to the total ERK signal.

Cell Viability assay: Malme-3M melanoma cells were plated in 96-wells and incubated with various concentrations of test compounds. On Day 3, the number of viable cells was measured using the MTS assay.

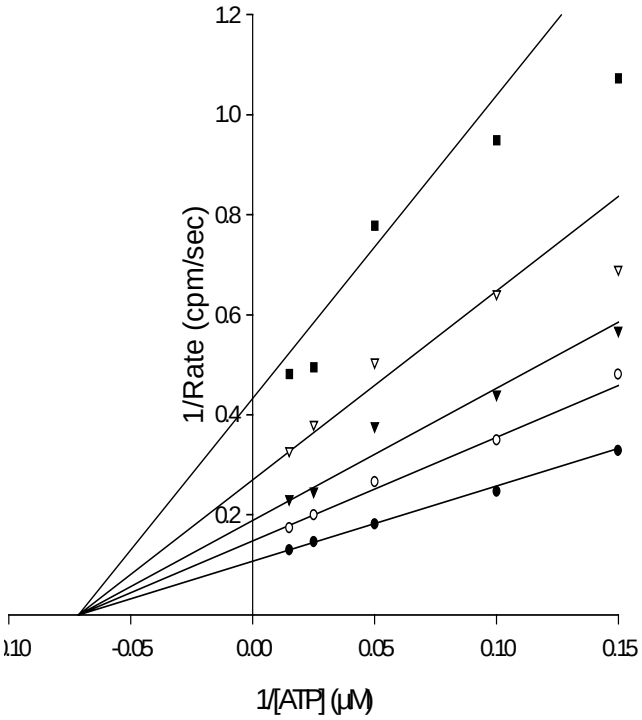
Selectivity Data

The activity of analogs **13**, **22**, **23** and **24** against a number of important kinases was evaluated at Upstate (Dundee). Compounds were tested at 10 uM.

Upstate Panel - % Inhibition @ 10 uM

Enzyme	**Cmpd 13**	**Cmpd 22**	**Cmpd 23**	**Cmpd 24**
MAPK2/ERK2	6	7	-9	6
JNK/SAPK1c	23	15	7	23
SAPK2a/p38	20	10	3	-3
SAPK2b/p38β2	16	9	5	13
MAPKAP-K1a	-9	6	-8	3
MAPKAP-K2	8	15	1	14
MSK1	2	7	6	5
PRAK	16	18	9	18
PKA(rec)	15	14	-7	-1
PBK	-8	-1	-4	3
PKCa	-3	6	0	7
PDK1	-7	-2	-9	-3
PKBa	-4	6	12	6
SGK	-15	-18	-39	-44
p70 S6K	18	13	-2	-1
GSK3b	17	21	12	15
ROCK-II	13	4	10	13
AMPK	19	8	-4	2
CHK1	11	13	-3	8
CK2	-1	4	-4	4
LCK	13	19	-1	9
CDK2/cyclin A	9	25	8	20
DYRK1a	10	12	2	15

Non-Competitive Inhibition of 16



Vmax = 9.3
Km = 14
Ki = 6.6

I=0 nM
I=2.5
I=5
I=10
I=20

To assess whether inhibitor **16** is a competitive inhibitor with respect to ATP, increasing concentrations of inhibitor **16** (0 – 40 nM) was tested against constitutively active MEK1 in the presence of ATP that ranged from 6.67 to 66.7 µM (γ-^{33}P-ATP added as tracer) and 1 µM ERK2. Reactions and method are as described. Data is analyzed using SigmaPlot.

In this plot, y-intercept = 1/Vmax and x-intercept = 1/Km. With increasing concentrations of inhibitor **16**, the Km for ATP remains unchanged (note the convergence on the x-axis) while the Vmax of the MEK1 reaction decreases (different y-intercepts with increasing [Inhibitor 16]). These results are characteristic of a noncompetitive inhibitor.

Hepatocyte Assay Methods

In vitro stability in the presence of hepatocytes was conducted as follows. Fresh or cryopreserved hepatocytes were thawed if necessary, isolated from shipping media and diluted to a viable cell density of 2 x 10^6 cells/mL according to the supplier's guidelines using Krebs-Henseleit buffer (KHB, pH 7.3, Sigma) supplemented with amikacin (84 µg/mL), calcium chloride (1 mM), gentamicin (84 µg/mL), HEPES (20 mM), heptanoic acid (4.2 µM) and sodium bicarbonate (2.2 mg/mL). Viability was determined by trypan blue exclusion using a hemacytometer (3500 Hausser, VWR). The 10 mM stock solution of test compounds in DMSO was diluted to 2 µM using supplemented KHB buffer to create the working standard. A 50-µL aliquot of test compound or control was added to each test well of a 96-well polypropylene plate (Costar) immediately followed by the addition of 50 µL of the hepatocyte suspension. One incubation plate was prepared for each timepoint with samples being prepared in duplicate. Incubations were conducted at 37 °C, 5% CO_2 and 100% relative humidity in an incubator (Model 2300, VWR). At each timepoint, one incubation plate was removed from the incubator, and a solution containing internal standard (100 µL, 2 µM labetalol) was added to each well. The plate was mixed at 700 rpm for 2 minutes on a plate shaker (IKA MTS 2/4 Digital Microtiter Shaker, VWR) and immediately spun in a centrifuge at 2,000 x g for 10 minutes using an Allegra benchtop centrifuge (Beckman Coulter). A 150-µL aliquot of the supernatant was transferred from each well to a 96-well shallow plate (Costar). The plates were sealed using reusable plate mats.

The relative concentrations of test compounds were quantitated using an LC-MS/MS system comprised of an HTS-PAL autosampler (Leap Technologies), an HP1100 HPLC (Agilent), and an API4000 triple quadrupole mass spectrometer (Sciex). Chromatographic separation of the analyte and internal standard was achieved using an Aqua C_{18} column (72 x 2 mm, 3 µm particle size, Phenomenex) in conjunction with gradient conditions using mobile phases A (aqueous 0.1% formic acid containing 1% isopropanol) and B (0.1% formic acid in acetonitrile containing 1% isopropanol). The total run time, including re-equilibration time, for a single injection was 6 minutes. Mass spectrometric detection of the analytes was accomplished using ESI+ ionization mode. Analyte responses were measured by multiple reaction monitoring (MRM) of transitions unique to each compound.

Data were acquired and peak areas were calculated for test compounds and the internal standard using the Analyst 1.4.1 software (Sciex). Peak area tables were exported to Excel 2000, where the average analyte-to-internal standard peak area ratios were used to calculate percent remaining (%rem), half-life ($t_{1/2}$), predicted intrinsic hepatic clearance (CL_{int}), predicted hepatic clearance (CL_h) and predicted hepatic extraction ratio (ER).

Caco-2 Methods

Upon receiving the plated Caco-2 cells, differentiation media was added to each transwell and well of the 24-well plate. The Caco-2 plates are incubated overnight at 37 °C and 5% CO_2 with saturating humidity. The pH of the transport buffer was adjusted to 6.5 or 7.4, using 1 M HCL for the apical and basolateral sides, respectively, and warmed to 37 °C in a water bath. Dosing solutions were prepared using the apical and basolateral transport media at a concentration of 10 µM for test compounds and containing 25 µM lucifer yellow to monitor monolayer integrity.

Prior to conducting the assay, to ensure the integrity of Caco-2 monolayers, trans epithelial electrical resistance (TEER) was measured using a Millipore Millicell-ERS TEER device. A reading of at least 1,000 Ω from the TEER device indicated that the cells were acceptable for the permeability assay.

Additionally, the Caco-2 cultures were considered acceptable for transport studies if the following criteria were met:

- A low mannitol permeability transport rate (P_{app}) of ≤ 20 nm/s.
- A propranolol:mannitol permeability ratio of ≥ 5.
- A vinblastine with verapamil:vinblastine permeability ratio of ≥ 1.5.

The typical experimental conditions were as follows (conducted at In Vitro Technologies on a Caco-2 plate from the same culture):

1. Mannitol permeability: A → B transport was measured using [^{3}H]mannitol at approximately 152 nM and 4 µCi/mL in a 2-hour incubation.
2. Propranolol permeability: A → B transport was measured using [^{3}H]propranolol at approximately 190 nM and 4 µCi/mL in a 2-hour incubation.
3. Vinblastime permeability: A → B transport was measured using [^{3}H]vinblastine at approximately 44 nM and 0.5 µCi/mL in a 2-hour incubation.
4. Vinblastine permeability in the presence of verapamil: A → B transport was measured using [^{3}H]vinblastine at approximately 22 nM and 0.25 µCi/mL in a solution containing 100 µM of verapamil.

Verapamil and progesterone, both high permeability compounds, metolazone, a medium permeability compound, and erythromycin, a low permeability compound, were used as controls in the assay. Stock solutions of each of these controls, as well as ARRY-334543 were prepared at 10 mM in DMSO. Compounds were diluted 1:1000 to 10 µM using the appropriate transport buffer.

For the apical to basolateral determination, 100 µL of the test compound in apical transport buffer was added to the apical side of the individual transwells and 600 µL of

basolateral media was added to each well. For the basolateral to apical determination, 600 µL of test compound in basolateral transport buffer was added to each well and 100 µL apical transport buffer added to each transwell. All compounds were tested in duplicate wells to assess transport in both directions. The plates were incubated for 2 hours on a Lab-Line Instruments Titer Orbital Shaker (VWR, Cat No. 57019-600) at 50 rpm and 37°C with 5%CO_2. Plates were removed from the incubator and 75 µL of media is removed from the apical and basolateral portion of each well and added to 75 µL of internal standard (4 µM labetalol) in acetonitrile.

The plates were first read using a Molecular Devices Gemini Fluorometer to evaluate lucifer yellow concentrations at an excitation/emission wavelength of 425/535 nm. Lucifer yellow transport values were below 1% for apical to basolateral and below 2% for basolateral to apical when compared to the dosing solutions. The plates were sealed with reusable plate mats and the contents of each well analyzed by LC-MS/MS. The compound concentrations were determined from the ratio of the peak areas of the compound to the internal standard (labetalol) in comparison to the dosing solution.

Instrumentation comprised of a CTC PAL autosampler coupled with a Finnigan Surveyor LC system. Mass selective detection was achieved using a Finnigan LCQ Deca ion trap mass spectrometer. When performing LC/MS analysis of test compounds, 10-20 µL of the sample from each well was injected for analysis. Separations were achieved using a XTerra C18 column (2.1 or 3.0 x 30mm, 3.5 µm particle size, Waters) and a 0.4 mL/min. linear gradient. The gradient was from 95% aqueous buffer (formic or NH_4OH) to 95% acetonitrile over 4-5 minutes. An optimized, dedicated MS/MS method was created by maximizing the MS/MS fragment ion signal during infusion of the stock solutions into a mobile phase equivalent to that present midway through the HPLC gradient.

Aqueous Solubility Assay

The thermodynamic aqueous solubility of compounds was measured using a modified shake-flask method. Crystallinity of each compound was confirmed using a polarizing light microscope (Olympus BX51). For each compound, 0.5 mL of aqueous buffer (10 mM potassium phosphate), pH 6.5, was added to 0.5 mg of dry compound and the mixture was swirled at 350 rpm at room temperature for 24 hours. Aliquots were subsequently removed and filtered for HPLC analysis. Standards of known concentration were also prepared and analyzed for each compound in order to create a calibration curve. Analysis was accomplished using a HPLC/PDA system comprised of an Alliance 2795 Separations System (Waters) and a 2996 Photodiode Array Detector (Waters).

Rodent Pharmacokinetics:

In vivo pharmacokinetic studies were performed in male Sprague Dawley rats (6-10 weeks of age) allowed food and water ad lib. Intravenous dose solutions were prepared in 10% DMSO/90% normal saline (0.9% NaCl) at a concentration of 1 mg/mL to yield a dose of 1 mg/kg at a dose volume of 1 mL/kg, IV. Oral dose solutions were prepared in 1% CMC/0.5% Tween 80 at a concentration of 2 mg/mL to yield a dose of 10 mg/kg at a dose volume of 5 mL/kg, PO. Whole blood samples (in EDTA) were obtained vial the tail vein at the following timepoints post dose administration:

IV: 1, 5, 15 and 30 min; 1, 2, 4, 8, 12 and 24 hrs

PO: 15 and 30 min; 1, 2, 4, 8, 12, 24, 48 and 72 hrs

Blood samples were centrifuged and resulting plasma was analyzed for compound concentration using a LC/MS/MS method. The method utilized protein precipitation by methanol of the plasma sample followed by centrifugation and detection of each compound using reversed phase liquid chromatography with triple quadrapole MS/MS selective detection. Labetalol was used as the internal standard. The method had a validated quantitation range of 9 ng/mL to 20 µg/mL. Higher sample concentrations were quantitated by diluting samples by up to 10-fold using plasma from naïve animals. Pharmacokinetic (PK) parameters were calculated by established non-compartmental methods using WinNonlin Pharsight, Inc).

PK / PD Assay

Tumor growth and harvest. HT-29 cells (ATCC # HTB-38) were grown as monolayer cultures at 37°C in a humidified tissue culture incubator with a 5% CO_2 atmosphere. Growth medium was McCoy's 5A (ATCC #30-2007) with 10% FBS (ATCC #30-2020), GlutaMAX (Gibco #35050-061) and antibiotic-antimycotic (Gibco #15240-062). Cells were harvested at approximately 80% confluency using 0.05% trypsin/EDTA (Cellgro #25-052-C1), pelleted, and resuspended at $3x10^7$/ml in PBS. 100 ul of cell suspension ($3x10^6$ cells) was implanted subcutaneously in the right flank of female athymic Ncr-nu/nu mice (Taconic – NCI stock, 6 – 10 weeks of age). 10 - 14 days after implantation, tumor volume reached at average of 250-350 mm^3 (volume determined by caliper measurement, using the formula volume = length x width2 / 2). Mice with tumors of the appropriate size were selected and randomized into groups (n=4 per group) of similar average volume.

Compounds were administered by oral gavage, at a volume of 10 ml/kg, in a dosing vehicle containing 1% methyl cellulose and 1% Tween-80. At the appropriate timepoints after compound administration, mice were euthanized by CO_2 inhalation and tumor and blood collected for analysis. Blood was collected by cardiac puncture, using sodium citrate as an anticoagulant, and plasma collected and stored at –20°C until analysis. Quantitation of compound in plasma was carried out as described in the rodent PK section. Tumors were excised, flash frozen in liquid nitrogen, and stored at –80°C until analysis for levels of phospho-ERK and total ERK.

Analysis of phospho-ERK and total ERK in tumor lysates. All steps in preparation of tumor lysate were carried out at 4°C. Tumor fragments were mixed with an equal volume of lysis buffer (50mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.05% SDS, 2mM EDTA) with 1% of each of the following phosphatase and protease inhibitors: Sigma #P8340, Sigma # P2850, Sigma #P5726. Samples were homogenized using a Tissue Tearor Model 398 tissue homogenizer (Biospec Products). The homogenized lysate was centrifuged for 10 min at 18,000 x g and the supernatant

collected. Protein concentration of the lysate supernatant was determined using the Comassie Plus protein assay (Pierce #1856210). Lysate was stored at –80^{o}C

Samples for gel analysis contained the volume of lysate supernatant needed for 150ug of protein diluted to 65ul with lysis buffer, plus 25ul of 4X gel loading buffer (NuPAGE LDS sample buffer, Invitrogen #NP0007) and 10ul of beta-mercaptoethanol. Samples were heated to 65^{o}C for 10 min prior to loading onto gels.

Fifteen ul of the above samples (containing 22.5 ug of total protein) were loaded onto 12-well NuPAGE precast 10% bis-tris gels (Invitrogen #NP0302). Electrophoresis was carried out in a NOVEX X-cell gell apparatus (Invitrogen), using conditions and buffers recommended by the supplier. After completion of electrophoresis, proteins were transferred to nitrocellulose membrane using a NOVEX transblot apparatus, using conditions and buffers recommended by the supplier.

After completion of electrophoresis, nitrocellulose membranes were washed 2X in TBST pH 8.0, followed by blocking in Odyssey blocking buffer (Licor Biosciences #927-40000) at room temperature for 1h or overnight at 4^{o}C. Membranes were then incubated with phospho-p44/42 MAPK (thr202/tyr204) antibody (Cell Signaling Technology #9101) diluted 1:1000 in Odyssey blocking buffer with 0.1% Tween-20, for either 1h at room temperature or 4^{o}C overnight. Membranes were then washed 3X with TBST, followed by incubation with Alexa-fluor 680 conjugated goat anti-rabbit IgG (Molecular Probes #A-21109), diluted 1:2000 as above, for 1h at room temperature. Membranes were then washed 2X with TBST, followed by a final wash with TBS pH 8.0.

Membrane images were captured using a Licor Odyssey imaging system (Licor Biosciences), with image capture on the 700 nm channel. Quantitation of band intensity was carried out using Odyssey version 1.2 software (Licor Biosciences).

Total ERK was quantitated on the same membranes. Dilutions and washes were as described above, using ERK-1 rabbit polyclonal IgG (Santa Cruz Biotechnology #K-23), IRdye 800 conjugated affinity purified goat anti-rabbit IgG (Rockland Immunochemicals #611-132-122), and Licor image capture on the 800 nm channel.

Data analysis. Each gel contained vehicle control samples. The phospho-ERK / total ERK signal ratio was calculated for each sample. These values were then normalized to the average vehicle control value for that gel, and reported as % of control value (mean and standard error for each treatment group and timepoint).