Discovery of RAF265: A Potent mut-B-RAF Inhibitor for the Treatment of Metastatic Melanoma

Teresa E. Williams, Sharadha Subramanian, Joelle Verhagen, Christopher M. McBride, Abran Costales, Leonard Sung, William Antonios-McCrea, Maureen McKenna, Alicia K. Louie, Savithri Ramurthy, Barry Levine, Cynthia M. Shafer, Timothy Machajewski, Paul A. Renhowe, Brent A. Appleton, Payman Amiri, James Chou, Darrin Stuart, Kimberly Aardalen, and Daniel Poon*

Global Discovery Chemistry, Oncology and Exploratory Chemistry, Novartis Institutes for Biomedical Research
5300 Chiron Way, Emeryville, California 94608, USA

Supporting Information

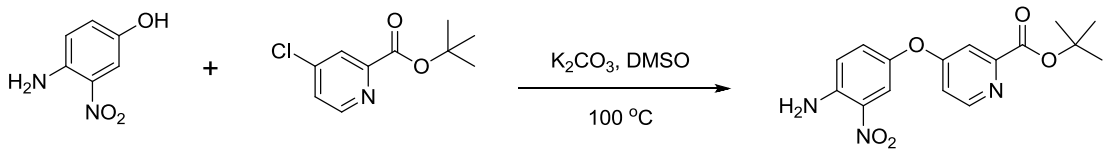
Contents

General Methods. All reagents and solvents were of commercial quality and used without further purification. Column chromatography was performed using Merck silica gel 60 (230-400 mesh). The compounds and/or intermediates were characterized by high performance liquid chromatography (HPLC) using a Waters Millenium chromatography system with a 2695 Separation Module (Milford, MA). The analytical columns were reversed phase Phenomenex Luna C18 -5 μ, 4.6 x 50 mm, from Alltech (Deerfield, IL). A gradient elution was used (flow 2.5 mL/min), typically starting with 5% acetonitrile/95% water and progressing to 100% acetonitrile over a period of 10 minutes. All solvents contained 0.1% trifluoroacetic acid (TFA). Mass spectrometric analysis was performed according to two different liquid chromatography / mass spectroscopy (LCMS) methods. Method A employed a Waters System (Alliance HT HPLC and a Micromass ZQ mass spectrometer for the LCMS instrument, an Eclipse XDB-C18, 2.1 x 50 mm for the chromatography column, and a solvent system that was a 5-95% gradient of acetonitrile in water with 0.05% TFA over a 4 min period (flow rate 0.8 mL/min; molecular weight range 200-1500; cone Voltage 20 V; column temperature 40 °C). Method B employed a Hewlett Packard System Series 1100 HPLC and a Micromass ZQ mass spectrometer for the LCMS instrument, an Eclipse XDB-C18, 2.1 x 50 mm for the chromatography column, and a solvent system that was a 5-95% gradient of acetonitrile in water with 0.05% TFA over a 4 min period (flow rate 0.8 mL/min molecular weight range 150-850; cone Voltage 50 V; column temperature 30 ^{o}C). All masses were reported as those of the protonated parent ions. HR-MS data employed a Waters Synapt G2 QTof Mass spectrometry. Ionization mode: ESI positive, cone voltage: 25V, capillary voltage: 3.0 kV, desolvation temperature: 350 ^{o}C, acquisition range: 100 to 2000 m/z, Scan time: 0.2s. Gas chromatography / mass spectroscopy (GCMS) analysis was performed on a Hewlett Packard instrument (HP6890 Series gas chromatograph with a Mass Selective Detector 5973; injector volume: 1 μL; initial column temperature: 50 ^{o}C; final column temperature: 250 ^{o}C; ramp time: 20 min; gas flow rate: 1 mL/min; column: 5% phenyl methyl siloxane, Model No. HP 190915-443, dimensions: 30.0 m x 25 m x 0.25 m).

^{1}H and ^{13}C NMR spectra of all compounds were recorded at 300 and 75 MHz, respectively. ^{1}H shifts are referenced to the residual protonated solvent signal (δ 7.25 for $CDCl_3$) and ^{13}C shifts are referenced to the deutrated solvent signal (δ 77 for $CDCl_3$). All compounds where biological data is presented have >95% purity as determined by HPLC and were prepared in similar manner as described for **1**.

Synthesis of RAF265 (1)

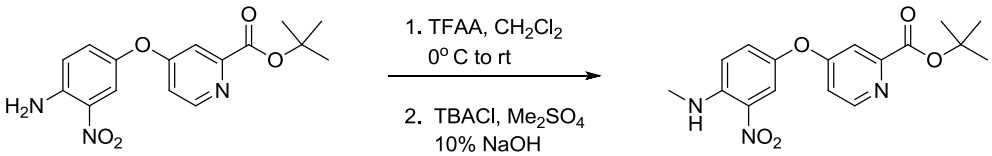
***tert*-butyl 4-(4-amino-3-nitrophenoxy)picolinate (6):**



5 **6**

A 500 ml three-neck flask was fitted with a mechanical stirrer and charged with K_2CO_3 (4.15 g, 30 mmol). The vessel was sealed, evacuated, and flame dried. The apparatus was allowed to cool to rt and purged with argon. To the reaction flask was added 4-amino-3-nitrophenol 1 (3.08 g, 20 mmol), *tert*-butyl 4-chloropyridine-2-carboxylate **3** (5.2 g, 24 mmol) and dry DMSO (30 mL). The resulting mixture was stirred vigorously and heated to 100 ^{o}C for ~14 h. The reaction was poured over iced phosphate buffer (pH = 7), and the reaction flask was rinsed well with MTBE and water. The combined biphasic mixture was filtered through Celite. The collected filtrate was partitioned and separated, and the aqueous phase was extracted with MTBE (3 X 100 mL). The combined organic layers were washed with water (5 X 100 mL), dried ($MgSO_4$), and evaporated. The crude residue was adsorbed onto SiO_2 and purified by flash chromatography (4:1, 2:1, 1:1 hexanes-EtOAc) to furnish 4.92 g (14.9 mmol, 74% yield) of **6** as a yellow brown solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 (d, *J* = 5.8 Hz, 1 H), 7.90 (d, *J* = 2.8 Hz, 1 H), 7.56 (d, *J* = 2.5 Hz, 1 H), 7.17 (dd, *J* = 2.8, 8.8 Hz, 1 H), 6.94 (dd, *J* = 2.8, 5.8, Hz, 1 H), 6.91 (d, *J* = 9.1 Hz, 1 H), 6.15 (br s, 2 H), 1.62 (s, 9 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.8, 164.0, 151.8, 151.5, 143.4, 143.2, 131.5, 129.8, 121.0, 118.0, 114.2, 113.1, 83.0, 28.4; mp 163-166 ^{o}C.

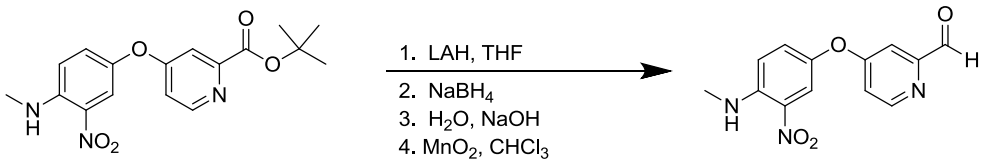
***tert*-butyl 4-(4-(methylamino)-3-nitrophenoxy)picolinate (7):**



6 **7**

To a solution of the nitroaniline **6** (5.62 g, 17 mmol) in CH_2Cl_2 (85 mL) at 0 ^{o}C was added TFAA (2.4 mL, 3.6 g, 17 mmol). The cooling bath was then removed, and the reaction maintained at rt for 2 h. The reaction was cooled to 0 ^{o}C and TBACl (2.5 g, 8.5 mmol), Me_2SO_4 (3.2 mL, 4.3 g, 34 mmol), and 10% NaOH (34 mL) were added. The resulting mixture was stirred vigorously for 4 h at rt. The reaction was diluted with water, and the resulting layers were partitioned and separated. The aqueous phase was extracted with CH_2Cl_2 (3 X 100 mL), and the combined organic layers were washed with brine (2 X 100 mL), dried ($MgSO_4$), and evaporated. The crude residue was adsorbed onto SiO_2 and purified by flash chromatography (4:1, 2:1, 1:1, 1:2 hexanes-EtOAc) to give 4.5 g (13.0 mmol, 76%) of **7** as a yellow-orange solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (d, *J* = 5.5 Hz, 1H), 8.04 (br d, *J* = 4.7 Hz, 1 H), 7.93 (d, *J* = 2.8 Hz, 1 H), 7.53 (d, *J* = 2.5 Hz, 1 H), 7.25 (app dd, *J* = 2.8, 9.1 Hz, 1 H), 6.91 (m, 2 H), 3.04 (d, *J* = 4.9 Hz, 3 H), 1.59 (s, 9 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.9, 164.1, 151.5, 144.7, 142.1, 130.4, 118.8, 115.5, 114.1, 112.9, 82.9, 30.4, 28.5; mp 187-189 ^{o}C.

(4-(4-(methylamino)-3-nitrophenoxy)pyridin-2-yl)methanol:

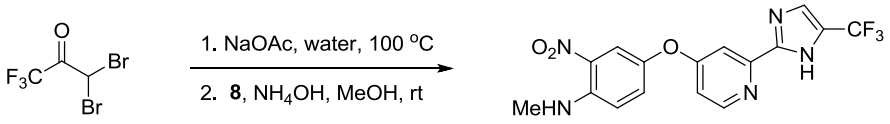


7 **8**

A flame dried 500 mL three necked round bottom flask purged with N_2 was charged with LAH (3.0 g, 75 mmol) and dry THF (240 mL). The resulting suspension was cooled to 0 °C, and *t*-butyl ester **7** (20.7 g, 60 mmol) was slowly added while keeping the internal reaction temperature under 5 °C. The reaction mixture was stirred at 0 °C for 2 h followed by stirring at rt overnight. $NaBH_4$ (2.27 g, 60 mmol) was added, and the reaction mixture was stirred for an additional hour at rt. After the reaction was judged complete, the reaction mixture was treated with successive dropwise addition of water (3 mL), 15% NaOH (3 mL), and water (9 mL). The resulting mixture was filtered through Celite, and the remaining solids were washed with EtOAc and methanol. The combined organic portions were evaporated and the resulting crude residue was adsorbed onto SiO_2 and purified by flash chromatography (97:3 CH_2Cl_2-MeOH) to afford 7.63 g (27.7 mmol, 46%) of a red-orange solid as (4-(4-(methylamino)-3-nitrophenoxy)pyridin-2-yl)methanol: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40 (d, *J* = 5.5 Hz, 1 H), 8.05 (br s, 1H), 7.96 (d, *J* = 2.75 Hz, 1 H), 7.29 (d, *J* = 2.75 Hz, 1 H), 6.92 (d, *J* = 9.35 Hz, 1 H), 6.75 (m, 2 H), 4.68 (s, 2 H), 3.07 (d, *J* = 5.23 Hz, 3 H).

A 100 mL round bottom flask was charged with (4-(4-(methylamino)-3-nitrophenoxy)pyridin-2-yl)methanol (1.38 g, 5.0 mmol), MnO_2 (6.52 g, 75 mmol) and $CHCl_3$ (20 mL). The resulting suspension stirred at rt for 2 d. The reaction mixture was filtered through Celite, and the remaining solids were washed successively with $CHCl_3$ and EtOH. The combined organic portions were evaporated, absorbed onto SiO_2, and purified by flash chromatography (98:2 CH_2Cl_2-MeOH) to give 790 mg (2.89 mmol, 58%) of an orange solid as **8**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.01 (s, 1 H), 8.64 (d, *J* = 5.5 Hz, 1 H), 8.09 (br s, 1 H), 7.96 (d, *J* = 2.75 Hz, 1 H), 7.37 (d, *J* = 2.48 Hz, 1 H), 7.29 (d, *J* = 2.75 Hz, 1 H), 7.08 (dd, *J* = 2.47, 5.5 Hz, 1 H), 6.94 (d, *J* = 9.35 Hz, 1 H), 3.08 (d, *J* = 5.23 Hz, 3 H).

***N*-methyl-2-nitro-4-((2-(5-(trifluoromethyl)-1H-imidazol-2-yl)pyridin-4-yl)oxy)aniline (9):**

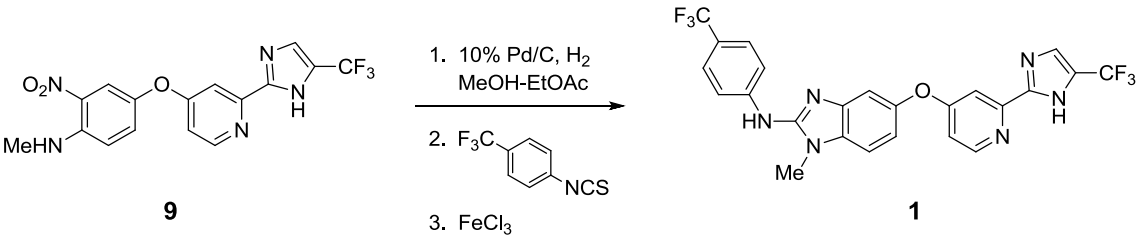


9

Dibromo-3,3,3-trifluoroacetone (25.75 mL, 136.5 mmol) was added to a solution of NaOAc (22.4 g, 273 mmol) in water (60 mL) and the resulting solution heated to 100 °C for 40 min. After cooling to rt, the solution was added to a suspension of **8** (25 g, 91 mmol) in 28% aqueous NH_4OH solution (150 mL) and MeOH (450 mL). The resulting mixture was stirred at rt overnight. TLC (95:5 CH_2Cl_2-MeOH) showed complete consumption of **8**. The crude reaction mixture was concentrated into an aqueous slurry, and partitioned between saturated aqueous Na_2CO_3 solution and CH_2Cl_2. The resulting layers were separated. The aqueous phase was extracted with CH_2Cl_2 (3X), and the combined organic portions were washed with brine, then

dried ($MgSO_4$), and concentrated to give 31.6 g of **9** (83 mmol) as an orange solid (91 % yield). No further purification was required.

1-methyl-5-((2-(5-(trifluoromethyl)-1H-imidazol-2-yl)pyridin-4-yl)oxy)-N-(4-(trifluoromethyl)phenyl)-1H-benzo[d]imidazol-2-amine (1):



A slurry of nitroaniline **9** (45.76 g, 120 mmol) in MeOH (220 mL) and EtOAc (200 mL) was sparged with N_2 for 20 min, and then charged with a suspension of 10 % Pd/C (12.77 g, 120 mmol) in MeOH (60 mL). The reaction was purged with H_2 and maintained under a H_2 atmosphere for 2 d. The reaction was filtered through Celite and the remaining solids were washed successively with MeOH and EtOAc. The combined organic filtrates were evaporated, and the resulting solid was dried overnight under vacuum to give 40.17 g (115 mmol) of N^1-methyl-4-((2-(5-(trifluoromethyl)-1H-imidazol-2-yl)pyridin-4-yl)oxy)benzene-1,2-diamine as a tan solid (96% yield). LCMS m/z 336.1 (MH+), t_R = 1.81 min.

4-Trifluoromethylphenyl isothiocyanate (23.37 g, 115 mmol) was added to a stirring solution of N^1-methyl-4-((2-(5-(trifluoromethyl)-1H-imidazol-2-yl)pyridin-4-yl)oxy)benzene-1,2-diamine (40.17 g, 115 mmol) in MeOH (460 mL) at rt. The reaction was maintained at rt for 16 h. After the reaction was judged complete, a solution of $FeCl_3$ (20.52g, 126.5 mmol) in MeOH (50 mL) was added to the reaction, and the resulting mixture was stirred at rt overnight. The crude reaction mixture was partitioned between EtOAc and water, and the resulting layers separated. The aqueous phase was extracted with EtOAc, and the combined organic layers were washed with saturated aqueous Na_2CO_3 solution, water, and brine, then dried ($MgSO_4$) and concentrated. The aqueous phase was made basic (pH = 10) by addition of saturated aqueous Na_2CO_3 solution, and the resulting slurry was combined with EtOAc. The resulting mixture was agitated, and the resulting emulsion was filtered through filter paper.The resulting layers were then separated. The aqueous portion was extracted with EtOAc (2 X). The organic layers were combined, washed with brine, dried ($MgSO_4$) and concentrated with the previously extracted material. The combined product was triturated with CH_2Cl_2 (500 mL), adsorbed onto SiO_2 and purified by flash chromatography (100:0, 99:1, 98:2, 97:3, 96:4 CH_2Cl_2-MeOH). A final trituration of material with CH_2Cl_2 produced **1** as a colorless solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (d, J = 5.5 Hz, 1 H), 7.75 (d, J = 8.8 Hz, 2H), 7.61 (dd, J = 2.2, 8.5 Hz, 1 H), 7.59 (d, J = 8.8 Hz, 2 H), 7.56 (d, J = 2.5 Hz, 1 H), 7.38 (app d, J = 8.5 Hz, 1 H), 7.23 (d, J = 1.9 Hz, 1 H), 6.96 (dd, J =

2.2, 8.5 Hz, 1 H), 6.93 (dd, *J* = 2.5, 5.5 Hz, 1 H), 3.76 (s, 3 H); LCMS m/z = 519.0, t_R = 2.57 min (MH^+); Anal. calc'd for $C_{24}H_{16}F_6N_6O$: C 55.6, H 3.11, N 16.21; Found: C 55.81, H 3.43, N 16.42; mp: 217 – 220 ^oC (dec.).

HRMS Analysis.

Compound	Molecular Formula	Calculated Mass	Mass
1	C24 H17 N6 O F6	519.1368	519.1361
2	C25 H28 N5 O2	430.2243	430.224
3	C25 H28 N5 O2	430.2243	430.2242
4	C26 H27 N6 O	439.2246	439.2242
10	C32 H31 N6 O	515.2559	515.2563
11	C27 H26 N6 O F3	507.212	507.2126
12	C27 H26 N6 O F3	507.212	507.2123
13	C27 H25 N6 O F4	525.2026	525.2027
14	C24 H17 N6 O F6	519.1368	519.137
15	C24 H16 N6 O F7	537.1274	537.1276
16	C24 H19 N6 O F4	483.1556	483.1558

Crystallography Methods

Wild-type BRAF kinase domain (residues 445-723) was expressed using the baculovirus expression system, purified by Ni-NTA chromatography and concentrated to 2 mg/mL in 20 mM Bis-Tris propane pH = 7.0, 15% glycerol, 1 mM TCEP and 0.1% CHAPS. Crystals were grown by the vapor diffusion method at 4 ℃ using equal volumes protein incubated with 0.1 mM compound for 30 minutes and a reservoir solution composed of 12% PEG8000, 100 mM NaCl, and 100 mM Tris pH 8.4. A protein crystal was transferred to a cryosolution of 15% PEG8000, 20% glycerol and 100 mM Tris pH 8.4. Data were collected at beam-line 5.0.2 at the Advanced Light Source (Berkeley, CA) and processed with autoPROC from the Global Phasing package (1). The structure was refined with BUSTER (2) with iterative model building using COOT (3).

(1) Vonrhein, C., Flensburg, C., Keller, P., Sharff, A., Smart, O., Paciorek, W., Womack, T. & Bricogne, G. (2011).Data processing and analysis with the autoPROC toolbox. Acta Cryst. D67, 293-302.

(2) Smart OS, Womack TO, Flensburg C, Keller P, Paciorek W, Sharff A, Vonrhein C, Bricogne G. (2012). Exploiting structure similarity in refinement: automated NCS and target-structure restraints in BUSTER. Acta Cryst D68, 368-80.

(3) "Coot: model-building tools for molecular graphics" Emsley P, Cowtan K Acta Crystallographica Section D-Biological Crystallography 60: 2126-2132 Part 12 Sp. Iss. 1 DEC 2004.

Assays

***In Vitro* Kinase and Cell Based Assays.** The biochemical assays have been previously reported in the literature and denoted in the manuscript References 6 and 7. The cell based target modulation and proliferation assays have been reported in the literature and is denoted in the manuscript References 5 and 9.

***In Vitro* Cell Assays from Figure 4**. The MV-4-11, Malme-3M, SW620, KM12, WM1799 and A375 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Unless otherwise indicated, all media and supplements were obtained from Mediatech, Inc. (Herndon, VA). SW620 cells were cultured in DMEM. A375M cells were cultured in McCoy's 5A medium; MV-4-11 cells were cultured in Iscove's modified DMEM. KM12, A375, Malme-3M cells were cultured in RPMI-1640. All media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1X MEM vitamins, 1X MEM amino acids and 1X MEM nonessential amino acids. Media for MV-4-11 cells was further supplemented with 4 mM L-glutamine and 10 ng/ml rhGM-CSF (R&D Systems, Minneapolis, MN). The assays were conducted in a manner similar to those reported in References 7 and 8.

In Vitro CYP Inhibition Assays. Inhibition of human cytochrome P450 (CYP) enzyme activity was assessed using pooled human liver microsomes. Pre-incubation times (0-48 min) and concentrations (0-50 μM) of compound were tested on the *in vitro* metabolism of several probe substrates whose metabolism is known to be cytochrome P450 isoenzyme-selective. Probe substrate metabolism was assessed by LC-MS/MS analysis of specific metabolite formation. CYP1A2 (phenacetin: *O*-de-ethylation), CYP2C9 (diclofenac: 4'-hydroxylation), CYP2D6 (bufuralol: 1'-hydroxylation) and CYP3A4 (midazolam: 1'-hydroxylation). Cassette analysis by LCMS in positive ion mode. Area ratio of the substrate metabolite to internal standard (IS) is determined. Then it is compared to the area ratio of substrate metabolite/IS at 0 uM compound (100% activity).

CYP Isoform Data for 1 in IC_{50}: CYP3A4 (midazolam, > 40 μM), CYP3A4 (testosterone, > 40 μM), CYP1A2 (phenacetin, > 40 μM), CYP2C9 (diclofenac, 3.4 μM), CYP2D6 (bufuralol, 1.7 μM).

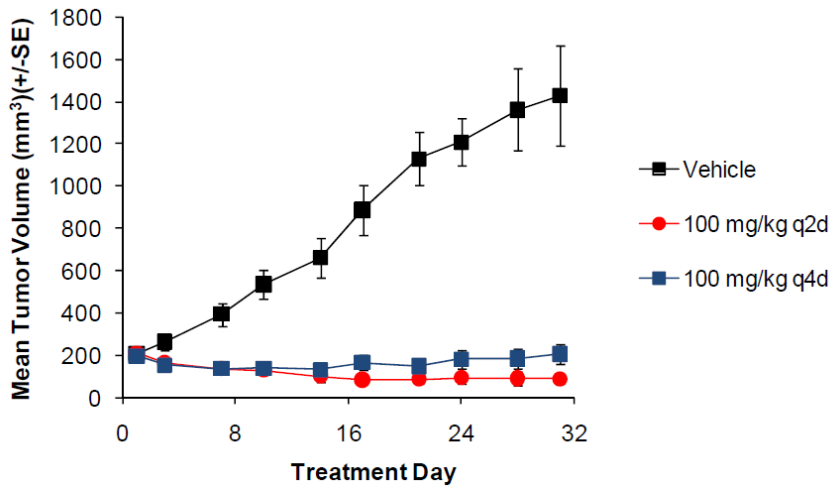
***In Vivo* Studies.** Female nu/nu or SCID-NOD mice (7-12 weeks) from Charles River Laboratories (Wilmington, MA) were housed in a barrier facility in sterile filter-top cages with 12-hour light/dark cycles and received sterile rodent chow and water *ad libitum*. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals (National Research Council). For tumor xenograft studies, mice were implanted with 3 x 106 A375M cells subcutaneously (s.c.) in the right flank. Approximately fourteen days after implantation when tumors reached the volumes indicated on day 1 of each graph, mice were randomized into groups based on tumor volume and treatment was initiated. Tumors were measured using electronic calipers, and volume was determined using the following formula: ½ (L x W x W). Compound **1** was formulated in 100% PEG400 for xenograft studies.

For tumor pharmacodynamic analyses, tumors were excised and pulverized over liquid nitrogen. Frozen tumor powder was thawed briefly, re-suspended in RIPA lysis buffer and homogenized on ice. Protein concentration was determined using a BCA protein assay (Pierce Biotechnology, Rockford, IL).

SDS-PAGE was carried-out on cell lysate or tumor homogenate using 10% or 4-20% gradient Tris-glycine gels (Invitrogen, Carlsbad, CA) using equal amounts of protein, then transferred to nitrocellulose membranes. Membranes were placed in blocking buffer (5% non-fat milk powder in Tris buffered saline containing 0.1% Tween-20 (TBST)) for 1 hour at room temperature and probed overnight at 4 °C using a rabbit polyclonal anti-phospho-MEK1/2 (Ser 217/221) antibody (Cell Signaling, Danvers, MA). Western blots for these targets were normalized using a mouse anti-actin antibody (Sigma, St. Louis, MO).

Efficacy and PKPD Evaluation of 1 in A375M Xenograft Model from Figure 5. Mice bearing A375M tumor xenografts were randomized by tumor volume (~200 mm^3) and then treated by oral gavage with vehicle (100% PEG-400), or RAF265 (**1**) at 100 mg/kg q2d or q4d. Tumor volume was measured twice weekly (n = 10/group). Tumors from mice treated using the same dosing regimen were harvested from mice at various time points following three doses of 100 mg/kg on a q4d regimen and lysates prepared for Western blot analysis of phospho-MEK. Plasma samples were collected from mice treated on q2d and q4d regimen and analyzed for RAF265 concentration using a liquid chromatography/mass spectroscopy assay. The efficacy results from this study are shown in Figure A.

Figure A. Efficacy of **1** in a 32 Day A375M Mouse Xenograft Study



Efficacy and PD of 1 in A375 Xenograft from Figure 6.
Tumor growth inhibition and modulation of mutant BRAF and downstream markers in A375M xenografts. Mice bearing A375M tumor xenografts were randomized by tumor volume (~ 150 mm3) and then treated by oral gavage with vehicle (100% PEG-400), or RAF265 at 10, 30 or 100 mg/kg q2d. Tumor volume was measured twice weekly (n = 10/group). Tumors from

mice treated using the same dosing regimen were harvested at four hours following the third dose and lysates prepared for Western blot analysis of phospho-MEK. Efficacy , PD, and body body weight change are shown in Figure B.

Figure B. Efficacy , PD, and Body Weight Change of **1** in a 28 Day A375M Mouse Xenograft Study