Quantification of a Pharmacodynamic ERK Endpoint in Melanoma Cell Lysates: Towards Personalized Precision Medicine.

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SUPPLEMENTARY

FIGURES

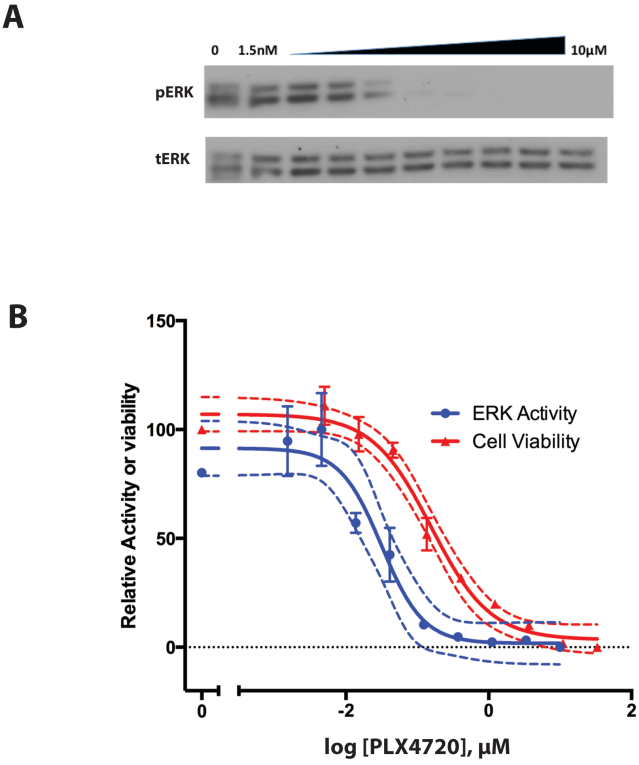


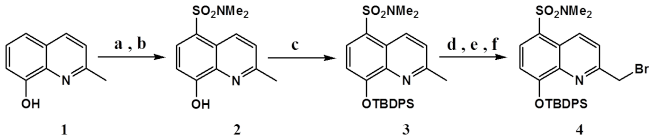
Figure S1. Correlating ERK activity to melanoma cell viability. **A)** Dose-dependent inhibition of ERK in BRAF-mutant A375 melanoma cells treated with PLX4720 by western blot. **B**) Correlation of the degree of ERK inhibition (measured by the sensor) to the inhibition of cell proliferation (Cell Titer Glo).

EXPERIMENTAL PROCEDURES

Reagents - NovaSyn TGR resin was purchased from Novabiochem (Gibbstown, NJ). Fmoc-6-aminohexanoic acid was purchased from AnaSpec (Fremont, CA). Other Fmoc-amino acids, HBTU, and HOBT were obtained from Advanced ChemTech (Louisville, KY). Ultrapure grade Tris and HEPES were obtained from Sigma (St. Louis, MO). MP Biomedicals (Irvine, CA) supplied [γ-^{32}P]-ATP. P81 Ion Exchange Cellulose Chromatography Paper was purchased from Whatman (Piscataway, NJ). The remaining molecular biology reagents, including protein molecular mass standards, were obtained from Invitrogen Corp. All other buffer components and chemicals were obtained from Sigma. A375 cells were obtained from Michael Davies, MD, PhD – MD Anderson Cancer Center Dept of Melanoma Medical Oncology. Mel1617 and 4105lu resistant and sensitive pairs were generously provided by the Heryln lab.[1]

Preparation of Proteins - Activated (Tagless) ERK2 was generated essentially as described previously.[2] Human JNK2α2 was expressed, purified and activated as described previously.[3] Active N-terminal His tagged p38MAPKα, p38MAPKβ, p38MAPKγ, and p38MAPKδ were generated according to the previously published procedure with the modification of 8h longer activation time for p38MAPKβ.[4] Expression and purification of Ets-1 (1-138) was followed by the method described in the previously published literature.[5] GST-ATF2 (1-115) was expressed and purified as previously reported.[4]

Peptide Synthesis and Purification - Sub-D was synthesized as reported earlier.[6] ERK-sensor-D1 was generated after alkylation of resin bound peptides with Sox-Br (2-bromomethyl-8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamide quinolone). Sox-Br was synthesized according to the following Scheme according to the reported procedure[7] with several modifications:



[a]Reagent and conditions: (a) Chlorosulfonic acid, 0 °C, 2h, 85%; (b) 2M dimethylamine in THF, rt, 8h, 90 %; (c) tert-butylchlorodiphenylsilane, NaH/THF, reflux, 4h, 90%; (d) Selenium dioxide/dioxane, 95 °C, 14h (e) $NaBH_4$, $EtOH/CH_2Cl_2$, 0 °C, 15 min (f) Carbontetrabromide, PPh_3/diethyl ether, 0 °C, 28% over three steps.

Briefly, 8-hydroxyquinaldine **1** was first converted to the sulfonamide **2** as reported.[8] Hydroxyl protected sulfonamide **3** was obtained with improved yield by treatment of **2** with tert-butylchloro-diphenylsilane using sodium hydride as the base. While methyl group of hydroxyl protected sulfonamide **3** was oxidized and reduced to obtain hydroxyl methyl quinoline as reported [8], Sox-Br **4** was obtained by treatment of hydroxyl methyl sulfonamide with carbontetrabromide and triphenylphosphine**.** Sox-Sub-D and Sox-Sub-F were generated after alkylation of resin bound peptides with Sox-Br (2-bromomethyl-8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamide quinolone) as reported.[8]

Detailed methods for steps a-f.

a) Synthesis of (2-bromomethyl-8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamidoquinoline) – 8-Hydroxy-2-methyl-quinoline-5-sulfonyl chloride**.** 8-hydroxy-2-methyl-quinoline (2.0g, 12.6 mmol) was dissolved in chlorosulfonic acid (10 mL) and stirred at 0 °C for 2 hours. The reaction mixture was added to a separation funnel containing dichloromethane (DCM 400 mL) followed by a slurry of ice in brine (400 mL). The layers were shaken 10-20 seconds and the DCM layer poured into a flask containing a bed (10 g) of anhydrous potassium carbonate. The resulting solution was filtered and the solvent evaporated *in vacuo* to obtain the crude product as a yellow powder (2.75 g, 85%), which was used in the next step without further purification.

b) Synthesis of 8-Hydroxy-2-methyl-quinoline-5-(N,N-dimetyl)sulfonamide. N,N-dimethylamine in dry tetrahydrofuran (THF, 2M, 100 mL) was dissolve in THF (500 mL). 8-Hydroxy-2-methyl-quinoline-5-sulfonyl chloride (12g, 46.5 mmol in 100 mL THF) was added to the N,N-dimethylamine in THF dropwise over 3 hours. After adding another portion of N,N-dimethylamine (20 mL , 2M in THF) the reaction mixture was stirred a further hour. After removing the solvent in vacuo excess N,N-dimethylamine was removed by dissolving the residue in DCM (100 mL x 3) and rotary evaporation. After purification by flash chromatography (ethylacetate/hexane 1:2) the product was obtained as an off-white powder (11.14 g, 90%) $R_f = 0.35$.

c) 8-tert-Butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamide-2-methylquinoline. 8-Hydroxy-2-methyl-quinoline-5-(N,N-dimetyl)sulfonamide (6 g, 22.53 mmol) was dissolve in 30 mL of anhydrous THF and brought to 0 °C. Sodium hydride (983 mg 24.78 mmol) was added slowly and stirred for 1 hour. tert-Butyldiphenylsilyl chloride (17.92 mL, 67.6 mmol in 30 mL of THF) was slowly added to the mixture and stirred for one hour. The reaction mixture was stirred for one hour and heated at 55 °C overnight. The solvent was removed in vacuo and the residue dissolved in hexane/ethyl acetate (2:1). The mixture was filtered through celite to remove insoluble material. After purification by flash chromatography (ethyl acetate/hexane 1:7) the product was obtained as a white powder (90%). R_f = 0.5 (silica, ethyl acetate/hexane, 1:4).

d) 8-tert-Butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamide-2-formylquinoline. 8-tert-Butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamide-2-methylquinoline (3 g, 5.95 mmol) was dissolved in 50 mL of anhydrous dioxane and molecular sieves added (4 Å, 1 g). Selenium dioxide (750 mg, 6.75 mmol) was added and the reaction stirred at 95 °C overnight. The reaction mixture was brought to room temperature and filtered through celite to remove a black residue and molecular sieves. The celite was washed with dioxane (60 mL, until no chromophore was detected by UV spotting on TLC plates). The dioxane was removed by rotary evaporation and the resulting yellow oil (3 g) used for the next step without further purification.

e) 8-tert-Butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamide-2-(hydroxymethyl) quinoline. 8-tert-Butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamide-2-formylquinoline (3.59 g) in DCM (90 mL) was added dropwise to a solution of sodium borohydride in ethanol (1g in 45 mL) at 0 °C. The reaction mixture was stirred for 30 minutes then diluted with diethyl ether (300mL), washed with saturated ammonium chloride (200 mL), water (2 x 100 mL), brine (100 mL) and dried with anhydrous magnesium sulfate ($MgSO_4$). A pale yellow sticky solid (2.87 g) was obtained after removing the solvent in vacuo. This material was used in the subsequent step without purification.

f) 2-Bromomethyl-8-tert-butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamide quinoline. 8-tert-Butyldiphenylsilyloxy-5-(N,N-dimethyl)sulfonamide-2-(hydroxymethyl)quinoline (2.3 g) was dissolved in diethyl ether (40 mL) and brought to 0 °C. Carbon tetrabromide (1.52 g, 4.5 mmol) and triphenylphosphine (1.14 g, 4.35 mmol) were added and the reaction mixture stirred for 36 hours at 0 °C. A yellow color precipitate was filtered off. The solvent was removed from the filtrate under reduce pressure. The product was obtained as a clear oil after purifying by flash column chromatography with hexane/ethyl acetate as the solvent (9:1, 750 mg, 28 % after three steps). Compounds were characterized by ^1H and ^{13}C NMR and compared with previously published data.[8]

Alkylation of peptides with Sox-Br – Peptides were synthesized on rink resin (NovaSyn TGR resin) using a solid phase peptide synthesizer (Liberty CEM Automated Microwave Peptide Synthesizer, or a Rainin Quartet Peptide Synthesizer) by utilizing an Fmoc solid-sate peptide synthesis protocol as reported.[6] The resin with bound peptide (500 mg, 0.095 mmol, 1 equiv.) incorporating Cys(Mmt) was swelled in DMF (30 min each). The Mmt protecting group was removed from the resin-bound peptide by bubbling argon through a solution of 1% TFA, 5% TIS in CH_2Cl_2 (until most of the yellow color due to the Mmt cation had disappeared). The resin was then subjected to rigorous washing with CH_2Cl_2 (5×) and DMF (5×). Anhydrous DMF (200 µL) was added to the resin followed by freshly distilled tetramethylguanidine (59.6 µL, 0.475 mmol, 5 equiv.). The mixture was incubated for 2-3 min. Sox-Br (170 mg, 0.285 mmol, 3 equiv.) was dissolved in anhydrous DMF (1.5 mL) and added to the resin. After 12 hours of shaking under argon, the excess reagents were drained and the resin was washed with DMF, CH_2Cl_2, MeOH and ether (5 x each). The sox modified peptide was cleaved by exposing the resin-bound peptide to TFA/H_2O/TIS (95:2.5:2.5% v/v) for 3h. Peptides were purified as reported [6] with UV detection at 214 nm (amide bond absorption) and 316 nm (Sox absorption). Peptide products were characterized using an ESI (LCQ, Thermo Finnigan). Concentrations of the peptide solution was determined by UV-Vis as reported earlier[9] (based on the determined extinction coefficient of the fluorophore unit, 5-(N,N-dimethylsulfonamido)-8-hydroxy-2-methylquinoline, $\varepsilon_{355} = 8247\ M^{-1}\ cm^{-1}$ at 355 nm in 0.1 M NaOH with 1 mM Na_2EDTA).

Preparation of cell lysates - HEK293T were maintained in DMEM (Invitrogen) supplemented with 10% (v/v) FBS-US grade (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U mL^{-1} penicillin (Sigma), and 100 g mL^{-1} streptomycin (Sigma). Cells were cultured in a humidified 5% CO_2 incubator at 37 °C. Cells were seeded in a 15 mm plate and incubated until 90% confluence.

Then the cells were serum starved for 24 hours before incubation with or without inhibitors including U0126 (Tocris Biosciences), BIRB796 (Selleck Chemicals), SCH772984 (Chemscene) and JNK-IN8 (synthesized in house according to a previously published synthetic route[10]) for 1 hour then cells were treated with/without phorbol-12 myristate 13-acetate (PMA) (10 nM) (MP Biomedicals) for 10-15 min and lysed. To lyse cells were washed in PBS (Invitrogen), and lysates were prepared in M-PER Protein Extraction Reagent supplemented with 'Halt Protease and Phosphatase Inhibitor Single-use Cocktail' (Pierce) with gentle shaking for 15 min at 4 °C. The lysates were cleared by centrifugation at 4 °C, and the protein concentration of the supernatant measured by Bradford analysis (Bio-Rad). Melanoma cell lines were propagated in RPMI 1640 (Thermo) supplemented with 5% FBS (Thermo) and 1X Glutamine (Corning). Experiments were plated overnight in 5% FBS. Cells were treated for 1 hour with PLX4720 (Plexxikon) or AZD2644 (Selleck, sold as selumetinib) and lysed in MPER mammalian extraction reagent (Thermo) with 1X Halt Protease Phosphatase inhibitor (Pierce).

Steady-state kinetic experiments - Kinase assays were performed at 28 °C with assay buffer (25 mM HEPES, pH 7.6, 50 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% glycerol, 2.5 mg/mL BSA), enzyme (2 nM of ERK2 or 20 nM JNK2, or 10 nM p38), 5 mM $MgCl_2$ and various concentrations of protein or peptide substrates. Rates were measured under conditions where total product formation represented less than 10% of the initial substrate concentrations. The reaction was incubated for 10 min before initiation by addition of enzyme and quantified as described previously.[11] Initial rates were determined by linear least squares fitting to plots of product against time.

$$\frac{k_{obs}}{k_{cat}^{app}} = \frac{s}{K_{mS}^{app} + s} \quad \text{eqn. 1}$$

$$F = (F_\infty - F_0)\left(1 - e^{-k_{obs}t}\right) + F_0 \quad \text{eqn. 2}$$

The parameters used in deriving equations are defined as follows; k_{obs}, observed rate constant; k_{cat}^{app}, apparent catalytic constant; S, concentration of substrate S; K_{mS}^{app}, apparent Michaelis constant for substrate S; F, fluorescence with time; F_0 , fluorescence of the substrate; F_α , fluorescence of the product; t, time.

Fluorescence assays for substrate kinetics - Kinase assays were performed at 28 °C with assay buffer: 25 mM HEPES, pH 7.6, 50 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% glycerol, 2.5 mg/mL BSA, 10 mM $MgCl_2$, containing various concentrations of peptide substrates with or without inhibitors. 2 nM ERK2, 20 nM JNK2, or 10 nM p38 were used in the assays. The reaction was initiated by adding 10 µL of 5 mM MgATP to 90 µL of assay mixture and fluorescence intensity was taken at each time point. Fluorescence spectra were measured on a Fluorolog fluorometer from Jobin Yvon in quartz cuvettes (Starna Cells) of 10 mm path length with 100 µL reaction volume. The sample was excited at 360 nm and fluorescence was monitored at 492 nm with excitation and emission slit widths of 3 nm. To relate the change in fluorescence to the progression of the reaction, the fluorescence intensity change was divided by the appropriate conversion factor (FU μM^{-1} of product). This conversion factor Sox-Sub-D ($f_{Sox\text{-}sub\text{-}D}$) was determined as reported earlier.[12] The fluorescent spectra of the observed fluorescence change versus time at 40 µM of fluorescent peptide were fitted using equation 2 to obtain $F_{Sox\text{-}sub\text{-}D}$ (72165± 455 FU) (Figure 2B). The conversion factor Sox-Sub-D ($f_{Sox\text{-}sub\text{-}D}$ = 1804 FU μM^{-1}) was obtained after dividing by the peptide concentration (40 µM).

Fluorescence assays for Cell lysates – Fluorescence kinase assay with cell lysate was performed at 28 °C with assay buffer: 25 mM HEPES, pH 7.6, 50 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM $MgCl_2$, 20 µg protein of cell lysate, 0.5 mM ATP and with or without kinase inhibitors (1µM of JNK-IN-8 and 1 µM of BIRB 796). The reaction was performed at 60 µL total reaction volume. The reaction was initiated by adding 20 µM Sox-Sub-D to the assay mixture and the fluorescence intensity was taken for 30 min at each 10 sec time point. The reaction rate was measured by taking the slope of the 30 min data series.

Correlating ERK activity and cell viability - ERK activity: Mel1617 cells were plated in media with 5% FBS overnight and treated with PLX4720 for six hours. Cells were lysed in "DXB lysis buffer" (0.25% 3-(N,N-dimetyl myristyl ammonio) propanesulfonate, 0.25% BrijC10, 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM Sodium pyrophosphate, 50 mM beta-glycerophosphate, 30 mM NaF, 2 mM EGTA, 1X Calbiochem protease inhibitor cocktail III, and 1× Santa Cruz Biotech phosphatase inhibitor cocktail B). This buffer contains surfactants reported to have promise for in vivo isolation of active protein[13]. Total protein loading was normalized using BCA assay (Thermo). 30 µL of 1µg/µL protein was loaded into a low-volume 96-well non-binding white microtiter plate (Corning). A 70 µL final reaction volume was prepared by adding kinase assay buffer (Final: 25 mM HEPES, pH 7.5, 500 µM ATP, 10 mM $MgCl_2$, 2 mM DTT, 20 µM Sox-Sub-D, 1 µM BIRB796, 1 µM JNK-IN-8). Assays were run on a SpectraMax M5e (Molecular Devices) at 28 °C for 1 hour with sampling every minute (Ex: 365, Em: 485). Linear regression was used to quantify the observed rate. Shown are three independent replicates. Sigmoid model fitting was obtained using GraphPad Prism where the dotted lines represent 95% confidence. Cell viability – Mel1617 cells were plated in a black-walled 96-well microtiter plate (Corning) and treated for 72 hours with PLX4720. Viability was assessed with Cell Titer Glo (Promega) according to manufactures instructions. Shown are three independent replicates. Sigmoid model fitting was with GraphPad Prism. Dotted lines represent 95% confidence interval.

Western Blotting – HEK293 cells lysates containing 60 µg of total protein were fractionated on a 12% SDS polyacrylamide gel (Bio-Rad) and transferred to Hybond-P PVDF Membrane (GE Healthcare). Primary antibodies were incubated overnight at 4 °C using 1:2000 anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (E10) mouse mAb (Cell Signaling Technology); 1:1000 anti-p44/42 MAPK (Erk1/2) (137F5) rabbit mAb (Cell Signaling Technology); 1:2000 anti-phospho-SAPK/JNK (Thr183/Tyr185) (G9) mouse mAb (Cell Signaling Technology); 1:10,000 anti-phospho-p38α (Thr180/Tyr182), clone 8.78.8 rabbit mAb (Millipore). Either anti-rabbit (Bio-Rad) or anti-mouse (Cell Signaling Technology) horseradish peroxidase-conjugated secondary antibodies and ECL Plus™ Western Blotting Reagents (GE Healthcare) were used to develop the blots. Melanoma cells lysates containing 15 µg of protein was loaded per well in a 10% polyacrylamide SDS-PAGE gel and run using Tris-Glycine buffer (25 mM Tris, 192 mM Glycine) with 0.1% SDS. Electrophoretic transfer to a PVDF Immunobilon Membrane (Millipore) was performed in Tris-Glycine buffer with 20% ethanol. Primary antibodies for western blotting were from Cell Signaling: rabbit phosphor-ERK (D13.14.4E) and mouse total-ERK (3A7). Secondary antibodies were from Li-Cor: IRDye 680RD goat anti-rabbit

IgG and IRDye 800CW goat anti-mouse IgG. Fluorescent western double staining was developed on the Odyssey fluorescent western system (Li-Cor). All blotting experiments were generally performed a minimum of two times.

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