**Combination therapy with potent PI3K and MAPK inhibitors overcomes adaptive kinome resistance to single agents in preclinical models of glioblastoma**

Robert S. McNeill, Demitra A. Canoutas, Timothy J. Stuhlmiller, Harshil D. Dhruv, David M. Irvin, Ryan E. Bash, Steven P. Angus, Laura E. Herring, Jeremy M. Simon, Kasey R Skinner, Juanita C. Limas, Xin Chen, Ralf S. Schmid, Marni B. Siegel, Amanda E.D. Van Swearingen, Michael J. Hadler, Erik P. Sulman, Jann N. Sarkaria, Carey K. Anders, Lee M. Graves, Michael E. Berens, Gary L. Johnson, and C. Ryan Miller

**SUPPLEMENTAL METHODS**

**Murine astrocyte cultures**. Cortical, genetically engineered mouse (GEM)-derived astrocytes were cultured as previously described.[1-3](#_ENREF_1) Briefly, mice harboring conditional *TgGZT121* (T), *KrasG12D* knock-in (R), and *Pten* knock-out (P) alleles were crossed to generate compound TRP mice.[1](#_ENREF_1),[4-6](#_ENREF_4) Astrocytes harboring the floxed alleles (T/R, heterozygous; P, homozygous) were harvested from neonatal mice. Recombination was induced *in vitro* with a recombinant adenoviral vector encoding cytomegalovirus promoter-driven Cre recombinase (Ad5CMVCre, University of Iowa Gene Transfer Vector Core). TRP astrocytes expressing luciferase were generated as previously described.[3](#_ENREF_3) Astrocytes were maintained as adherent cells at 37 °C and 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The UNC Institutional Animal Care and Use Committee approved all animal studies (16-112).

**Human ECL and PDX cultures**. D32, D65, D54, U251, and U87 ECL were a kind gift from G. Yancey Gillespie at the University of Alabama at Birmingham. U373, LN229, and LN18 ECL were obtained from ATCC (Manassas, VA). ECL were maintained as adherent cells in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and 5% CO2 as previously described.[7](#_ENREF_7),[8](#_ENREF_8) GSC20, GSC23, GSC6-27, GBM6, GBM12, GBM46, and GBM59 PDX were maintained at 37 °C and 5% CO2 as non-adherent spheroids in DMEM/F12 supplemented with 1x B27 (ThermoFisher, Waltham, MA), 20 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich, St. Louis, MO or ThermoFisher) and basic fibroblast growth factor (bFGF) (Sigma-Aldrich or ThermoFisher), with or without N-2 supplement (ThermoFisher) and 1% penicillin/streptomycin, as previously described.[9](#_ENREF_9),[10](#_ENREF_10) Subcutaneous PDX were maintained as previously described.[11](#_ENREF_11)

**Human GBM samples**. Newly diagnosed human GBM tumors were resected prior to any treatment. These samples were obtained from the UNC Tissue Procurement Facility in compliance with institutional guidelines under a protocol approved by the UNC Office of Human Research Ethics (Institutional Review Board (IRB) 15-0923).

**Drugs**. See **Table S1** for details on drugs used in this study.[12-19](#_ENREF_12) For *in vitro* experiments, working stocks of buparlisib, LY, temsirolimus, everolimus, selumetinib, PD01, and trametinib were dissolved in dimethyl sulfoxide (DMSO), while dactolisib was dissolved in N,N-Dimethylformamide (DMF).

**Dose response**. To examine relative growth in response to drugs, TRP astrocytes were plated in technical replicates (N=3-6) in 96-well tissue culture plates and allowed to adhere overnight. Cells were treated the following day with increasing concentrations of drug or an equal concentration of solvent (control). Cell growth was assessed 5 days after treatment with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI) according to manufacturer’s instructions. Absorbance was measured on an Emax plate reader (Molecular Devices, Chicago, IL) equipped with SoftMax Pro 5 software. Baseline absorbance (MTS reagent plus media) was subtracted from the absorbance in each well and relative growth was calculated as absorbance of treated relative to control cells.

Drug response of cultured PDX (GBM6, GBM12, GBM46, and GBM59) was performed as previously described with minor modifications.[20](#_ENREF_20) Briefly, PDX were seeded (3000 cells/well) in technical replicates (N=7) in 96-well plates and cultured for 72 hours to form neurospheres. Increasing drug concentrations were added and treated cells were incubated for 96 hours. Vehicle only and staurosporine (a drug toxic to most cell lines at 5 µM) were included as negative and positive controls, respectively. CellTiterGlo® (Promega, San Luis Obispo, CA) was used to assess cell viability. Luminescence was measured using a Perkin Elmer Envision 2104 Multilabel Reader.Raw values were normalized on a plate-by-plate basis such that 100% cell viability was equivalent to the mean of vehicle wells and 0% cell viability was equivalent to the mean of the staurosporine positive control.

Data from independent TRP (N=2-5) and PDX (N=1) drug treatment experiments were pooled and fit to a non-linear, log [inhibitor] versus response curves with variable slope. IC50, GI50, Imax and Hill slopes were calculated as previously described.[3](#_ENREF_3),[21](#_ENREF_21),[22](#_ENREF_22) Genotype and drug potency effects on IC50 were calculated by two-way ANOVA. The pairwise effects of PDX model and drugs on IC50 were compared using the extra-sum-of-squares F test.

**Cell cycle analysis**. TRP astrocytes were plated in 12-well tissue culture plates, allowed to adhere overnight, and then treated with drugs or vehicle control. Cell cycle analysis was performed 2 days post-treatment using Guava Cell Cycle Reagent according to the manufacturer’s instructions (EMD Millipore, Billerica, MA). Briefly, cells were harvested by trypsinization, fixed for >1 h in 70% ethanol on ice, and relative DNA content was determined on a Guava EasyCyte Plus. Percent cells in each cell cycle phase were analyzed using ModFit LT v3.2 (Verity, Topsham, ME). Mean percent cells in G0/G1, S, and G2/M were calculated from 1-2 technical replicates from 2 independent experiments. Drug effects on cell cycle distribution were analyzed by two-way ANOVA.

**Kinome profiling**. For dynamic kinome profiling of TRP astrocytes, cells were treated with either 1 µM of buparlisib for 4, 24, and 48 h or DMSO (control) for ~20 min (0 h). Multiplexed inhibitor bead chromatography was performed as previously described for astrocytes and untreated, snap-frozen GBM PDX samples.[23](#_ENREF_23),[24](#_ENREF_24) Briefly, cells or frozen PDX samples were lysed in a HEPES/NaCl/Triton X-100 buffer and lysates gravity-flowed through columns containing inhibitor-conjugated beads (CTx-0294885, VI-16832, PP58, Purvalanol B, and two custom-synthesized compounds UNC-2147A and UNC-8088A). Beads were then washed and kinases eluted. Kinases were purified by chloroform/methanol extraction, suspended in 50 mM HEPES (pH 8), digested with trypsin, labeled with iTRAQ (AB SCIEX, Framingham, MA) or TMT (Thermo Scientific) according to manufacturer’s instructions, and cleaned with PepClean C18 Spin Columns (Thermo Scientific). Peptide samples were analyzed by LC/MS/MS using an Easy nLC1000 coupled to a QExactive or QExactive HF mass spectrometer (Thermo Scientific). Peptides were injected onto an Easy Spray PepMap C18 column (75 µm x 25 cm, 2 µm particle size) (Thermo Scientific) and separated over a 300 min (QExactive) or 165 min (QExactive HF) gradient. The gradient for separation consisted of 5–40% mobile phase B at a 250 nl/min flow rate, where mobile phase A was 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in ACN. Mass spectrometer parameters were as follows: 3e6 AGC MS1, 80ms MS1 max inject time, 1e5 AGC MS2, 100ms MS2 max inject time, 20 loop count, 1.8 m/z isolation window, 45s dynamic exclusion. Spectra were searched against the Uniprot/Swiss-Prot database with Sequest HT on Proteome Discoverer software with 5% false discovery rate (FDR). Peptides with greater than 25% coisolation interference were omitted. Multiplexed inhibitor beads (MIB)-binding of treated samples was set relative to controls and pooled from two independent experiments. For baseline PDX comparison, scaled abundance values in biological triplicate from Proteome Discoverer were used for principal components analysis using Perseus software (Max Planck Institute of Biochemistry) and hierarchical clustering (one minus Pearson correlation) using GENE-E software (Broad Institute).

Baseline kinome profiling of ECL and human samples were performed as described above. Baseline spectra were searched with MaxQuant software using default parameters and Label Free Quantification (LFQ) intensity was used as the normalized level for each kinase. D54, U373, LN18 and LN229 were selected and pooled as a standard reference for MIB and mass spectrometry (MIB-MS) analysis of the human GBM samples based on their differentially enriched kinases. All kinome tree illustrations reproduced courtesy of Cell Signaling Technology, Inc. ([www.cellsignal.com](http://www.cellsignal.com)). One minus Pearson hierarchical clustering was performed on LFQ intensities using GENE-E software. Principal components analysis (PCA) was performed using Cluster 3.0.

**Immunoblots**. TRP astrocytes were treated with drug(s) or solvent (control). Cells were mechanically harvested 4 or 24 h after treatment and proteins were extracted. Briefly, proteins (20 µg for immunoblots to detect PI3K and MAPK signaling and 50 µg for immunoblots to detect cleaved caspase-3) were resolved by gradient (8-16%) gel electrophoresis (SDS-PAGE, Bio-Rad, Hercules, CA) then transferred to PVDF membranes (EMD Millipore). Immunoblots were probed at 4 °C overnight using primary antibodies against Gapdh (#AB2302, EMD Millipore) and Akt (#2967), p-Akt (Ser473, #9271), p-S6 (Ser240/244, #2215), p-Erk1/2 (Thr202/Tyr204, #9101), Erk1/2 (#4696), and cleaved caspase-3 (#9664) all from Cell Signaling Technology (Danvers, MA). Blots were incubated with species specific Alexa 488, 568, or 633 conjugated secondary antibodies at room temperature for 30 min, imaged on a GE Typhoon Trio (GE Healthcare), and band intensities were quantified using ImageJ (NIH, Bethesda, MD). Signaling levels were assessed by normalization of phosphoproteins to the corresponding total protein or Gapdh and then set relative to control-treated cells. Alternatively, normalized proteins from tumor samples were set relative to a TRP protein standard contained on all blots, and then were normalized to untreated tumors.

**Whole exome sequencing**. DNA from PDX tumors were isolated from subcutaneous tumor chunks using Qiagen’s AllPrep Kit. Isolated DNA was used to generate whole exome sequencing libraries. Genomic tumor and normal DNAs (1.1 µg) for each sample were fragmented to a target size of 150–200 bp; 100 ng of fragmented product was run on TAE gel to verify fragmentation. The remaining 1 µg of fragmented DNA was prepared using Agilent's SureSelectXT Human All Exon 50 Mb kit (catalog# G7544C). Exome libraries were prepared with Agilent's SureSelectXT Human All Exon V4 library preparation kit (catalog# 5190-4632) and SureSelectXT Human All Exon V4+UTRs (catalog# 5190-4637) following the manufacturer's protocols. Libraries were paired-end sequenced on the Illumina HiSeq 2000 and analyzed after Fastq generation and alignment using the Burrows-Wheeler Alignment against the human reference genome and matched patient germline DNA sequence for identification of somatic events.[25](#_ENREF_25)

**Drug synergism**. Relative growth of TRP astrocytes was determined for single agent and combination treatments at a constant molar ratio as described above and synergism was determined using the Chou-Talalay method.[26](#_ENREF_26) Briefly, fraction affected (1 – relative growth, FA) was determined and the combination index was calculated using CompuSyn (ComboSyn, Inc., Paramus, NJ). Data were pooled from 2–4 independent experiments. Combination index (CI) values >1, 1–0.86, and <0.86 were considered antagonistic, additive, and synergistic, respectively.

**EdU incorporation**. EdU incorporation assays were performed as previously described with the following modifications.[27](#_ENREF_27) TRP astrocytes were seeded on poly-D lysine (Sigma-Aldrich) coated Nunc Lab-Tek II chamber slides (ThermoFisher). Cells were allowed to adhere and at ~60% confluence were treated with vehicle control (DMSO), selumetinib (2.5 μM), buparlisib (1.5 μM) or both selumetinib (2.5 μM) + buparlisib (1.5 μM). At 24 h post-treatment, 10 µM 5-ethynyl-2′-deoxyuridine (EdU, Invitrogen, Grand Island, NJ) was supplemented to the culture + drug containing media and cells were incubated in the presence of EdU for 6 hr. Cells were then fixed with 4% para-formaldehyde for 5 min, blocked with 0.1% Triton X-100, 1% BSA, 5% horse serum, and 0.01 M glycine in PBS at 4 °C for 30 min. EdU was detected by labeling with Alexa 555 or 647 using the Click-iT EdU Imaging Kit (ThermoFisher) according to manufacturer’s instructions. Cells were washed, counter stained with DAPI, and coverslipped with PermaFluor (ThermoFisher). Three images per well were taken with a LSM 780 confocal microscope (Zeiss, Oberkochen, Germany) equipped with 20X objective and ZEN 2012 software. Percentage of EdU positive cells per image was quantified using ImageJ (NIH) and the mean (±SEM) percent EdU+ cells was calculated from the 3 images per 2 technical replicates from 2 independent experiments. Drug effects on EdU incorporation were analyzed by Students’s t tests.

**Apoptosis**. TRP astrocytes were plated in 12-well tissue culture plates, allowed to adhere overnight, and then treated with vehicle control (DMSO), selumetinib (2.5 μM), buparlisib (1.5 μM) or both selumetinib (2.5 μM) + buparlisib (1.5μ M). Apoptosis was measured 24 h post-treatment by flow cytometry using the Guava Viacount Assay according to the manufacturer’s instructions (EMD Millipore). Briefly, cells were harvested by trypsinization, stained with the Guava Viacount reagent and dye exclusion was determined on a Guava EasyCyte Plus. Gates for viable, apoptotic and dead cells were set according to the manufacture’s instructions and percent apoptotic cells (mean ± SEM) was calculated from 1 technical replicate from 4 independent experiments. Drug effects on apoptosis were analyzed by Students’s t tests.

**Orthotopic TRP allografts**. TRP astrocytes expressing luciferase were injected orthotopically into syngeneic C57Bl/6 mice as previously described.[1](#_ENREF_1),[2](#_ENREF_2) Briefly, astrocytes were harvested by trypsinization, counted, and suspended in serum free DMEM with 5% methyl cellulose. Adult mice (mean ~6 months) were anesthetized with Avertin (250 mg/kg) and 105 astrocytes were injected into the right basal ganglia using the coordinates 1, -2, and -4 mm (A, L, D) from bregma. Beginning on day 3 post injection, isoflurane anesthetized mice were imaged bi-weekly by bioluminescence imaging (BLI) on an Ivis Kinetic (PerkinElmer, Waltham, MA) as previously described.[2](#_ENREF_2),[3](#_ENREF_3) Briefly, mice were injected peritoneally with D-luciferin (PerkinElmer), images were acquired after 10-15 min, and bioluminescence flux (photons/sec/cm2) was quantified using PerkinElmer Living Image software. Mice were randomized 7 days after cell injection into 4 groups (13-18 mice per group) and treatment with buparlisib, selumetinib, or buparlisib/selumetinib on a 5 days on, 2 days off schedule (**Table S2**) was initiated via chow on day 10 and continued until signs of neurologic morbidity. BLI flux was expressed relative to initial flux (day 3). Relative flux from treatment initiation (day 10) through day 21 was fit to an exponential growth equation and the rate constant k and doubling time [ln(2)/k] were calculated. Animals were monitored for neurological symptoms and upon onset, mice were sacrificed, brains were harvested, and cut sagittally through the needle track. A portion of grossly visible tumor was harvested and snap frozen while the remaining brain was immersion fixed in 10% neutral buffered formalin overnight and stored in 70% ethanol prior to paraffin embedding. Immunoblots on frozen tumor lysates (N=3 per group) were performed as described above.

**Orthotopic PDX.** PDX were established in athymic mice (Taconic) as described previously.[11](#_ENREF_11) Briefly, 3 x 105 glioma cells (GBM6, GBM12, and GBM46) were implanted in the right striatum and tumors established for 15 days. Mice were then randomized to receive vehicle control (n = 10) or 45 mg/kg dactosilib (n = 10) on a 5 days on/2 days off schedule every 28-day cycle. Mice were observed daily and euthanized upon neurological morbidity. Studies were approved by the Translational Drug Development Management Animal Care and Use Committee (Scottsdale, AZ).

**Histopathology**. Histopathological evaluation was conducted as previously described.[3](#_ENREF_3) Briefly, formalin-fixed, paraffin-embedded brains were cut in serial 4-5 µm sections on a rotary microtome, placed on glass slides, and stained with hematoxylin and eosin (H&E) on a Leica Microsystems Autostainer XL (Buffalo Grove, IL). Stained slides were scanned on an Aperio ScanScope XT (Vista, CA) using a 20X objective and svs files were imported into an Aperio eSlideManager web database. Histopathological analyses and tumor grading was conducted according to the WHO 2007 criteria for human astrocytomas for each mouse within the control (n=15) or selumetinib (n=13) cohorts.[28](#_ENREF_28) Tumor area (mm2) on scanned H&E-stained slides (2 sections per brain) was measured using the annotation tools in Aperio ImageScope v12.2.1.5005. Photomicrographs were taken on an Olympus BX41 microscope and DP70 digital camera (Center Valley, PA). Effects of treatment on diagnosis were compared by two-way ANOVA with Bonferroni multiple comparisons post-tests. Effects of treatment on tumor size were compared using unpaired t-tests.

**Subcutaneous TRP allografts**. TRP astrocytes expressing luciferase were harvested by trypsinization, counted, and suspended in serum free DMEM with 5% methyl cellulose. Adult syngeneic C57Bl/6 mice (mean ~9.5 months) were anesthetized with Avertin (250 mg/kg) and 5 x 106 astrocytes were injected into the right flank. Tumors were established for 14 days, volume (mm3) was determined with calipers using the formula (L\*W2)/2, mice were randomized into treatment groups (**Table S2**) containing 4-6 mice each with statistically insignificant differences in tumor volume, and treatment via oral gavage was initiated and continued for five days (14–19) with the following exception. Dactolisib- and dactolisib/selumetinib-treated mice showed signs of drug-induced toxicity and were thus not treated on day 19. Tumor volume was measured 5 days per week from days 14–25 and expressed relative to treatment initiation (day 14). Tumor volume data were then fit to an exponential growth equation and the rate constant k and doubling time [ln(2)/k] were calculated. For pharmacokinetic experiments, subcutaneous allografts and treatment were performed as described above, except that mice (n=2-3 per group) were treated for two days and then sacrificed 4 h following the second treatment. Subcutaneous tumors were harvested, snap frozen, and then lysed for immunoblots. Buparlisib, dactolisib, and selumetinib were dissolved in 10/90 V/V of N-methyl-2-pyrrolidone (NMP)/poly(ethylene glycol) 300 (PEG300) at 15 mg/ml, 15 mg/ml, and 10.8 mg/ml respectively. Trametinib was dissolved in 0.5% methylcellulose and 0.2% Tween-80 in deionized water at 0.3 mg/ml.

**Statistics**

Statistical analyses were conducted in GraphPad Prism 5 (La Jolla, CA). P≤0.05 were considered statistically significant unless otherwise stated. Error bars are SEM. Log10 (Log) IC50 was graphed unless otherwise stated. Effects of treatment on survival of allograft or xenograft mice were determined by Kaplan-Meier analyses and were compared by log-rank tests. The effects of treatment on orthotopic or subcutaneous allograft growth (k value) was compared using the extra sum of squares F test.

**SUPPLEMENTAL REFERENCES**

1. Vitucci M, Karpinich NO, Bash RE, et al. Cooperativity between MAPK and PI3K signaling activation is required for glioblastoma pathogenesis. *Neuro Oncol.* 2013;15(10):1317-1329.

2. McNeill RS, Schmid RS, Bash RE, et al. Modeling astrocytoma pathogenesis in vitro and in vivo using cortical astrocytes or neural stem cells from conditional, genetically engineered mice. *J Vis Exp.* 2014(90):e51763.

3. Schmid RS, Simon JM, Vitucci M, et al. Core pathway mutations induce de-differentiation of murine astrocytes into glioblastoma stem cells that are sensitive to radiation but resistant to temozolomide. *Neuro Oncol.* 2016;18(7):962-973.

4. Jackson EL, Willis N, Mercer K, et al. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev.* 2001;15(24):3243-3248.

5. Xiao A, Wu H, Pandolfi PP, Louis DN, Van Dyke T. Astrocyte inactivation of the pRb pathway predisposes mice to malignant astrocytoma development that is accelerated by PTEN mutation. *Cancer Cell.* 2002;1(2):157-168.

6. Xiao A, Yin C, Yang C, Di Cristofano A, Pandolfi PP, Van Dyke T. Somatic induction of Pten loss in a preclinical astrocytoma model reveals major roles in disease progression and avenues for target discovery and validation. *Cancer Res.* 2005;65(12):5172-5180.

7. Miller CR, Buchsbaum DJ, Reynolds PN, et al. Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. *Cancer Res.* 1998;58(24):5738-5748.

8. Miller CR, Williams CR, Buchsbaum DJ, Gillespie GY. Intratumoral 5-fluorouracil produced by cytosine deaminase/5-fluorocytosine gene therapy is effective for experimental human glioblastomas. *Cancer Res.* 2002;62(3):773-780.

9. Bhat KP, Balasubramaniyan V, Vaillant B, et al. Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. *Cancer Cell.* 2013;24(3):331-346.

10. Gupta SK, Kizilbash SH, Carlson BL, et al. Delineation of MGMT Hypermethylation as a Biomarker for Veliparib-Mediated Temozolomide-Sensitizing Therapy of Glioblastoma. *J Natl Cancer Inst.* 2016;108(5).

11. Carlson BL, Pokorny JL, Schroeder MA, Sarkaria JN. Establishment, maintenance and in vitro and in vivo applications of primary human glioblastoma multiforme (GBM) xenograft models for translational biology studies and drug discovery. *Curr Protoc Pharmacol.* 2011;52(14):1-14.

12. Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem.* 1994;269(7):5241-5248.

13. Maira SM, Pecchi S, Huang A, et al. Identification and characterization of NVP-BKM120, an orally available pan-class I PI3-kinase inhibitor. *Mol Cancer Ther.* 2012;11(2):317-328.

14. Maira SM, Stauffer F, Brueggen J, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Mol Cancer Ther.* 2008;7(7):1851-1863.

15. Yeh TC, Marsh V, Bernat BA, et al. Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase kinase 1/2 inhibitor. *Clin Cancer Res.* 2007;13(5):1576-1583.

16. Sebolt-Leopold JS, Merriman R, Omer C, et al. The biological profile of PD 0325901: A second generation analog of CI-1040 with improved pharmaceutical potential. *Cancer Res.* 2004;64(7 Supplement):925.

17. Yoshida T, Kakegawa J, Yamaguchi T, et al. Identification and characterization of a novel chemotype MEK inhibitor able to alter the phosphorylation state of MEK1/2. *Oncotarget.* 2012;3(12):1533-1545.

18. Shor B, Zhang WG, Toral-Barza L, et al. A new pharmacologic action of CCI-779 involves FKBP12-independent inhibition of mTOR kinase activity and profound repression of global protein synthesis. *Cancer Res.* 2008;68(8):2934-2943.

19. Sedrani R, Cottens S, Kallen J, Schuler W. Chemical modification of rapamycin: the discovery of SDZ RAD. *Transplant Proc.* 1998;30(5):2192-2194.

20. Barretina J, Caponigro G, Stransky N, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature.* 2012;483(7391):603-607.

21. Reinhold WC, Sunshine M, Liu H, et al. CellMiner: a web-based suite of genomic and pharmacologic tools to explore transcript and drug patterns in the NCI-60 cell line set. *Cancer Res.* 2012;72(14):3499-3511.

22. Pawaskar DK, Straubinger RM, Fetterly GJ, Ma WW, Jusko WJ. Interactions of everolimus and sorafenib in pancreatic cancer cells. *AAPS J.* 2013;15(1):78-84.

23. Duncan JS, Whittle MC, Nakamura K, et al. Dynamic reprogramming of the kinome in response to targeted MEK inhibition in triple-negative breast cancer. *Cell.* 2012;149(2):307-321.

24. Stuhlmiller TJ, Miller SM, Zawistowski JS, et al. Inhibition of lapatinib-induced kinome reprogramming in ERBB2-positive breast cancer by targeting BET family bromodomains. *Cell Rep.* 2015;11(3):390-404.

25. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics.* 2009;25(14):1754-1760.

26. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* 2010;70(2):440-446.

27. Irvin DM, McNeill RS, Bash RE, Miller CR. Intrinsic astrocyte heterogeneity influences tumor growth in glioma mouse models. *Brain Pathol.* 2017;27(1):36-50.

28. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, eds. *WHO classification of tumours of the central nervous system.* 4th ed. Lyon: IARC; 2007. WHO Classification of Tumours.

29. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* 2008;455(7216):1061-1068.

30. Sarkaria JN, Yang L, Grogan PT, et al. Identification of molecular characteristics correlated with glioblastoma sensitivity to EGFR kinase inhibition through use of an intracranial xenograft test panel. *Mol Cancer Ther.* 2007;6(3):1167-1174.

31. Yang L, Clarke MJ, Carlson BL, et al. PTEN loss does not predict for response to RAD001 (Everolimus) in a glioblastoma orthotopic xenograft test panel. *Clin Cancer Res.* 2008;14(12):3993-4001.

**Supplemental Figure Legends**

**Figure S1**. **Increased PI3Ki or MEKi potency potentiates growth inhibition *in vitro***. Mutations in core GBM signaling pathways and MAPK (p-ERK) and PI3K (p-AKT) signaling status as determined by immunoblots in a panel of EGFR-amplified PDX (**A**).[10](#_ENREF_10),[29-31](#_ENREF_29) Multiple PI3Ki reduced growth of cultured TRP astrocytes and PDX (R2≥0.96) (**B**). Multiple MEKi reduced growth of cultured TRP astrocytes (R2≥0.97) and PDX (R2, 0.2-0.97) (**C**). IC50 calculated from these curves are graphed in **Fig 1**.

**Figure S2**. ***In vitro* efficacy of PI3Ki**, **MEKi**, **and mTORi in TRP astrocytes**. Micromolar doses of the PI3Ki buparlisib induced G2/M cell cycle arrest within 48 h post-treatment (\*, P≤0.001) (**A**). Summary of data from **Figs** **1AC**, **S1BC**. N represents replicate biologic experiments (**B**). Micromolar doses of the MEKi selumetinib induced G1/S cell cycle arrest within 48 h post-treatment (\*, P≤0.05) (**C**)

**Figure S3**. ***In vitro* efficacy of PI3Ki and MEKi in PDX**. Summary of data from **Figs 1BD, S1BC**. P≤0.01 for all PI3Ki IC50 in GBM59 vs. GBM12 or GBM6. P<0.001 for selumetinib IC50 in GBM59 vs. GBM12. P≤0.02 for trametinib IC50 in GBM46 vs. GBM12 or GBM6. P≤0.0002 for all MEKi IC50 in GBM12 vs. GBM6. Some IC50/GI50 could not be calculated (NA, not applicable) or were ambiguous (~).

**Figure S4**. **Buparlisib induces dynamic kinome reprogramming in TRP astrocytes.** Buparlisib induced dynamic changes in numerous kinase families over 4-48 h (**AB**). A kinome tree view of relative MIB-binding showed detected, but unchanged kinases (black) and those ≥1.5x (red) or ≤0.6x (blue) 48 h after buparlisib (**A**).

**Figure S5**. **Human GBM have diverse kinome profiles**. Tree views of all subtype K1 (**A**) and K2 (**B**) human GBM tumors from **Fig 3B-F** highlighted relative kinome hyper-activation of K1 vs. K2 tumors. Tree views of subcutaneous PDX models from **Fig 3GH** (**C**). Kinases with ≥2x (red) greater or ≤0.4x (blue) less MIB-binding relative to a pooled, standard reference consisting of established GBM cell lines are indicated. Other kinases detected by MIB-MS are indicated in black (**ABC**).

**Figure S6**. **PDX cultures have diverse kinome profiles**. Hierarchical clustering demonstrates heterogeneous kinome activity in cultured human GBM PDX (**A**). Tree views of the cultured PDX models, GSC20, GSC23, and GSC6-27 (**B**). The top (red) or bottom (blue) 25 kinases detected by MIB-binding in each model are indicated. Other kinases detected by MIB-MS are indicated in black.

**Figure S7**. **Subcutaneous** **PDX have diverse genetic profiles**. Circos plots summarizing somatic events in GBM59 (**A**), GBM46 (**B**), and GBM12 (**C**). A summary of all identified somatic genomic alterations is shown. Translocations are marked by yellow (interchromosomal) and black (intrachromosomal translocations) lines; for intrachromosomal translocations, the gray connecting line may appear as a single line if the joined regions lie within 2000 kb. CNVs are shown along the thick black ring encircling the translocations (green, regions of loss; red, regions of gain; black, no change); on the ring encircling CNVs, somatic indels (insertion/deletions) are marked by light blue tick marks and on the ring encircling the indels, somatic point mutations are marked by dark blue tick marks. Gene labels associated with point mutations are shown along the outermost area of the plot.

**Figure S8**. **Dual PI3Ki and MEKi treatment inhibits proliferation and induces apoptosis in TRP astrocytes *in vitro***. Representative images of EdU incorporation (red) in TRP astrocytes treated with vehicle (DMSO), selumetinib (2.5 μM), buparlisib (1.5 μM), or both (**A**). DAPI is shown in blue. Scale bars = 50 μm. Original magnifications 20X. Proliferation (EdU incorporation) was decreased by single agent selumetinib (\*, P=0.02), but not buparlisib treatment (**B**). Combination treatment decreased proliferation compared to DMSO (\*. P=0.003) or buparlisib alone (ǂ. P=0.02). Apoptosis was increased by single agent buparlisib (\*, P=0.02), but not selumetinib (**C**). Combination treatment increased apoptosis compared to DMSO (\*. P=0.05) or selumetinib alone (#. P=0.05).

**Figure S9**. **Single agent PI3Ki or MEKi delayed growth of subcutaneous TRP allografts**. The PI3Ki buparlisib or dactolisib alone delayed tumor growth (\*, P<0.0001) (**AB**). Neither buparlisib nor dactolisib significantly improved median survival (**C**). The MEKi selumetinib or trametinib alone delayed tumor growth (\*, P<0.0001) (**DE**), but trametinib was more effective (P=0.0008). Trametinib improved median survival (\*, P=0.01), but the less potent MEKi selumetinib did not (**F**). Doubling times (days) are indicated in white (**BE**).

**Figure S10**. **PI3Ki and MEKi inhibit their targeted pathways in subcutaneous TRP allografts**. Representative immunoblots of acutely isolated bulk subcutaneous TRP tumors (**A**) and quantification showed a trend (~50%) towards PI3K inhibition in PI3Ki treated samples (P=0.10 - 0.13) (**B**) and a significant decrease (64-91%) in MAPK signaling in MEKi treated samples (\*, P≤0.04) (**C**). While alternate pathway activation was not apparent in these bulk tumor samples (**BC**), this may be due to either the kinetics of pathway inhibition and alternate activation *in vivo* and/or changes in cellular composition of the bulk tumor tested. Mean ± SEM of 3 biologic replicates are graphed. Representative immunoblots of acutely isolated bulk subcutaneous TRP tumors (**D**) and quantification (**E**) showed increased cleaved caspase-3 in most single agents and all combination treatments. Mean ± SEM of 2 biologic replicates are graphed

**Figure S11**. **Selumetinib influences malignant progression of orthotopic TRP allografts**. Histology of representative control (**A**) or selumetinib-treated (**B**) mice showed GBM and anaplastic astrocytoma (grade III, A3), respectively. Arrows indicate mitoses (black) and necrosis (white). No significant difference in tumor area was evident (**C**). Controls developed GBM more frequently than selumetinib-treated mice (P<0.05) (**D**). Original magnifications 40x (HI, left panels) or 10x (HI, right panels). Scale bars = 100 µm.