**Supplemental materials and methods**

Wei BR, et al.Synergistic MEK and mTORC1/2 inhibition in melanoma

**Antibodies and inhibitors**

Antibodies (catalog number) against p‐AKT-Ser473 (4060), p‐mTORSer2448 (5536), p‐4E‐BP1-Thr37/46 (2855), p‐S6-Ser235/236 (4857), ERK1/2, p‐ERK 1/2 (4370), Bcl‐xl (2764), p-Bad (5284), Mcl-1 (94296), Bim (2933), and cyclins D1 (2978) and B1 (4138) were obtained from Cell Signaling Technologies (Danvers, MA). p-HH3 antibody (ab32107) was from Abcam (Cambridge, MA). Anti‐β‐actin (A1978) was from Sigma‐Aldrich (St. Louis, MO). Anexin-V-alexa 488 (Thermo Fisher Scientific). The PI3K/mTOR inhibitor library, sapanisertib and Torin 2 used were purchased from MedChemExpress (Monmouth Junction, NJ), and the MEK inhibitor trametinib was from ChemieTek (Indianapolis, IN).

**Apoptosis**

M1 and M5 cells were treated with 10 nM of trametinib, sapanisertib, the two-drug combination at 1:1 molar ratio, or equal volume of DMSO for 24 hours.  Apoptosis was determined using Annexin V Alexa Fluor™ 488 (Thermo Fisher Scientific, Waltham, MA) labeling according to manufacturer’s suggestions. Cell images were taken using EVOS FL AUTO 2 imaging system (Thermo Fisher Scientific). Numbers of green fluorescent annexin V positive cells and total cells in three randomly chosen fields were counted for each treatment group. A Wilcoxon signed-rank test was used to compare the % annexin V positive cells between treatment groups.

**Western blotting**

Mouse xenografts of canine melanomas created in this study were prepared for western blot. Specimens were homogenized in RIPA buffer (Cell Signaling, Danvers, MA) using a biovortexer (Bellco Glass, Inc., Vineland, NJ). Cell lysates of all cultured cell lines were generated by lysing cells in cell lysis buffer (Cell Signaling) for 20 min on ice. Tumor and cell lysates were cleared by centrifugation at 18 000 X g for 10 min; supernatants were separated from cell debris for western blot (WB) analyses. The cleared tumor and cell lysates were separated on 4–20% Tris‐glycine gels (Thermo Fisher Scientific, Waltham, MA) and transferred onto PVDF membranes (Bio‐Rad, Hercules, CA). Membranes were probed with primary antibodies at room temperature for approximately 1 hour, followed by respective horseradish peroxidase (HRP)‐conjugated secondary antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA). Immunoreactive bands were detected using chemiluminescence (GE Healthcare Bio-Sciences, Pittsburgh, PA).

**Immunohistochemistry and image analysis**

Target modulation was also evaluated ex vivo from melanoma xenografts collected from the flanks of mice given three days of treatment. Tissues were processed for routine preparation of formalin‐fixed paraffin‐embedded histology glass slide‐mounted tissue sections. Hematoxylin and Eosin (H&E) staining and p-ERK, p-S6 and p-HH3 immunohistochemistry (IHC) were performed as previously described (manuscript reference # 5). IHC labeling signals were developed with peroxidase‐catalyzed brown chromogen 3,3′‐diaminobenzidine (DAB, Agilent Technologies, Inc., Santa Clara, CA). Negative assay control included slides processed for IHC by omitting primary antibodies. H&E and IHC tissue sections were digitally scanned at 20× magnification using an Aperio ScanScope AT2 digital slide scanner (Leica Biosystems, Buffalo Grove, IL). To assess metastatic tumor growth, five H&E-stained step sections were obtained at approximately 400 um intervals through each sample. All tissue sections were analyzed for tumor vs. both tumor and nonneoplastic thoracic tissue area in each section (excluding heart and great vessels). The tumor burden in thoracic tissues was quantified using a pattern classifier algorithm (Indica labs, Halo version 2.3, Corrales NM). Analysis was specifically designed to delineate between tumor and non-tumor bearing thoracic tissues and performed on whole slide images. The algorithm was trained on representative lesions and post image processing mark-up image segmentation was quality assured through visual examination. Anti-p-HH3 immunolabeled tumor cell nuclei were segmented, quantified and compared to numbers of unlabeled nuclei, in steps which were computationally executed downstream of the pattern classifier in identified tumor regions of interest (Halo, cytonuclear algorithm, version 1.6, Indica Labs).

**Pharmacokinetics**

Sapanisertib was quantified in mouse plasma with a validated assay by first precipitating plasma proteins by addition of 3x volume of acetonitrile and pushing the contents through a phospholipid removal plate (Ostro®, Waters Corp, Milford, MA) with additional dilution before assaying with a validated LC-MS/MS assay with a calibration range of 1-1000 ng/mL. Sapanisertib was chromatographically resolved on an Agilent Polaris 3 C18 column (2.0 x 5mm) and detected by MRM of m/z 310.2 🡪 268.1. Tumor concentrations of sapanisertib were measured by adding 10 uL of water per mg of weighed tissue mass, followed by homogenization (100 mg/mL tissue homogenate). Fifty microliters of tissue homogenate was then mixed with 150 uL acetonitrile in Eppendorf tubes, vortexed and centrifuged, and the resulting supernatant further diluted then injected into the LC-MS/MS with the same instrument conditions as the plasma assay.

Trametinib plasma concentrations were measured using a validated LC-MS/MS assay with a calibrated range of 0.25 – 500 ng/mL. Briefly, 100 uL of plasma was diluted with 250 uL of water containing internal standard (IS; deuterated olaparib) and loaded into wells of a solid-phase extraction plate (ABN Express®, Biotage). The clean eluent was evaporated to dryness and reconstituted prior to injected onto a Waters ACQUITY HSS PFP (2.1 x 50 mm, 1.7 um) column with detection by MRM (m/z 616.0 🡪 491.6 for trametinib; m/z 442.5 🡪 281.1 for IS).