Supplementary figure legends

Suppl. Fig. S1. MEK and BRAF inhibitors induce expression of EDNRB mRNA transcript and protein in melanoma cell lines. EDNRB mRNA transcript levels, relative to reference transcript RPL19, were evaluated in the designated cell lines treated overnight with 0, 0.01, 0.1 and 1 μM of MEKi GDC-0623 (A) or GDC-0973 (C) or BRAF inhibitor G590945 (E). Error bars were derived from measurements performed in triplicate for each sample. EDNRB protein was evaluated in lysates from the designated cells treated overnight with the indicated concentrations of GDC-0623 (B) or GDC-0973 (D) or BRAF inhibitor G590945 (F). GAPDH was used a loading control.

Structure of MEK inhibitor GDC-0623 is also shown in Suppl.Fig. S1A.

Suppl. Fig. S2. Administration of PLX4032 does not affect clearance of the EDNRB ADC. Levels of total anti-EDNRB were monitored in mice dosed at 3 mg/kg EDNRB ADC in the absence of presence of either 10 or 30 mg/kg of PLX4032. Blood samples were withdrawn for analysis at Days 1, 7 and 15 post-dosing.

Suppl. Fig. S3. BRAF inhibition enhances EDNRB expression and anti-EDNRB ADC activity in a melanoma model with a BRAF V600E mutation. A. Mice bearing UACC-257X2.2 tumor xenografts were administered the indicated mg/kg dose of BRAF inhibitor G590945 once a day for 21 days (blue lines), a single IV injection of 1 or 3 mg/kg of anti-EDNRB ADC on day 1 (red lines), or the combination of both agents (black lines). Base-line tumor growth is shown for animals administered vehicle only (gray line). Average tumor volumes with standard deviations were determined from 10 animals per group. The data was generated from a single group of mice for each dose level or vehicle control. For clarity, each unique dose combination is presented as an individual graph. B. EDNRB, phosphorylated ERK (Perk) and total ERK (erk) proteins were evaluated in

lysates from UACC-257X2.2 cells treated overnight with the indicated concentrations of G590945.

Suppl Fig S4. Relative Cell Viability of A2058 and COLO 829 cells in a PLX4032 titration cell-killing assay. Data was transferred to GraphPad Prism (version 4; GraphPad Software; San Diego, CA) for graphic display. The indicated concentrations (uM on the x-Axis) of BRAFi (dark blue line), DMSO (Vehicle) control (light blue line), or equivalent amount of PBS vehicle (gray line) were incubated with cells for 5 days and relative cell viability (**y**-axis) assessed.

Suppl. Fig. S5. Kinetics of EDNRB elevation in tumors exposed to MEK inhibitor GDC-0973 or BRAF inhibitor PLX4032. A. EDNRB transcript levels normalized to GAPDH; phosphorylated ERK (Perk) and total ERK (erk) proteins, were monitored in SK23-mel tumor xenografts exposed to daily dosing of vehicle or GDC-0973 (MEKi) at 7.5 mg/kg (MPK) for 21 days followed by a 7-day washout. B. Day 1 and day 3 mRNA transcript levels for EDNRB in COLO 829 tumor xenografts exposed to daily dosing of vehicle or PLX4032 (BRAFi) at 30 mg/kg (MPK). Each bar or lane corresponds to an individual tumor monitored at the indicated time point and error bars were derived from measurements performed in triplicate for each sample.

Suppl. Fig S6. A. Caspase 3/7 activation is depicted on the Y-axis as a percent of the luminescence read out for each assay condition. Luminescence read out obtained with "No addition of drugs" is subtracted as background. B and C. Percent of gated events for each sample (x-axis) in the early apoptotic or late apoptotic/necrotic phase depicted on Y-axis. Cells in early apoptotic phase uptake are counted as events in the FL1 +, FL2 -

gate; Cells in late apoptotic/Necrotic phase are counted as events in the FL1 +, FL2 + gate. Read out obtained with "No addition of drugs" is subtracted as background.

Supplementary Materials and Methods.

In vivo efficacy data analysis in Supplementary Table S1- To analyze the repeated measurement of tumor volumes from the same animals over time, a mixed-modeling approach was used (Pinheiro et al. 2009). This approach can address both repeated measurements and a modest drop-out rate due to non-treatment-related termination of animals prior to study end.

Cubic regression splines were used to fit a non-linear profile to the time courses of log2 tumor volume at each dose level. These non-linear profiles were then related to dose within the mixed model. Tumor growth inhibition (TGI) as a percentage of vehicle was calculated as % area under the fitted curve (AUC) per day in relation to the vehicle, using the following formula:

$$\%TGI = 100x\left[1 - \left(\frac{AUC_{treatment} / day}{AUC_{vehicle} / day}\right)\right]$$

Using this formula, a TGI value of 100% indicates tumor stasis, of $>1\%$ but $<100\%$ indicates tumor growth delay, and of $>100\%$ indicates tumor regression.

To get uncertainty intervals (UIs) for %TGI, the fitted curve and the fitted covariance matrix were used to generate a random sample as an approximation to the distribution of %TGI. The random sample is composed of 1000 simulated realizations of the fitted-mixed model, where the %TGI has been recalculated for each realization. Our reported UI is the values for which 95% of the time, the recalculated values of %TGI will fall in this region given the fitted model. The 2.5 and 97.5 percentiles of the simulated distribution were used as the upper and lower UIs.

Partial response (PR) for an animal is defined as a tumor regression of $>50\%$ but $<100\%$ of the starting tumor volume, and complete response (CR) is defined as 100% tumor regression (i.e., no measurable tumor) on any day during the study. Time to tumor doubling (TTD) is defined as the time in days for the fitted tumor volume of a treatment group to double, compared with the Day 0 fitted tumor volume of the vehicle group.

Plotting was performed using KaleidaGraph 4.0 (Synergy Software; Reading, PA). Data were analyzed using R, version 2.10.1 (R Foundation for Statistical Computing; Vienna, Austria), and the mixed models were fit within R using the nlme package, version 3.1–96 (Pinheiro et al. 2009).

Reference:

Pinheiro J, Bates D, DebRoy S, et al. nlme: Linear and nonlinear mixed effects models. R package version 3.1-96. 2009.

Pharmacokinetics of the ADC (Suppl. Fig. S2)

To evaluate whether the clearance of ADC was affected by the administration of PLX4032, serum samples were collected from mice in the COLO 829 xenograft study depicted in Fig. 2C. The extracellular domain (ECD) of EDNRB protein, generated in baculovirus as a C-term His-tagged construct, was used for capture of anti-EDNRB antibodies in an ELISA assay format. The antibody assay format was a bridging ELISA utilizing NeutrAvidin as the coat reagent, which captured bridging of anti-EDNRB antibodies with biotinylated ECD protein of EDNRB to goat anti-human IgG

horseradish peroxide (HRP) as the detection reagent. The minimum sample dilution was 100-fold. The minimum reportable value was 0.062 ug/mL. The Total Antibody assay measured both conjugated and unconjugated anti-EDNRB antibodies.

Apoptosis Assays-

The effects of anti ET_BR ADC and MAPK inhibitors on caspase activation in tumor cells were assessed using Caspase 3/7 Glo Assay (G8092, Promega Corp). 10,000 cells were plated in 50 µLs volume in black-walled 96-well plates and allowed to adhere overnight at 37^0C in a humidified atmosphere of 5% CO2. Fifty uLs of medium containing 2 X desired concentrations of anti ET_BR ADC and MAPK inhibitors were added and the cells incubated for 24 h. (All cells are treated to 10 µg/mL anti ET_BR ADC, 1 uM MEKi-973. A2058, COLO 829 and SK23MEL are treated to BRAFi 10 µM PLX4032 whereas UACC257 X2.2 cells are treated to BRAFi 1 µM G590945).

Caspase activation was assessed by adding Caspase-Glo 3/7 reagent for 30 min at room temperature, and the luminescence was recorded using Envison Plus instrumentation and software.

For evaluation of Early and Late Apopotic cells using Annexin V/Propidium Iodide cells were plated in 6-well dishes and treated overnight to combinations of ADC and MAPK inhibitors. (All cells are treated to combinations of 10 µg/mL anti ET_BR ADC and 1 uM MEKi-973. A2058, COLO 829 and SK23MEL are treated to BRAFi 10 µM PLX4032 whereas UACC257 X2.2 cells are treated to BRAFi 1 µM G590945). Harvested cells are washed and stained with Annexin V FITC (Early Apoptosis marker) and Propidium iodide (Late Apoptic/Necrotic cell marker) using BD Pharmingen kit 556547 per manufacturer's suggested protocol. Cells are evaluated for the uptake of Annexin V and Propidium Iodode by Flow Cytometry analysis using FACS Calibur instrumentation. (BD Biosciences)