Supplementary Table and Figure Legends

Supplementary Tables 1A-C. Immunohistochemical analysis indicates correlation between pAKT(Ser473), pERK1/2(Thr202/Tyr204) and pS6(Ser235/236) in primary tumours: Contingency tables for IHC staining of rhabdomyosarcoma primary tumour TMA. Cores were scored as **0**: negative, **1**: weak, **2**: moderate, and **3**: strong staining and were considered to be positive if at least 10% of cells in the core showed staining. **A:** pAKT and pERK, trend statistic: 4.0912 (p=0.0431). **B**: pAKT and pS6, trend statistic: 8.5413 (p=0.0035) **C:** pERK and pS6, trend statistic: 11.1429 (p=0.0008), (Chi squared test for trend).

Fig S1. High level expression of p110δ in two of four ARMS cell lines. Western immunoblots of p110α, p110β and p110δ in a panel of seven RMS cell lines

Fig S2. *PIK3CA* KD does not induce cell cycle arrest or apoptosis in the growth inhibited RMS cells. Cell cycle FACS analysis of RMS cells on days 2, 5, and 8 following lentiviral transduction.

Fig S3. Downregulation of PTEN expression appears to be associated with delayed inhibition of AKT phosphorylation in RH30 cells following p110α KD. Quantitation of the western blots shown in Fig 1C using ImageQuant software showing **a.** reduced expression of PTEN and delayed inhibition of AKT phosphorylation in RH30 cells, **b.** increased expression of PTEN with inhibition of AKT phosphorylation in RMS-1 cells, and **c.** and **d.** increased expression of PTEN with inhibition of AKT phosphorylation by d5 but recovery to control levels by d8 following p110α KD in RD and RMS-YM cells

Fig S4 Increased sensitivity of the RMS-1 *PIK3CA* KD line to TGX221, and of the RMS-1, RD and RMS-YM KD lines to ZSTK474. A. Class 1 PI3K isoform and mTOR IC_{50} values of three PI3K inhibitors: NVP-BKM120,TGX221 and ZSTK474. **B, C** and **E.** Representative growth inhibition curves (MTS assays) following treatment of the CONSH and *PIK3CA* KD lines with NVP-BKM120. TGX221, and ZSTK474 respectively, showing **B:** no difference in sensitivity of the two lines to NVP-BKM120, **C:** increased sensitivity to the p110β-selective inhibitor TGX221in the RMS-1 KD line lacking both p110α and δ expression and **E:** increased sensitivity to ZSTK474 in the KD lines with elevated IRS2 expression. **D.** Western immunoblots of selected elements of the PI3K and MAPK pathways in the stable p110α KD lines showing cell line-specific increased phosporylation of GSK3β correlating with increased p^{Thr308}AKT but no consistent change in $p^{ser235/6}$S6.

Fig S5 Equivalent changes in the biomarkers of MAPK and PI3K activity at 6h *in vivo,* to those seen *in vitro* following acute dosing with AZD6244 and AZD8055 alone, and in combination, with recovery to control levels correlating with plasma and tumor clearance of both drugs. Aa. pERK, **b.** pAKT and **c.** pS6 levels (MSD electrochemi-luminescence immunoassay) in RD xenografts at the indicated time points following AZD6244 and AZD8055 (10mg/kg po) alone or in combination, showing recovery to control levels at 16h. **Ba.** PK analysis of plasma AZD6244 and **c.** AZD8055 levels at the same time points as shown in **A .** showing plasma clearance by 16h following dosing of each drug individually. Peak plasma concentrations of AZD6244 were several logs higher than AZD8055. Evidence of drug–drug interaction is seen with AZD6244 when in combination with AZD8055, but not conclusively with AZD8055 in combination. **Bb.** Tumor and normal tissue levels of AZD6244 showing efficient AZD6244 tumor uptake. When in combination, tumor concentrations of AZD6244 were not detected. **Bd.** Tumour AZD8055 levels following dosing alone or in combination showing therapeutically inadequate concentrations of AZD8055 and an indication of drug-drug interaction.

Fig S6 Tolerability study of twice daily dosing of 10mk/kg po AZD6244 and AZD8055 indicates reversible weight loss with combination treatment, while PK analysis of tail vein blood spots confirms drug-drug interactions. Non tumor-bearing mice were dosed, as indicated, twice daily (6h between dosing) for four days. Mice were weighed before the first dosing each day and for a further 10 days following cessation of treatment. Tail blood spots for PK analysis were taken on days 1, 2 and 4 at 1h, 3h and 6h following the first daily dose. **A.** Mice body weights expressed as % weight on day 0 showing that twice daily dosing of 10mg/kg AZD6244 and AZD8055 p.o. was well tolerated when given individually but revealed rapid weight loss following treatment in combination. Weight loss was reversed following cessation of treatment implying metabolic disruption as opposed to systemic toxicity. **Ba and b.** Tail vein blood spot PK analysis of AZD6244 and AZD8055 respectively showing reduced peak levels and an extended elimination phase of AZD6244 when given in combination with AZD8055, and higher levels of AZD8055 when given in combination with AZD6244. Over the four days of treatment, the levels of AZD6244 achieved reduced while the levels of AZD8055 increased with repeat dosing regardless of whether each was given individually or in combination.

Fig S7 Dose-finding therapeutic study defines a once daily treatment schedule of AZD6244, 10mg/kg p.o., and AZD8055, 20mg/kg p.o., as well tolerated and active when given in combination. A. Mean tumour volumes following once daily dosing with AZD6244 (10mg/kg po) and AZD8055, 10mg/kg po for the first 6 days increasing to 20mg/kg from day 7 to day 19 inclusive as indicated. **B.** Final tumour weights in gms showing significantly increased therapeutic efficacy of the combination AZD6244/AZD855 compared to AZD6244 alone ($p=0.0087$) but not compared to AZD8055 alone ($p=0.24$) (Mann Whitney t test). **C.** Tumour concentrations of AZD6244 and AZD8055 3 hours following the final dosing showing decreased levels of AZD6244 when given in combination with AZD8055, and increased levels of AZD8055 when given in combination With AZD6244. * In three of the tumor samples (n=6) AZD6244 was not detected for reasons unknown. **D.** Mice body weights as % of weights at day 0 during treatment with AZD6244 and AZD8055 as indicated.