Dual Blockade of the PI3K/AKT/mTOR (AZD8055) and RAS/MEK/ERK (AZD6244) Pathways Synergistically Inhibits Rhabdomyosarcoma Cell Growth In Vitro and In Vivo

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Abstract

Purpose: To provide rationale for using phosphoinositide 3-kinase (PI3K) and/or mitogen-activated protein kinase (MAPK) pathway inhibitors to treat rhabdomyosarcomas, a major cause of pediatric and adolescent cancer deaths.

Experimental Design: The prevalence of PI3K/MAPK pathway activation in rhabdomyosarcoma clinical samples was assessed using immunohistochemistry. Compensatory signaling and cross-talk between PI3K/MAPK pathways was determined in rhabdomyosarcoma cell lines following p110α short hairpin RNA-mediated depletion. Pharmacologic inhibition of reprogrammed signaling in stable p110α knockdown lines was used to determine the target-inhibition profile inducing maximal growth inhibition. The in vitro and in vivo efficacy of inhibitors of TORC1/2 (AZD8055), MEK (AZD6244), and P13K/mTOR (NVP-BEZ235) was evaluated alone and in pairwise combinations.

Results: PI3K pathway activation was seen in 82.5% rhabdomyosarcomas with coactivated MAPK in 36% and 46% of alveolar and embryonal subtypes, respectively. p110α knockdown in cell lines over the short and long term was associated with compensatory expression of other p110 isoforms, activation of the MAPK pathway, and cross-talk to reactivate the PI3K pathway. Combinations of PI3K pathway and MAP–ERK kinase (MEK) inhibitors synergistically inhibited cell growth in vitro. Treatment of RD cells with AZD8055 plus AZD6244 blocked reciprocal pathway activation, as evidenced by reduced AKT/ERK/S6 phosphorylation. In vivo, the synergistic effect on growth and changes in pharmacodynamic biomarkers was recapitulated using the AZD8055/AZD6244 combination but not NVP-BEZ235/AZD6244. Pharmacokinetic analysis provided evidence of drug–drug interaction with both combinations.

Conclusions: Dual PI3K/MAPK pathway activation and compensatory signaling in both rhabdomyosarcoma subtypes predict a lack of clinical efficacy for single agents targeting either pathway, supporting a therapeutic strategy combining a TORC1/2 with a MEK inhibitor. Clin Cancer Res; 19(21): 1–12. ©2013 AACR.

Introduction

Rhabdomyosarcomas are the most common soft tissue sarcomas in children of ages 0 to 14 years, accounting for around 50% of all cases in this age group (reviewed in ref. 1). The two major histologic subtypes, alveolar rhabdomyosarcoma and embryonal rhabdomyosarcoma carry distinct morphologic and genetic alterations: The majority of alveolar rhabdomyosarcomas harbor PAX3-FOXO1 or PAX7-FOXO1 chimeric transcription factors (2), and the presence of the PAX3-FOXO1 fusion transcript has been associated with poor prognosis (3). Embryonal rhabdomyosarcomas generally have a more favorable outcome and carry no consistent chromosomal translocations, although allelic imbalances at 11p15.5 are commonly identified (1, 2). Dysregulation of the RAS signaling pathway is likely relevant to the pathogenesis of embryonal rhabdomyosarcoma (4, 5), and also insulin-like growth factor (IGF) signaling to the pathogenesis of all rhabdomyosarcomas (6). The use of multiagent chemotherapy, surgery, and radiation has improved the outcome for patients with rhabdomyosarcoma with favorable features, although at a cost of disfigurement and long-term sequelae...
Translational Relevance

Molecular rationale to underpin the therapeutic use of PI3K/AKT/mTOR and RAS/RAF/MAPK pathway inhibitors is critical. Here, we show coactivation of these pathways in 43% of primary rhabdomyosarcomas with most of the remainder exhibiting activation of the phosphoinositide 3-kinase (PI3K), but not mitogen-activated protein kinase (MAPK), pathway. The former predicts resistance to single pathway–targeted agents and the latter, potential sensitivity to PI3K pathway inhibitors. However, we also show extensive compensatory signaling and cross-talk between the PI3K/MAPK pathways on inhibition of either pathway alone, both in vitro and in vivo. Therefore, simultaneous inhibition of both pathways is essential for effective treatment of rhabdomyosarcomas. The in vivo synergistic antitumor response achieved with the combination AZD8055/AZD6244, but not NVP-BEZ235/AZD6244, was reflected in reduction of the pharmacodynamic biomarkers pERK/pS6/p(Ser473)AKT. Preliminary pharmacokinetic analyses revealed drug–drug interactions between the PI3K/MAPK inhibitors studied, indicating a requirement for pharmacokinetic analyses after repeat dosing in future clinical trials of the promising AZD8055/AZD6244 combination.

(7). Unfortunately, the prognosis for metastatic and relapsed rhabdomyosarcoma tumors remains poor, and the need for novel, less toxic therapeutic strategies is pressing. High levels of phosphorylated AKT, reported in rhabdomyosarcoma cell lines and a significant proportion of primary tumors, are indicative of constitutive activation of the PI3K/AKT/mTOR pathway and suggest that rhabdomyosarcoma may be sensitive to the targeted inhibition of this pathway (8, 9). However, previous reports in other tumor types have indicated that activated mitogen-activated protein kinase (MAPK) signaling mediates resistance to phosphoinositide 3-kinase (PI3K) inhibitors (10, 11). Indeed, NRAS mutations, identified in approximately 20% of patients with embryonal rhabdomyosarcoma (4), may identify those tumors unlikely to respond to PI3K inhibitors, as has been shown for KRAS-mutated lung cancers (10). Conversely, intrinsic resistance to MAP–ERK kinase (MEK) inhibitors has been associated with strong PI3K signaling in colorectal and breast cancer cell lines (12, 13). Thus, dual activation of both PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways is likely to result in resistance to the targeting of either pathway alone. Encouragingly, coinhibition of both pathways has shown use in reducing tumor growth in a variety of xenograft cancer models (10, 13–15), and clinical trials of such combinations are under way in adults.

Here, we assessed the prevalence of coactivation of the PI3K and MAPK pathways in a large series of well-characterized rhabdomyosarcoma clinical samples by conducting immunohistochemistry for phosphorylated markers of both pathways, as well as downstream S6. We used short hairpin RNA (shRNA)–induced p110α knockdown to explore compensatory signaling and cross-talk between the PI3K/MAPK pathways in rhabdomyosarcoma cell lines and conducted comprehensive preclinical in vitro and in vivo studies of PI3K and/or MAPK pathway–targeted therapies. We provide evidence to support the class of PI3K inhibitor that is most effective, identify mechanisms likely to be responsible for treatment failure, and undertook combination studies that address this problem. Our results predict a general lack of clinical efficacy of PI3K/AKT/mTOR or RAS/RAF/ERK pathway monotherapy in rhabdomyosarcoma, and we propose a dual-targeted strategy for clinical testing.

Materials and Methods

Rhabdomyosarcoma tissue from patients and immunohistochemistry

A tissue microarray (TMA) of formalin-fixed, paraffin-embedded diagnostic tumor material from 79 patients with rhabdomyosarcoma (25 alveolar and 54 embryonal by histology), with approval for the study (Local Research Ethics Committee protocol Nos 1836 and 2015 and Multi-Regional Research Ethics Committee/06/4/71), has been described previously (16). Following antigen retrieval (0.1 mol/L sodium citrate, pH 6.0, microwave 600 W, 30 minutes), and blocking (TBS with 5% milk, 2% normal rabbit serum, 30 minutes), TMA sections (4 μm) were immunostained using antibodies against phospho-AKT (Ser473), phosphor-ERK1/2 (Thr202/Tyr204), and phospho-S6 (Ser235/236; 1:200 dilution; Cell Signaling Technology) with detection using the avidin–biotin complex method (DAKO) visualized by 3,3′-diaminobenzidine (DAB). Slides were lightly counterstained with hematoxylin. Cores were scored blind by a pathologist as negative, weak, moderate, or strong for intensity and were considered to be positive if at least 10% of cells in the core showed staining.

Cell culture, compounds, and GI50 estimation

The source of the human tumor cell lines RH30, RMS-1, SCMC, and RH41 derived from alveolar rhabdomyosarcoma, and RD, RMS-YM, and CT10 derived from embryonal rhabdomyosarcoma is described elsewhere (16). All cell lines were grown in Dulbecco’s modified Eagle medium (DMEM; Sigma) supplemented with 10% FBS (Biosera; Labtech International Ltd.) in 5% CO2 in air at 37°C. Cell line identity was validated by analyzing short-tandem repeat using the Promega Power Plex 1.2 system, according to the manufacturers’ instructions, within 6 months of the experiments described. Results were matched with those available from repositories which hold the lines (RH30 and RD) or those produced immediately after delivery of the cell lines to our laboratory. All compounds were purchased from AxonMed Chem, except NVP-BMK120 (Selleckchem). Compounds were dissolved in the appropriate solvent at 10 mmol/L and diluted in tissue culture medium to working
concentrations. GI50 values (concentrations causing 50% inhibition of cell proliferation after 72 hours of continuous exposure), were determined in 96-well plates using the MTS assay (Promega). For the construction of isobolograms, the GI50 values of compound A and B were determined along with the GI50 values of compound B in the presence of various fixed sub-GI50 concentrations of compound A. The GI50 values of compound A and B were then plotted on the x- and y-axis along with the GI50 values of compound B versus the fixed concentration of compound A.

**Lentiviral shRNA knockdown of PIK3CA, Western blotting, and FACS analysis**

Lentiviral particles for shRNA knockdown of PIK3CA were produced following transfection of the lentiviral packaging vectors pMD2.G (3 μg), pMD1g/RRE (5 μg), and pRSV-Rev (2.5 μg; Addgene), and either the PIK3CA shRNA vector (10 μg; TRCN0000039603; Sigma) or the control shRNA vector (10 μg; SHC002; Sigma) into HEK293T cells as described previously (16). Control (CONSH) or PIK3CA shRNA lentiviral particles were transduced into RH30, RMS-1, RD, and RMS-YM cells (in 2×6-well plates per line) at a multiplicity of infection of 10 in the presence of polybrene (4 μg/ml; Sigma-Aldrich) and transduced cells were selected with puromycin (2.5 μg/ml) from day 2. On days 2, 5, and 8, viable cells were counted (ViCell series automated Cell Viability Analyzer; Beckman Coulter), cells were fixed with 70% ethanol for fluorescence-activated cell sorting (FACS) analysis, and proteins were extracted for Western blotting.

For Western blotting, following quantitation (BCA Protein Assay Kit; Pierce), 10 μg of each lysate was separated using NuPage Bis-Tris or Tris-Acetate gels (Invitrogen), transferred onto Hybond polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences) and probed with 1:1,000 dilution of primary antibody (Cell Signaling Technology) overnight at 4°C, and 1:10,000 dilution of horseradish peroxidase secondary antibody (Amersham Biosciences) for 2 hours at room temperature. Signal was detected with ECL Plus reagent (Amersham) and visualized and analyzed using a STORM 860 PhosphorImager and ImageQuant software (AmershamBiosciences).

For FACS analysis, fixed cells were spun, resuspended in 800 μl PBS, 100 μl RNase (0.1 mg), and 100 μl propidium iodide (PI; 40 μg), and incubated at 37°C for 30 minutes. Labeled cells were stored at 4°C until cell-cycle analysis on a BDLSRII FACS analyzer (BD) using BDFACSDiVa software with doublet discrimination. For each sample 10,000 events were recorded.

**Pharmacodynamic, pharmacokinetic, and xenograft efficacy studies**

All animal experiments were carried out in accordance with United Kingdom Home Office Regulations under the Animals (Scientific Procedures) Act 1986 and United Kingdom Co-coordinating Committee on Cancer Research guidelines (17). RD cells (5 × 10^6 cells in 25% Matrigel; BD Biosciences) were subcutaneously implanted bilaterally (for initial pharmacodynamic and pharmacokinetic studies), or in one flank (for tumor efficacy studies) into female 6- to 8-week-old CrTac:Ncr-Fox1(1u; Ncr) athymic mice (Charles River Laboratories). AZD6244 [10 mg/kg in 10% dimethyl sulfoxide (DMSO) water], AZD8055 (10 or 20 mg/kg in acidified water), and NVP-BEZ235 (25 mg/kg in 10% NMP in 90% PEG300) were given orally (0.1 ml/10 g body weight of vehicle) alone, or in combination. Control animals received the equivalent volume of appropriate vehicle(s). For initial pharmacokinetic and pharmacodynamic analysis, mice (n = 3) bearing tumors of sufficient size were euthanized at various times following dosing, tumors were excised, bisected and snap-frozen, and blood was collected, centrifuged, and the plasma was frozen for storage at −80°C. For tumor-efficacy studies, dosing commenced when the tumors were well established (>5 mm mean diameter; n = 6/treatment group) and continued daily for 19 to 22 days. Animals were weighed at regular intervals and observed for adverse effects. Tumors were measured across two perpendicular diameters, and volumes were calculated using the formula V = 4/3πr²(d1+d2/2)³. On termination of the experiment, mice were bled, tumors were excised, and weighed, and samples were processed as described earlier.

Quantitative pharmacokinetic analysis was conducted by liquid chromatography/tandem mass spectrometry and multiple reaction monitoring, using a modification of the previously described method (ref. 18; Supplementary Methods). Tumor pharmacodynamic biomarkers were assessed by a Meso Scale Discovery (MSD) multiplex electrochemiluminescence immunoassay system to detect phospho (T/Y:202/204:185/187)ERK1/2/total ERK1/2, phospho (Ser473)AKT/total AKT, and phospho(p38)MAPK/total p38 (Ser473) in liver lysates. Tumor tissues were fixed in 10% neutral buffered formalin and paraffin embedded. 3-μm-thick sections were cut and stained using a Dako Autostainer Link 48 automated stainer (Dako). Staining was performed using mouse monoclonal anti-p-Akt (Ser473), rabbit polyclonal anti-p-S6 (Cell Signaling Technology), rabbit polyclonal anti-p-ERK1/2 (Cell Signaling Technology), and mouse monoclonal anti-α-SMA (Sigma). Immunohistochemistry was scored using a V surroundings the majority of rhabdomyosarcomas. The expression of p-Akt and p-S6 was assessed independently and in combination. The proportion of positively staining tumor cells was scored on a 0 to 4+ scale. The correlation between resistance to single-agent inhibitors and the levels of p-Akt and p-S6 was assessed using regression analysis. The correlation between staining using χ² tests for trend showed a significantly positive correlation.
in all pairwise analyses: pAKT and pERK trend statistic: 4.0912 ($P = 0.0431$), pAKT and pS6 trend statistic: 8.5413 ($P = 0.0035$), pERK and pS6 trend statistic: 11.1429 ($P = 0.0008$; Supplementary Table S1A–S1C).

PIK3CA knockdown induces widespread compensatory signaling and reciprocal cross-talk between the MAPK and PI3K pathways

Consistent with previous reports that the PI3K p110α and β isoforms are expressed ubiquitously (reviewed in ref. 19), p110α was found to be expressed at similar levels in a panel of seven rhabdomyosarcoma cell lines, whereas p110β expression was more variable (Supplementary Fig. S1). Unexpectedly, p110β, expressed predominantly in leukocytes (19), was expressed at high level in two (RH30 and RH41) of four alveolar rhabdomyosarcoma but none of the embryonal rhabdomyosarcoma lines studied here. PI3K p110α (encoded by PIK3CA) has been suggested to be the isoform that is predominantly involved in insulin, IGF signaling, and cell growth in many cell types (reviewed in ref. 19). To assess the importance of PI3K p110α to the growth and survival of rhabdomyosarcoma cell lines, two alveolar rhabdomyosarcoma lines (RH30 and RMS-1) and two embryonal rhabdomyosarcoma lines (RD and RMS-YM) were transduced with lentiviral shRNA particles targeted to PIK3CA. None of these lines carry an oncogenic PIK3CA mutation. Only the alveolar rhabdomyosarcoma lines, RMS-1, was growth inhibited in response p110α knockdown (Fig. 1A). Growth inhibition did not involve cell-cycle arrest as evidenced by FACS analysis (Supplementary Fig. S2), but did not seem to be increased apoptosis (PARP cleavage) or autophagy (LC3BII production; Fig. 1B) indicating cytotostasis.

Knockdown of p110α induced varying degrees of compensatory upregulation of p110β expression in all four cell lines (Fig. 1B), whereas p110β expression was minimally affected. Downstream, AKT phosphorylation was not consistently inhibited following knockdown of p110α (Fig. 1C). Of note, increased $p^{Thr308}$AKT to above control levels (not mirrored by an equivalent increase in $p^{Ser473}$AKT) was seen on day 5 following lentiviral transduction of RH30 cells, with gross inhibition of AKT phosphorylation being delayed until day 8. In contrast, both $p^{Thr308}$AKT and $p^{Ser473}$AKT were reduced to below control levels in RMS-1, RD, and RMS-YM cells by day 5 with recovery toward control levels in the embryonal rhabdomyosarcoma lines by day 8. In addition, PTEN expression was downregulated in RH30 cells but increased in the remaining three cell lines (quantitation shown in Supplementary Fig. S3). Inhibition of downstream S6 phosphorylation mirrored inhibition of AKT phosphorylation in all cell lines except RH30, where inhibition of S6 and AKT phosphorylation seemed disconnected. The latter may reflect signaling from p110α through only one of the three AKT isoforms to mTOR, leading to decreased S6 phosphorylation despite an apparent lack of inhibition of total AKT phosphorylation.

Classical relief of IRS2 (but not IRS1; data not shown), feedback repression on inhibition of mTOR signaling (20), was seen in three of the cell lines (exception, RH30), as well as evidence of reciprocal cross-talk between the PI3K/AKT/mTOR and RAS/RAF/ERK pathways. Varying degrees of upregulation of pERK1/2 by day 5 following lentiviral transduction were observed in the three p110α knockdown–resistant cell lines, but not until day 8 in the sensitive RMS-1 cells. Evidence of transient activation of AMPK co-occurring with upregulated pERK (21) was observed in three cell lines (exception RD, where pAMPK levels were reduced). Quantitation of the pERK1/2 and total ERK1/2 Western blot analyses confirmed both a higher basal activation of extracellular signal-regulated kinase (ERK) signaling in the embryonal rhabdomyosarcoma compared with the alveolar rhabdomyosarcoma lines and the stimulation of ERK phosphorylation following PIK3CA knockdown (Fig. 1D).

Pharmacologic inhibition of reprogrammed signaling pathways in PIK3CA knockdown stable cell lines establishes the rationale for dual blockade of the PI3K/MAPK pathways in rhabdomyosarcoma

To determine the long-term effects of reduced PIK3CA expression on PI3K and MAPK signaling, stable p110α knockdown, and control (CONSβ) cell lines were derived from the virally transduced cell lines over a period of around 4 weeks. The derived cell lines were then used to screen PI3K pathway inhibitors with a variety of inhibition profiles against the class 1 p110 isoforms and/or mTOR.

Both embryonal rhabdomyosarcoma stable knockdown lines showed increased expression of p110β, a novel observation consistent with p110β being downstream of growth factor signaling (19), and functional redundancy between the p110α and δ isoforms (22). No differences in sensitivity to the pan-PI3K inhibitor BMK120 [modestly selective for p110α versus the other isoforms (Supplementary Fig. S4A)] was observed in the stable p110α knockdown lines and their CONSβ counterparts (Supplementary Fig. S4B). In addition, only the RMS-1 stable knockdown cell line, with minimal p110α and δ expression, showed increased sensitivity to the p110β inhibitor, TGX221 (78-fold selective for p110β vs. p110α, less so for p110δ; Supplementary Fig. S4A and S4C). Taken together, these data show that all expressed isoforms must be inhibited for maximum inhibition of cell growth.

Assessment of selected elements of the PI3K/AKT/mTOR and RAS/RAF/ERK pathways in the stable knockdown lines (Fig. 2A) revealed increased $p^{Thr308}$AKT but not $p^{Ser473}$AKT levels in three of four stable knockdown lines (exception, RMS-YM), which was accompanied by altered PTEN expression in the RD stable knockdown line. Increased levels of $p^{Thr308}$AKT correlated with increased phosphorylation of GSK3β, a downstream AKT substrate, but did not have a consistent impact on pS6 levels (Supplementary Fig. S4D). IRS2 expression was upregulated in the RMS-1, RD, and RMS-YM stable knockdown lines suggesting that increased signaling through IGF-I receptor (IGF-IR) may contribute to increased $p^{Thr308}$AKT in some cell lines. Upregulated MAPK signaling, as evidenced by increased pERK levels, was observed in all the stable knockdown lines resulting
Figure 1. PIK3CA shRNA-mediated knockdown (KD) induces cell line–specific compensatory signaling and reciprocal cross-talk between the MAPK and PI3K pathways: A, representative growth curves following PIK3CA knockdown: two alveolar rhabdomyosarcoma (RH30 and RMS-1) and two embryonal rhabdomyosarcoma (RD and RMS-YM) cell lines were transduced with control (CONSH) or PIK3CA-targeted shRNA (PIK3CA knockdown) on day 0 or treated with polybrene only (CONUT). On day 2, 5, and 8, cells were counted, and, on day 2 and 5, an appropriate dilution was reseeded in fresh plates enabling accumulative cell counts to be calculated, and plotted for day 5 and 8. Puromycin (2.5 μg/mL) selection was introduced on day 2 and maintained for the entire experiment, the difference between the CONUT and CONSH growth curves being a reflection of viral transduction efficiency. B, Western immunoblot analyses of class 1A PI3K isoforms, concomitant with PTEN, pAKT, pS6, IRS2, pERK, and pAMPK in the above cells. *pS6 levels in RMS-1 cells were below the PhosphorImager detection levels. This image was therefore collected following 10 minutes of exposure to X-ray film. D, quantitation of pERK and total ERK immunoblots shown in C using ImageQuant software, expressed as the ratio pERK/total ERK. Numbers above each bar: in each cell line the ratio of pERK/total ERK in the CONUT cells was set as 1.0 and the relative fold ratio change calculated for each sample. LV, lentiviral.
in cross-talk that substantially increased pAMPK levels and, in the embryonal rhabdomyosarcoma knockdown lines, increased the basal rate of autophagy (LC3II production). These latter data are consistent with disruption of aspects of mTOR signaling in these cell lines. Treatment with NVP-BEZ235, which predominantly inhibits mTOR at concentrations of less than 100 nmol/L (23), and AZD8055, a specific TORC1/2 inhibitor, showed that the p110α stable knockdown cell lines with deregulated IRS2 expression are more sensitive to mTOR inhibition than their CONSH counterparts (Fig. 2B and C). In addition, stable knockdown lines exhibited increased sensitivity to ZSTK474, a pan PI3K inhibitor with selectivity for p110δ, and modest activity against mTor (IC50 of 0.377 μmol/L; ref. 24; Supplementary Fig. S4A and S4E). Although all the stable knockdown lines expressed increased pERK levels, only the RMS-1 knockdown line (lacking both p110α and δ) exhibited significantly enhanced sensitivity to the MEK inhibitor AZD6244 (Fig. 2D). This suggests that p110α and/or p110δ can support compensatory signaling through PI3K/AKT/mTOR on inhibition of the MAPK pathway, whereas p110β cannot.

Overall, these data suggest that not only should pan-PI3K (or AKT) and mTOR activity be inhibited to achieve maximal inhibition of the PI3K/AKT/mTOR pathway, but simultaneous inhibition of the RAS/RAF/ERK pathway to prevent compensatory cross-talk is also necessary to maximize the antiproliferative effect.

**Dual blockade of PI3K/AKT/mTOR and RAS/RAF/ERK signaling is synergistic in rhabdomyosarcoma cell lines**

To determine whether dual blockade of both the PI3K and MAPK pathways yields improved efficacy in
rhabdomyosarcoma cells, combination GI<sub>50</sub> isobolograms were constructed using the parental alveolar rhabdomyosarcoma and embryonal rhabdomyosarcoma cell lines and the combinations AZD8055/AZD6244, ZSTK474/AZD6244, and NVP-BEZ235/AZD6244. The combination AZD8055/AZD6244 was synergistic in the RH30, RD, and RMS-YM cell lines, but nearer additive in the RMS-1 line (the most sensitive line to single-agent PI3K pathway inhibition; Fig. 3A). The embryonal rhabdomyosarcoma cell lines were used to confirm synergy with the combinations

Figure 3. Dual blockade of both the PI3K and MAPK pathways is synergistic in rhabdomyosarcoma cell lines in vitro. A–C, combination isobolograms using the combinations: AZD8055/AZD6244, ZSTK474/AZD6244, and NVP-BEZ235/AZD6244, respectively. The GI<sub>50</sub> values of compound A and B are plotted on the x- and y-axis along with the GI<sub>50</sub> values of compound B obtained in the presence of various fixed concentrations of compound A. The diagonal line drawn between the GI<sub>50</sub> values for the two compounds on the y- and x-axis is the theoretical line of additivity. All GI<sub>50</sub> values to the left of this line indicate synergy. D, levels of pERK1/2, p<sup>Ser473</sup>AKT, and p<sup>Ser240/244</sup>S6 in RD cells following treatment with AZD8055, AZD6244, and AZD8055/AZD6244 in combination at 0.5 x GI<sub>50</sub> and 1.0 x GI<sub>50</sub>. Proteins from control- and drug-treated cells were extracted at the indicated time points following treatment. Control and treated samples from each time point were loaded side by side for Western immunoblot analyses and quantitated using ImageQuant software. Drug-treated/control phosphorylated biomarker levels at each time point are expressed as percentage of levels at time 0. NB, levels of pERK in control samples were below consistently quantifiable levels at 48 hours, when the cells were reaching confluence.
ZSTK474/AZD6244 and NVP-BEZ235/AZD6244 (Fig. 3B and C).

RD cells that have an NRAS mutation (5) and are relatively resistant to both AZD8055 and AZD6244 were selected to investigate the effect of single-agent and combination treatment with AZD8055 and AZD6244 on the biomarkers of PI3K/mTOR pathway (pSer473AKT and pS6) and MAPK pathway (pERK) activity over a 48-hour time course. To more easily assess any synergistic effects on the pathway biomarkers following combination treatment, cells were treated with 0.5× and 1.0× GI50 concentrations. AZD8055 increased pERK levels, and AZD6244 increased pSer473AKT levels after 16 hours of treatment, confirming reciprocal compensatory cross-talk between the PI3K and MAPK pathways following inhibition of each pathway individually (Fig. 3D). The extent of inhibition of S6, but not AKT phosphorylation, was concentration dependent following AZD8055 treatment, indicating that at low concentrations, and with activating cross-talk from the MAPK pathway, TORC1 was more effectively inhibited than TORC2. AZD6244 treatment ablated pERK levels at all time points, and both concentrations and the activation/inhibition profiles of both AKT and S6 phosphorylation, respectively, were similar at both concentrations. These data indicate that growth inhibition following AZD6244 does not correlate with MEK inhibition (IC50 concentration for MEK1/2 of 14 nmol/L; ref. 25), and may be due to off target effects.

AZD6244 in combination with AZD8055 reduced pERK levels to the same extent as AZD6244 alone, whereas pS6 levels were reduced earlier, to a greater extent, and for a longer duration than with either treatment alone (Fig. 3D). AKT phosphorylation was reduced to the same extent as treatment with AZD8055 alone but did not recover to control levels at later time points. These data imply that significant and protracted inhibition of mTORC1 activity is essential for maximal antitumor activity and that coinhibition of ERK and AKT signaling is necessary to achieve this.

Equivalent alterations to biomarkers of the PI3K and MAPK pathways occurred in vivo 6 hours following treatment of mice bearing RD xenografts with AZD8055 (10 mg/kg per os) and AZD6244 (10mg/kg per os) alone, or in combination (Supplementary Fig. S5A). However, levels of all three biomarkers recovered to control levels or above by 16 hours following treatment and correlated with plasma clearance of both drugs to close to, or below, the limit of detection at this time point (Supplementary Fig. S5B). This and further pharmacokinetic analysis (Supplementary Fig. S6) revealed an interaction between both drugs when given in combination. Peak plasma concentrations of AZD6244 were reduced and the elimination phase extended when given in combination with AZD8055, whereas higher plasma and tumor levels of AZD8055 were achieved when given in combination with AZD6244. These changes became more pronounced on repeat dosing. The possibility of interaction at the level of metabolism by P450 enzymes was eliminated using a drug–drug interaction assay (Supplementary Methods; data not shown).

Significantly enhanced antitumor efficacy following treatment of RD xenograft tumors with AZD8055, but not NVP-BEZ235, in combination with AZD6244

A preliminary dose-finding therapeutic study defined a once daily treatment schedule of AZD8055, 20 mg/kg per os, and AZD6244, 10 mg/kg per os, as well tolerated and active when given in combination (Supplementary Fig. S7A–S7D). Previous studies have shown that NVP-BEZ235 treatment of Her2-overexpressing breast cancer cells resulted in compensatory activation of ERK signaling, and that the combination of NVP-BEZ235 (25 mg/kg) and AZD6244 (8 mg/kg) was synergistic in vivo (26). NVP-BEZ235 is a dual PI3K/mTORC1/2 inhibitor at the concentrations (above 500 nmol/L) likely to be achieved in vivo (23), and had the potential to inhibit the compensatory increase in pAKT levels on MEK inhibition more effectively than the specific TORC1/2 inhibitor, AZD8055. Therefore, these two combinations were compared in a head-to-head therapeutic study.

When given alone, NVP-BEZ235 inhibited tumor growth to a greater extent than AZD8055. However, synergism was shown only when using AZD6244 in combination with AZD8055 and not when used in combination with NVP-BEZ235 (Fig. 4A and B). Pharmacokinetic analysis of all three drugs showed that when given in combination, NVP-BEZ235 reduced plasma concentrations of AZD6244 to a similar extent as AZD8055 (Fig. 4C). The plasma concentrations of AZD8055 and NVP-BEZ235 were in the same range when the agents were administered alone, and the concentration range of both drugs was increased when given in combination with AZD6244 (Fig. 4C).

Assessment of the pharmacodynamic biomarkers, pERK, pSer473AKT, and pS6 in treated tumors confirmed reciprocal compensatory signaling on inhibition of either the PI3K pathway by AZD8055 or NVP-BEZ235, or the MAPK pathway by AZD6244 in the in vivo therapeutic setting (Fig. 4D). Individualy, AZD8055 and NVP-BEZ235 were equally efficient in reducing AKT and S6 phosphorylation although AZD8055 increased pERK levels to a greater extent than NVP-BEZ235. However, while treatment with the combination AZD8055/AZD6244 reduced all three biomarkers to less than 30% of control levels, the combination NVP-BEZ235/AZD6244 failed to reduce pAKT below control levels and there was a corresponding reduction in the inhibition of S6 phosphorylation. Thus, the inhibitory potency of NVP-BEZ235 against PI3K and TORC2 was insufficient to block the compensatory upregulation of AKT phosphorylation induced by MEK inhibition in this tumor type in vivo. AZD8055/AZD6244 is therefore the combination indicated to take forward to the clinic.

Discussion

Previous reports have indicated that dual activation of the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways is likely to result in innate resistance to the targeting of either pathway alone (10–13). Although activation of both pathways, individually, has been documented in rhabdomyosarcoma (4, 5, 8, 9), dual activation status has not been assessed.
Using immunohistochemical analysis of TMAs, we have shown dual activation in 43% of primary rhabdomyosarcoma samples, and of these, 55% showed strong activation of mTOR signaling. A higher proportion of alveolar rhabdomyosarcoma than embryonal rhabdomyosarcoma samples stained positively for pAKT in the absence of pERK staining (59% and 29%, respectively). Thus, while theoretically, some patients with alveolar rhabdomyosarcoma might benefit from targeted inhibition of the PI3K pathway, it is less likely to be the case for patients with embryonal rhabdomyosarcoma. Importantly, we provide evidence for compensatory upregulation of the MAPK pathway following PI3K pathway inhibition in rhabdomyosarcoma, as has been shown in other tumor types (15). This has the potential to circumvent the antiproliferative effect of PI3K pathway inhibitors and result in treatment failure.

Small-molecule inhibitors of PI3K mainly target the class 1 PI3Ks, namely p110α, β, δ, and γ, of which only p110α is mutated in cancer (19). PI3K p110α is also the isozyme that is predominantly involved in IGF-IR signaling (commonly activated in rhabdomyosarcoma; ref. 6) and cell growth. Using lentiviral shRNA particles targeted to PIK3CA, we showed that only one of four rhabdomyosarcoma cell lines was growth inhibited following PI3K p110α knockdown, suggesting that a p110α-specific inhibitor would not have general use in the rhabdomyosarcoma clinic. Examination of basal class 1 PI3K isoform expression profiles in rhabdomyosarcoma cell lines revealed uniform expression of p110α, more variable expression of p110β, and no detectable expression of p110γ. Unexpectedly, previously unreported expression of p110δ, generally restricted to cells of hematopoietic origin, was also seen in two of four alveolar

Figure 4. Significantly enhanced antitumor efficacy following treatment of RD xenografts with AZD8055, but not NVP-BEZ235, in combination with AZD6244. A and B, head-to-head therapeutic study of RD xenografts treated with AZD6244 and either AZD8055 or NVP-BEZ235, respectively, at the indicated doses alone, and in combination. Tumor volumes are expressed as a percentage volume of each tumor on day 0. Final tumor weights (g) show significantly increased efficacy of the combination AZD8055/AZD6244 compared with AZD6244 alone (\(P = 0.0159\)) or AZD8055 alone (\(P = 0.038\)) and no significant difference in efficacy of the combination NVP-BEZ235/AZD6244 compared with AZD6244 alone \(P = 0.13\) or BEZ235 alone \(P = 0.82\); Mann–Whitney \(t\) test). C, plasma AZD6244 concentrations 3 hours following the final dose in mice treated with AZD6244 alone or in combination with AZD8055 or NVP-BEZ235 and plasma AZD8055 and NVP-BEZ235 concentrations from the same mice as above. D, tumor pharmacodynamic biomarkers pERK: phospho(TY:202/204:185/187)ERK/total ERK1/2, pAKT: phospho(Ser473)AKT/total AKT and pS6: phospho240/244S6/total S6, as determined by Meso Scale Discovery (MSD) immunoassay. p.o., per os.
Pik3ca knockdown was observed by day 5 in the three signaling (31), have also been reported. A cell lines resistant to the effects of p110
in rhabdomyosarcoma cell lines, neither the expression of p110α nor the overall isoform expression profiles was associated with sensitivity/resistance to p110α knockdown.

Compensatory upregulation and reprogramming of alternative signaling pathways in the short and long term offers clues to the mechanisms of innate and acquired resistance to targeted inhibitors. Here, we have shown that cell line–specific, widespread compensatory and adaptive signaling occurs following Pik3ca knockdown, even in the absence of any overt phenotypic effect. Using various PI3K pathway inhibitors and a MEK inhibitor to inhibit these bypass mechanisms in stable p110α knockdown lines, we have determined which elements of the PI3K and MAPK pathways must be inhibited to achieve a maximum anti-proliferative effect.

In the short term, p110α knockdown induced compensatory upregulation of p110β although this was not maintained in the p110α knockdown stable lines. However, significant upregulation of p110β was seen in the embryonal rhabdomyosarcoma stable knockdown lines, which highlights the plasticity of PI3K isoform expression and supports functional redundancy, particularly between the p110α and Δ isoforms. No difference in the sensitivity to the pan-PI3K inhibitor, BMK120, was seen in the stable p110α knockdown lines compared with their CONSH counterparts, and only the RMS-1 knockdown line, with minimal p110α and Δ expression, showed increased sensitivity to the selective p110β inhibitor, TGX2221. These data suggest that pan-PI3K inhibitors should possess potent activity against all class 1A isoforms to ensure maximum growth inhibition in rhabdomyosarcoma.

AKT phosphorylation was not consistently inhibited following p110α knockdown despite early inhibition of S6 phosphorylation in all four cell lines. Of note, we showed increased p
AKT, delayed gross inhibition of pAKT, and downregulation of the phosphatase, PTEN, in RH30 cells following Pik3ca knockdown, a novel observation. In contrast, early inhibition of AKT phosphorylation and upregulation of PTEN expression was seen in the other three lines. Transcriptional regulation of PTEN has been shown previously in various settings, but the functional roles and mechanisms responsible remain poorly understood. For example, decreased PTEN expression on inhibition of mTOR has been reported in cells with loss or inactivation of Tsc2 (29), whereas upregulation of PTEN in response to activation of c-Jun-NH2 kinase (JNK; ref. 30), and repression of PTEN expression by activated NFκB signaling (31), have also been reported.

Compensatory upregulation of pERK levels following Pik3ca knockdown was observed by day 5 in the three cell lines resistant to the effects of p110α knockdown, but this was delayed in the sensitive RMS-1 cells. However, increased levels of ERK phosphorylation were seen in all four stable knockdown lines. A lack of early stimulation of ERK signaling, together with extremely low levels of basal mTOR activity, may underpin the initial sensitivity of RMS-1 cells to Pik3ca knockdown, whereas the subsequent upregulation of ERK signaling may have contributed to their ultimate recovery and survival. Although previous reports have suggested that activated MAPK signaling mediates resistance to PI3K inhibitors, these studies indicate that it is not necessarily the basal rate of MAPK activity that dictates resistance to PI3K pathway inhibitors, but rather the degree and kinetics of compensatory cross-talk. Thus, while all four stable Pik3ca knockdown lines exhibited higher levels of pERK they were not more resistant to any of the PI3K pathway inhibitors than their CONSH counterparts. Similarly, increased activation of MAPK signaling did not alter the sensitivity of three of the four stable knockdown lines to MEK inhibition. Only the RMS-1 knockdown line, expressing 110β but not p110α or Δ, exhibited increased sensitivity to the MEK inhibitor AZD6244. This suggests that p110β does not support compensatory activation of the PI3K pathway following MEK inhibition, whereas p110α and Δ do.

Activated ERK signaling has been shown to activate mTOR by phosphorylation of both Tsc2 and Raptor (32), and also to activate AMPK resulting in increased retinoblastoma (Rb) phosphorylation and stimulation of cell growth (21). In addition, activated AMPK acts as a survival factor protecting cells from hypoxia and nutrient deprivation through increased glucose uptake and a shift to anaerobic respiration with altered intermediary metabolism (the Warburg effect), often accompanied by inhibition of protein synthesis and cell growth through suppression of mTOR signaling (reviewed in ref. 33). Activation of AMPK along with deregulated IRS2 expression, and an increased basal rate of autophagy (the latter prominent in the embryonal rhabdomyosarcoma lines), suggests that mTOR signaling is partially compromised in the stable p110α knockdown lines. Importantly, these cells are more sensitive to TORC1/TORC2 inhibitors, suggesting that mTOR is a central node for integrating cross-talk between the PI3K and MAPK pathways and an important target element for maximal PI3K pathway inhibition.

Dual blockade of both the PI3K and MAPK pathways was shown to be synergistic in vitro and was shown to be due to the reciprocal inhibition of the compensatory activation of the alternate pathway seen following inhibition of each pathway individually. As predicted, treatment of the NRAS-mutated RD tumor xenografts in vivo with the TORC1/TORC2 inhibitor AZD8055 or the MEK inhibitor AZD6244, showed no therapeutic benefit, whereas the dual PI3K/mTOR inhibitor NVP-BEZ235 was more active. However, in combination, AZD8055/AZD6244 resulted in significant inhibition of tumor growth, whereas NVP-BEZ235/AZD6244 resulted in no additional benefit over treatment with NVP-BEZ235 alone. This was shown to be due to the inability of NVP-BEZ235 to inhibit the compensatory activation of AKT induced by MEK inhibition. Therefore, the
biomarkers of effective activity are the simultaneous reduction of pAKT, pS6, and pERK.

Using data collected from phase I clinical trials, a recent study has evaluated the clinical outcome of dual targeting both the PI3K and MAPK signaling pathways compared with targeting either pathway alone (34). The results showed increased efficacy in many tumor types but at the cost of additional toxicity. Although the in vivo combination doses used here were well tolerated, pharmacokinetic analysis revealed an interaction between the two classes of compounds, resulting in lower plasma and tumor levels of AZD6244 but higher levels of the PI3K pathway inhibitors, both effects becoming more pronounced with repeat dosing. However, the concentrations of AZD6244 achieved in vivo were still in excess of levels required for MEK inhibition. Further pharmacokinetic/pharmacodynamic-driven preclinical studies to identify the lowest AZD6244 dose required for MEK inhibition, followed by escalation of AZD8055 to achieve therapeutic efficacy, will help inform early clinical trials. In addition, careful monitoring of plasma pharmacokinetics of both drugs following repeated dosing will be required to assess the extent of drug–drug interactions in patients.

In summary, in the preclinical proof-of-principle rhabdomyosarcoma xenograft studies presented here, the combination of AZD8055 and AZD6244 showed significantly increased therapeutic benefit over the combination NVP-BEZ235/AZD6244. We show that the three phosphorylated biomarkers of ERK, S6, and AKT must be reduced for synergistic activity. These studies confirm that dual inhibition of both the PI3K and MAPK pathways offers a way forward for the treatment of those tumor types such as rhabdomyosarcoma that are predicted to be resistant to blockade of either pathway alone. Even in innately sensitive tumors, addition of a MEK inhibitor to a PI3K inhibitor may forestall the emergence of resistance.

Disclosure of Potential Conflicts of Interest
S.A. Eccles is an employee of ICR, which has a commercial interest in the discovery and development of anticancer drugs, including PI3K and Akt inhibitors, and operates a Rewards to Inventors scheme. No potential conflicts of interest were disclosed by the other authors.

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