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Rapamycin Rescues ABT-737 Efficacy in Small Cell Lung Cancer

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Abstract

Overexpression of the antiapoptotic protein Bcl-2 is observed in the majority of small cell lung cancer (SCLC) cases and is associated with resistance to chemotherapy. While targeting Bcl-2 in hematologic malignancies continues to show signs of promise, translating the BH3 mimetic ABT-737 (or ABT-263; navitoclax) to the clinic for solid tumors has remained problematic, with limited single-agent activity in early-phase clinical trials. Here, we used patient-derived xenograft (PDX) models of SCLC to study ABT-737 resistance and demonstrated that responses to ABT-737 are short lived and coincide with decreases in HIF-1 α -regulated transcripts. Combining the mTOR inhibitor rapamycin with ABT-737 rescued this resistance mechanism, was highly synergistic *in vitro*, and provided durable tumor regressions *in vivo* without notable hematologic suppression. In comparison, tumor regressions did not occur when ABT-737 was combined with etoposide, a gold-standard cytotoxic for SCLC therapy. Rapamycin exposure was consistently associated with an increase in the proapoptotic protein BAX, whereas ABT-737 caused dose-dependent decreases in BAX. As ABT-737 triggers programmed cell death in a BAX/BAK-dependent manner, we provide preclinical evidence that the efficacy of ABT-737 as a single agent is self-limiting in SCLC, but the addition of rapamycin can maintain or increase levels of BAX

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protein and markedly enhance the anticancer efficacy of ABT-737. These data have direct translational implications for SCLC clinical trials.

Introduction

Small cell lung cancer (SCLC) represents 12% to 15% of all lung cancers and accounts for nearly 30,000 deaths in the United States annually (1). Etoposide plus cisplatin was established as the standard of care for SCLC in the 1980s and can confer high response rates (>60%) as initial therapy (2). However, therapy-refractory recurrence is nearly universal and, as a consequence, SCLC has one of the highest case fatality rates among cancer—a statistic that has not changed significantly over the past 30 years (3). Since 2003, only one agent, topotecan, has received approval by the U.S. Food and Drug Administration for SCLC. New therapies are in critical need.

One targetable opportunity is in the overexpression of the antiapoptotic protein Bcl-2. Between 60% to 90% of SCLC cases have been reported to express high Bcl-2 protein (4, 5) and this is thought to be a mechanism by which SCLC resists programmed cell death. Through structure-guided medicinal chemistry efforts, the Bcl-2 inhibitor ABT-737 emerged as the prototypic small-molecule candidate to exploit overexpression of Bcl-2 in cancer (6). ABT-737 most closely simulates the proapoptotic protein Bad from a functional standpoint and serves as a competitive BH3 mimetic with high affinity to the antiapoptotic proteins Bcl-2, Bcl-xL, and Bcl-w. Thus, when targeting Bcl-2 was possible with ABT-737 and the orally bioavailable derivative ABT-263 (navitoclax), efforts were made to translate preclinical findings in SCLC to patients (7, 8).

However, early-phase clinical trials investigating ABT-263 as a single agent in SCLC failed to show the dramatic responses that were observed in preclinical SCLC cell line xenograft models (7, 9, 10). In clinical studies of ABT-263, the inhibition of Bcl-xL in platelet populations yielded dose-dependent thrombocytopenia and has somewhat limited the utility of this high-affinity Bcl-2/Bcl-xL inhibitor. It is becoming increasingly evident that targeting certain Bcl-2 family members in a tumor must be considered in the context of other lineage-specific dependencies to minimize toxicities (11). Moreover, although lead-in dosing strategies with ABT-263 could lessen the observed thrombocytopenia, inhibiting both Bcl-2 and Bcl-xL with concurrent cytotoxic chemotherapy has added complexity to maximizing therapeutic combinations with ABT-263.

Strategies to model resistance to ABT-737/263 in SCLC and other tumor types have identified the antiapoptotic protein MCL-1 as a major culprit, in which MCL-1 can serve a functionally redundant role in sequestering the proapoptotic proteins BAX and BAK and is not targeted by ABT-737. Efforts to increase the sensitivity of various cancers to ABT-737 have focused on mechanisms that affect MCL-1, either through direct targeting or exploiting the relatively short half-life of the MCL-1 protein (12, 13). Many compounds shown to synergize with ABT-737 in SCLC and other cancers are global transcriptional or translational repressors, such as anthracyclines (14, 15); however, combining these agents in the clinic could prove challenging due to toxicity. Although other antiapoptotic proteins not targeted by ABT-737 have been implicated in resistance to ABT-737, the major

antiapoptotic players observed to be amplified across many cancer types are Bcl-2, Bcl-xL, and MCL-1 (16). How these antiapoptotic regulators govern the activity of proapoptotic BAX and BAK with other apoptotic effectors in the context of a given tumor help to define a cancer hallmark—resistance to programmed cell death (17).

We previously reported that ABT-737 could induce dramatic responses in SCLC cell lines, in agreement with what others have shown; however, ABT-737 alone failed to produce substantial tumor responses in SCLC patient-derived xenograft (PDX) models that express low levels of Bcl-2 (18). Here, we show that Bcl-2-expressing SCLC PDXs initially respond to ABT-737, but the responses are not durable and the addition of etoposide does not improve responses. We used these PDX models to investigate the transcriptional changes that occur during initial responses to ABT-737 to develop strategies to increase the efficacy of ABT-737. We found that the mTOR inhibitor rapamycin could enhance the antitumor activity of ABT-737 *in vitro* and *in vivo*, rescuing the self-limiting effects of single-agent ABT-737. We further observed that this combinatorial activity correlated with an increase in proapoptotic BAX.

Materials and Methods

Cell lines and reagents

SCLC cell lines were purchased from American Type Culture Collection and maintained as recommended. All cell lines tested negative for *mycoplasma* (Lonza; MycoAlert) and were short tandem repeat verified at the Johns Hopkins Fragment Analysis Facility within 6 months of use. ABT-737 was obtained from Abbott Laboratories (now Abbvie) and purchased from Active Biochem (A-1002). For *in vivo* use, ABT-737 and etoposide (Accord Healthcare, Inc.) were prepared as previously described (18). Rapamycin (LC Laboratories) was stored in 100% ethanol at 50 mg/mL. For *in vivo* use, rapamycin vehicle was 82% PBS/5% Tween-80/5% polyethylene glycol 400/8% ethanol. AZD8055 (S1555), BEZ235 (S1009), everolimus (S1120), LY294002 (S1105), and wortmannin (S2758) were purchased from Selleck Chemicals. Dimethyl sulfoxide (DMSO) was used as a vehicle for all *in vitro* experiments.

Cell proliferation and viability assays

Cells were plated 24 hours before treatments in 96-well plates at 1,000 to 5,000 cells per well. Proliferation (CellTiter 96 AQueous One; Promega) and viability (CellTiter-Glo; Promega) assays were quantified on a compatible plate reader (SpectraMax M2e; Molecular Devices). All combination and drug synergy experiments were performed for 72 hours before assaying.

Assessment of drug synergy

Drug synergy was determined quantitatively using the combination index (CI) method of Chou and Talalay (19). Viability was calculated across a wide range of doses for both ABT-737 and each compound of interest using a nonconstant ratio. CI was calculated as a function of response or fraction affected (Fa) using the formula $[CI = (D_1)/(D_x)_1 + (D_2)/(D_x)_2]$, in which D_1 and D_2 are the doses used to achieve a specific response in combination

and $(D_x)_1$ and $(D_x)_2$ are individual drug doses needed to achieve similar response. A CI >1 indicates antagonism, whereas a CI <1 indicates synergism.

Pimonidazole staining and immunohistochemistry

Mice were injected with Hypoxyprobe-1 solution (Hypoxyprobe, Inc.) approximately 1 hour before sacrifice. Tumors were fixed in formalin, embedded in paraffin, and sectioned at 5- μ m thickness. Sections were dewaxed and incubated in citrate, pH 6.0 (Vector Laboratories; H-3300) for 25 minutes. Sections were stained using the Dako EnVision Plus Detection System with rabbit PAb2627 (Hypoxyprobe; HP3-100Kit) for 45 minutes at 25°C and were visualized with 3,3'-diaminobenzidine reagent (Sigma; D423). Hematoxylin and eosin (H&E) staining was performed with an automated slide stainer (Leica) with Modified Harris Hematoxylin (Richard-Allan Scientific).

Isolation of heavy membranes

Separation of crude mitochondria ("heavy membranes") from cytosol in cell lines and tumor tissue was performed using a QProteome Mitochondrial Isolation Kit (Qiagen). Samples were lysed in radioimmunoprecipitation assay buffer supplemented with protease/phosphatase inhibitor cocktails (Sigma-Aldrich) for 30 minutes on ice before clarification at 13,000 rpm for 10 minutes at 4°C. Protein concentrations were determined using the bicinchoninic acid method (microBCA; Pierce). A list of antibodies used for Western blots is provided in Supplementary Methods.

SCLC PDX models and dosing

All *in vivo* experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University (Baltimore, MD). The LX33 and LX48 PDXs were isolated and passaged as previously described (18, 20, 21). LX47 PDXs were passaged as tumor tissue sections (~3 mm) coated in Matrigel and implanted via subcutaneous flap incisions. All treatment experiments were performed in female C.B-17 severe combined immunodeficient (scid) mice, 4 to 6 weeks old at time of PDX injection/implantation (Taconic; C.B-*Igh-1^b/IcrTac-Prkdc^{scid}*). The LX33 and LX47 PDXs were derived from chemotherapy-naïve patients with SCLC and the LX48 PDX from a patient previously treated with platinum plus etoposide. Tumor volumes were calculated from manual caliper measurements with an ellipsoid formula in which volume (mm^3) = $(xy^2)/2$. Once tumor volumes reached approximately 150 mm^3 , mice were randomized to treatment arms and treated daily via intraperitoneal injections with vehicles, ABT-737 (100 mg/kg), rapamycin (20 mg/kg), etoposide (12 mg/kg on days 1, 4, and 9), or combinations of two agents.

RNA isolation and genome-wide transcriptional profiling

Total RNA was prepared using an RNeasy Mini Kit (Qiagen) with on-column DNase I digestion. RNA quality was assessed with a Nanodrop-1000 for OD260/280 and OD260/230 ratios and an Agilent 2100 Bioanalyzer. Total RNA (500 ng) from each sample was amplified and labeled using an Illumina TotalPrep RNA amplification kit according to the manufacturer's protocol (Ambion). Biotinylated RNA (750 ng) was combined with

hybridization buffer and hybridized to HumanHT-12 v4 Expression BeadChip arrays (Illumina) for 16 to 20 hours at 58°C. Arrays were washed at 55°C and blocked at 25°C. Bound biotinylated RNA was stained with streptavidin-Cy3 and washed. Dried arrays were protected from light until scanning with an iScan System. Data were extracted using the Gene Expression Module in Genome-Studio Software. Gene expression data are deposited into Gene Expression Omnibus (GEO) under accession IDs pending.

Gene expression analysis

Using the Bioconductor suite for R (22) and the limma package (23), raw gene expression data were quantile normalized, then $\log_2$ transformed. Differentially expressed genes were identified by fitting a linear model for each feature. SEs of $\log_2$ transformed changes were moderated using an empirical Bayesian approach. For each feature, moderated t statistics, B statistics, raw, and adjusted P values (corrected for multiple testing by the Benjamini and Hochberg method) were calculated. Permutation-based gene set enrichment was performed using gene set enrichment analysis (GSEA), a sample permutation-based method, and CAMERA, a competitive gene set testing method that accounts for intergene correlation (24, 25). The gene set database used was MSigDB 3.1 from the Broad Institute (Cambridge, MA; ref. 26).

Hematology

Whole blood was collected immediately following cervical dislocation in C.B-17 scid mice via cardiac puncture using tuberculin syringes flushed with 1 mol/L EDTA, immediately transferred to K₂EDTA lavender tubes (BD Microtainer), and stored at 4°C for less than 24 hours before analysis. Complete blood count with differential parameters was measured on a Hemavet 950FS (Drew Scientific) at the Johns Hopkins Phenotyping Core.

Statistical analysis of drug efficacy experiments

All animal data are reported as average tumor volumes $\pm$ SD. Tumor volume comparisons between treatment groups used data from weekly time points using a two-sided Student t test; throughout the text unless indicated: *, $P < 0.01$ and **, $P < 0.001$. All graphs in figures were created using GraphPad Prism 6.0c (GraphPad Software, Inc.). Figures for drug combination matrix outputs and drug synergy were created in R, as previously reported (27).

Results

Treatment with ABT-737 results in short-lived tumor responses and is associated with a decrease in HIF-1 α transcripts

We previously reported that ABT-737 had limited activity alone and in combination with etoposide in SCLC PDXs that express low levels of Bcl-2 (18). To further assess the activity of ABT-737 in a more representative set of PDXs, we extended these studies to include three Bcl-2-expressing SCLC PDX models—LX44, LX47, and LX48—as well as a SCLC PDX model that does not express appreciable Bcl-2 for historic control (LX33). Treatment with ABT-737 resulted in modest antitumor activity with statistically significant tumor growth inhibition (TGI) in LX47 and LX48, but minimal effects in LX44 and LX33 over a period of 3 weeks where ABT-737 was administered daily (Fig. 1A). As shown in Fig. 1B,

all PDX models express other antiapoptotic targets of ABT-737, as well as MCL-1. Treatment with ABT-737 was more effective than etoposide, a standard of care therapeutic agent for SCLC (Fig. 1C). The addition of etoposide to ABT-737 did not improve responses in PDXs derived from chemotherapy-naïve (LX47) or recurrent (LX48) tumors and was poorly tolerated by mice as evidenced by weight loss while on treatment (Supplementary Fig. S1A and S1B). Notably, we observed that responses to ABT-737 in these PDXs were short-lived; minimal regressions during continuous treatment progressed at growth rates comparable with vehicle-treated tumors after treatment discontinuation.

To investigate potential acute resistance mechanisms to ABT-737, we selected LX47 and LX48 for further characterization of responses. We hypothesized that during tumor response, a sensitivity signature may exist and precede tumor outgrowth in such models. Using whole-genome transcriptional profiling, we compared the gene expression profiles of tumors from vehicle- and ABT-737-treated mice collected at points of maximal response (Supplementary Table S1). Transcriptional profiling results comparing ABT-737-treated with vehicle-treated tumors revealed that some of the most differentially expressed genes were well-established HIF-1 α transcriptional targets, such as *LDHA* and *BNIP3L* (Fig. 1D and Supplementary Table S2). GSEA revealed that HIF-1 α gene sets comprised the majority of the most statistically significant gene sets downregulated in ABT-737-treated tumors (Supplementary Table S3). To further illustrate the changes observed in HIF-1 α transcripts, we plotted the log ratio of treatment groups (M) by the average intensity (A) for each probe in order to visualize how genome-wide transcriptional profiling data from the ABT-737-treated PDX models compared with a benchmark HIF-1 α -regulated gene set (28). Many transcripts that are normally upregulated by hypoxia (Fig. 1D, bottom; green) were decreased upon ABT-737 treatment, whereas transcripts downregulated by hypoxia (Fig. 1D, bottom; magenta) were unchanged. There are data supporting that ABT-737 is more effective under hypoxic conditions *in vitro* as well as *in vivo* and that hypoxia is a negative regulator of MCL-1 half-life (29, 30). We did not observe changes in MCL-1 transcript or protein levels during acute treatments with ABT-737 in LX47 or LX48. Although transcriptional profiling data suggested that ABT-737-treated tumors lack expression of hypoxia-induced genes, pimonidazole staining to assess regions of physiologic hypoxia at time points within the window of maximal response to ABT-737 revealed no appreciable difference between treatment groups (Fig. 1E).

A rapamycin signature is predicted to connect with ABT-737 response and shows synergy with ABT-737 *in vitro*

To identify agents or pathways that are associated with responses to ABT-737 in Bcl-2-expressing PDX models, we queried the Broad Institute's publicly available Connectivity Map database (cMap build 2.0) using the most significantly differentially expressed genes between control- and ABT-737-treated tumors (Supplementary Table S2). The cMap database attempts to connect gene expression data to chemical perturbation by cataloging gene expression changes that result from exposures to bioactive compounds *in vitro* (31). This approach is advantageous in the setting of data from whole-genome profiling experiments in which the contributions of individual transcripts may not hold clear significance to an overarching pathway or group of pathways being affected, and thus it

provides more generalizable strategies to affect an observed profile. Several of the highest scoring bioactive compounds identified by our cMap query to align with an ABT-737 response were inhibitors of the phosphoinositide 3-kinase (PI3K)/mTOR pathway (Supplementary Table S4)—pathways known to regulate and interact with HIF-1 α (32).

Taken together with literature supporting the combined effect of PI3K/mTOR and Bcl-2 inhibition in other tumor types (33–36) and the mounting evidence for rationally cotargeting growth and survival pathways, we tested the combination of rapamycin and ABT-737 in two SCLC cell lines that express high levels of Bcl-2 (NCI-H146 and NCI-H187) and two lines that do not express appreciable Bcl-2 (NCI-H82 and NCI-H446; ref. 6). As expected, treatment with ABT-737 resulted in dose-dependent decreases in viability in lines that express Bcl-2 with little effect on lines that do not express Bcl-2 (Fig. 2A). Bcl-2-expressing lines were also more sensitive than non-Bcl-2-expressing lines to rapamycin. This effect was not dose-dependent after 72 hours of exposure in the dose ranges studied (low nanomolar to micromolar) and we confirmed this trend in a larger set of SCLC cell lines (Supplementary Fig. S2A). Combining increasing doses of ABT-737 with 100 nmol/L rapamycin had minimal effect on low expressing Bcl-2 lines, but was at least additive in Bcl-2-expressing lines. However, treating ABT-737-sensitive cell lines with 100 nmol/L rapamycin for 24 or 72 hours before exposure to ABT-737 did not dramatically alter the sensitivity of these lines to ABT-737 (Fig. 2B), suggesting that rapamycin does not acutely sensitize cell lines to ABT-737.

To demonstrate whether the combined effect of rapamycin and ABT-737 was synergistic, we focused on two cell lines that differed in their expression of Bcl-2 (NCI-H146 and NCI-H82) and treated cells with ABT-737 and drugs targeting PI3K (wortmannin; LY294002), mTOR (rapamycin; everolimus; AZD8055), or both (BEZ235), selected based on our cMap results (Supplementary Fig. S2B and S2C). Using broad dose combinations, we quantitatively calculated synergy for each drug combination across a range of doses using the method of Chou and Talalay and a nonconstant ratio approach (19). Representative dose-matrix outputs for the combination of ABT-737 and rapamycin in NCI-H82 and NCI-H146 are shown in Fig. 2C. The agents studied had varying potency that was generally segregated by inhibitor class (Fig. 2D). Synergism in NCI-H146 is summarized in Fig. 2E by plotting the CI as a function of Fa (37). For example, wortmannin showed no synergy at any dose, whereas all other compounds showed quantitative synergism. The mTOR inhibitors rapamycin and everolimus displayed strong synergism over a wide dose-response range. We felt that these data warranted testing rapamycin in combination with ABT-737 *in vivo*.

Rapamycin has combinatorial activity with ABT-737 in multiple SCLC PDX models

We next assessed the combination of ABT-737 and rapamycin in the same PDXs in which we observed acute resistance to ABT-737—LX47 and LX48—as well as in a SCLC PDX that does not express appreciable Bcl-2 protein (LX33) that we previously reported had little response to ABT-737 (18). All PDX models examined express PI3K/mTOR pathway components and exhibit pathway activation as demonstrated by basal levels of phospho-Akt (S473), phospho-4E-BP1 (T37/46), and phospho-ribosomal protein S6 (S235/6; Supplementary Fig. S3A). In both Bcl-2-expressing PDXs, treatment with either ABT-737

or rapamycin caused significant TGI and the combination of ABT-737 and rapamycin caused tumor regressions. Importantly, these responses were sustained well beyond the 14-day treatment period, with no detectable tumor masses in LX47 PDXs for several weeks after combination treatment was discontinued, though tumors eventually returned (data not shown). Consistent with our previous report, treatment of LX33 PDXs with ABT-737 did not affect tumor growth. Treatment of LX33 with rapamycin resulted in TGI and the addition of ABT-737 to rapamycin caused a modest increase in TGI over rapamycin alone (Fig. 3C). Although this combined effect lost statistical significance after 1 week of treatment, these data suggest there was some degree of combinatorial activity in the absence of high Bcl-2 expression.

Mice tolerated the combination well, with no apparent weight loss across all 3 PDX models (Supplementary Fig. S3B). As both ABT-737 (38, 39) and rapamycin (40) are known to affect platelets, we assessed hematologic measures in mice bearing LX47 after 7 days of treatment. Platelet counts in ABT-737-treated mice were significantly lower than those of vehicle-treated mice as expected; however, hematologic parameters from combination-treated mice were similar to vehicle and rapamycin alone (Fig. 3D, Supplementary Fig. S3C). All animals that received rapamycin were slightly anemic. Histologically, tumors from mice treated with ABT-737 or combination exhibited greater necrosis than did vehicle- or rapamycin-treated tumors as evidenced by H&E sections showing increased eosinophilic regions throughout the tumor core (Fig. 3E; ABT-737 H&E section). Pimonidazole staining of tumors from each treatment arm showed consistent, detectable hypoxic regions in all samples except tumors treated with combinatorial therapy. This striking difference was observed in multiple tumors treated with combination at early (after 3 days) and later (after 7 days) time points. Because this combination seemed to be well tolerated, we tested whether we could obtain similar tumor regressions in LX47 PDXs previously exposed to either ABT-737 or rapamycin. We observed tumor volume reductions of approximately 50% with 2 weeks of ABT-737 and rapamycin treatment that were sustained after treatment discontinuation, adding strength to the potential utility of this combination (Fig. 3F).

Rapamycin induces expression of HIF-1 α targets in SCLC PDXs

To study the effects of rapamycin alone or in combination with ABT-737 on HIF-1 α targets, we compared protein and message expression in LX47 and LX33 PDXs after 7 days of treatment. In LX33, there was a notable decrease in tumor levels of HIF-1 α protein and several HIF-1 α targets such as CXCR4, Glut1, and VEGF-A in ABT-737-treated compared with vehicle-treated mice (Fig. 4A). Treatment with rapamycin increased the protein levels of most targets examined; however, these changes were less evident in LX47. In LX47, but not LX33, combination treatment resulted in significant decreases in HIF-1 α , HIF-1 β , LDHA, and CXCR4 levels. One reason for these observations may relate to differences in tumor response: combination-treated LX47 tumors lose >70% of their starting volume by day 7, which may complicate analysis of protein samples.

Correlating these findings with transcriptional profiling data, we observed decreased levels of six HIF-1 α -regulated genes in LX47 after treatment with ABT-737 (Fig. 4B); this effect was less pronounced in LX33, but nonetheless, observed for most genes. Interestingly,

rapamycin increased transcript levels of nearly all highlighted genes. In the combination arm, levels of nearly all transcripts were less than vehicle controls, and in some cases (i.e., VEGF-A in LX33) lower than ABT-737–treated groups.

Although this paired analysis of changes highlighted several similarities in the few targets examined, we sought to better understand whether there was a more general transcriptional response to ABT-737. This led us to examine the overall concordance between gene expression changes observed in LX33 and LX47 in response to ABT-737, as treatment influenced protein and transcript levels in these SCLC PDXs independent of Bcl-2 expression. We generated a concordance at the top (CAT) plot to compare drug treatment effects in each model (41). This plot relates the concordance between the top differentially expressed gene lists of n length for each model compared with what would be expected by chance. The ABT-737 treatment signature was more concordant than rapamycin alone, combination treatment, or what would be expected by chance (Fig. 4C). These results were surprising, as we anticipated that there would have been a more pronounced concordance between models treated with rapamycin than ABT-737, as the effects on HIF-1 α –regulated genes were similar in the rapamycin-treated groups as compared with the ABT-737–treated groups. One potential explanation could be that this ranked approach does not account for the magnitude of gene expression differences, nor does it weigh P values for each comparison.

The differences observed prompted further investigation into how the mTOR pathway was affected in these two models. In LX33, treatment with ABT-737 decreased protein levels of several mTOR components, including near complete loss of G β L by Western blot (Fig. 4D). This may reflect the extent to which the mTOR pathway is influenced by HIF-1 α in this PDX model, as we observed a similar loss of HIF-1 α and HIF-1 β /ARNT in LX33. In both PDXs, treatment with rapamycin—alone or in combination with ABT-737— decreased levels of phospho-Akt (S473) versus the vehicle controls; this effect was most pronounced for the combination treatment in LX33. Although we observed mTOR inhibition at the level of Akt, the levels of phospho-ribosomal protein S6 differed between rapamycin-treated groups, but were similar between combination treatment groups. This difference may reflect the degree of Akt feedback that is present when treating with rapamycin alone or in combination with ABT-737 (42). Indeed, levels of phospho-Akt (T308) in LX47 treated with rapamycin seem to be elevated as compared with vehicle, but not in the context of the combination (Fig. 4D).

Rapamycin blocks ABT-737–induced decrease in BAX protein levels in SCLC

In parallel, we looked at protein expression of candidate Bcl-2 family members in LX33 and LX47 PDXs at day 7 by Western blot. We did not observe consistent changes in protein levels of previously reported determinants of ABT-737 sensitivity (MCL-1 or proapoptotic proteins Puma and Noxa) that correlated with response in our PDXs models. However, we did observe that the proapoptotic proteins BAX and BAK were increased in both PDXs treated with rapamycin alone or in combination with ABT-737 (Fig. 5A).

As ABT-737 is known to cause programmed cell death in a BAX/BAK-dependent manner, and BAX is a key proapoptotic binding partner of Bcl-2 (43), we assessed the effect of

ABT-737 or rapamycin on BAX protein levels *in vivo*. For these analyses, we selected LX33 that allowed for more control in size matching tumor volumes across treatment groups. We treated mice bearing LX33 for 7 days with vehicle, escalating doses of ABT-737 (5, 25, and 100 mg/kg), escalating doses of rapamycin (1, 5, and 20 mg/kg), or the combination of the highest doses of each agent. We observed a modest, but dose-dependent decrease in BAX protein from tumors treated with ABT-737 and an overall increase in BAX protein in tumors treated with rapamycin (Fig. 5B). This change was also observed in heavy membranes isolated from tumors, enriching for mitochondria. Although we had small numbers of mice per dose-escalation group, we noted that all rapamycin doses caused TGI to a similar degree, consistent with our *in vitro* data (Supplementary Fig. S4A). We also observed an increase in the antiapoptotic proteins Bcl-xL and Bcl-w with increasing doses of rapamycin (Fig. 5B). Levels of MCL-1 protein were not significantly decreased by rapamycin *in vivo*, where MCL-1 levels were actually greater in combination-treated tumors (Supplementary Fig. S4B). Furthermore, ABT-737 caused a dose-dependent decrease in HIF-1 β /ARNT (Supplementary Fig. S4C). We also found that in SCLC cell lines, exposure to rapamycin was associated with an increase in the amount of BAX present in the heavy membrane fraction of cells, most prominent in NCI-H146 (Fig. 5C). This increase in BAX occurred without an apparent release of cytochrome C into the cytosol, suggesting that an increase in membrane-associated BAX in these lines was not by itself associated with greater programmed cell death, but that multiple mechanisms may be at play for the combined effect of rapamycin and ABT-737.

Discussion

Here, we report that the BH3 mimetic ABT-737 has limited single-agent efficacy in Bcl-2-expressing SCLC PDXs, consistent with the modest clinical activity of ABT-263 observed in patients with recurrent SCLC. Using PDX models as a platform to study ABT-737 resistance, we found that acute treatment with ABT-737 was associated with a decrease in many HIF-1 α -regulated transcripts. Rapamycin effectively blocked this decrease and increased message and protein levels of many of these genes. Combining rapamycin with ABT-737 was highly synergistic *in vitro* and provided durable tumor regressions in Bcl-2-expressing PDX models *in vivo*. We observed a decrease in BAX protein levels upon exposure to ABT-737 that was prevented by rapamycin. As BAX protein levels are regulated by hypoxia through HIF-1 α -dependent and independent mechanisms (44), we were surprised to see changes in BAX levels upon exposure to ABT-737 or rapamycin were not associated with observable changes in physiologic hypoxia in tumors as compared with controls. This may in part be due to the basal levels of hypoxia in these SCLC PDX models, as heterogeneity within a given PDX may complicate mechanistic analysis.

Loss of BAX is a known mechanism of resistance to several classes of anticancer agents (45, 46) and can promote tumorigenesis in certain models (47). In human cancers, loss of BAX through mutation (48) or regulation of protein stability (49) is associated with events promoting tumor development and correlates with poor prognosis. Here, the observed changes in BAX protein after rapamycin exposure align well with known mechanisms by which ABT-737 induces apoptosis and with studies of genetic determinants of ABT-737 lethality (6, 50). The most straightforward interpretation of these data is that acute treatment

with ABT-737 is self-limiting in nature, possibly through global decreases in critical HIF-1 α -regulated transcripts and the proapoptotic protein BAX, both of which are effectively rescued or restored upon the addition of rapamycin (Fig. 5D). However, multiple mechanisms are likely involved, warranting further investigation.

Of clinical relevance, the LX47 and LX48 PDXs were derived from chemotherapy-naïve and recurrent SCLC patient tumors, respectively; thus, the combination of ABT-737 and rapamycin was active independent of prior chemotherapy exposure and sensitivity to etoposide in our models. The observations made here have clear implications for clinical trials in patients with SCLC. The efficacy of single-agent ABT-737 or its orally bioavailable derivative ABT-263 is limited, both in PDX models and in patients with SCLC. Previous efforts to combine ABT-263 with cytotoxic chemotherapy in patients with SCLC were stopped due to unacceptable levels of hematologic suppression. The combination of ABT-737 with rapamycin results in sustained antitumor activity in PDX models, without significant hematologic toxicity or weight loss, and in fact, the thrombocytopenia of single-agent ABT-737 was abrogated in the presence of rapamycin. The mechanisms responsible for this potent combinatorial activity may include maintained tumor expression of BAX, a key regulator of apoptotic induction. These data strongly support assessment of combined targeted inhibition of Bcl-2 and mTOR pathway in patients with SCLC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Responses to ABT-737 in PDX models of SCLC are short-lived and progression is preceded by a decrease in HIF-1α transcripts. A, tumor response curves in three Bcl-2–expressing (left to right; LX44, LX47, and LX48) and one Bcl-2–nonexpressing (LX33) SCLC PDX models treated with ABT-737 or vehicle. Dashed line along x-axis indicates duration of treatment (21 days), with shaded area corresponding to SD from the indicated averages; $n = 5$ to 6 mice per arm. B, Western blot for protein expression of select Bcl-2 family members in PDX models used in this study. PDX tumor lysates were prepared from banked tumor tissue that had been propagated in untreated nonobese diabetic scid mice without evidence of tumor necrosis. β-Actin provided for loading control. C, response of Bcl-2–expressing SCLC PDX models LX47 (top) and LX48 (bottom) to etoposide, ABT-737, or the combination of agents. D, heatmap visualization of differentially expressed (log₂) genes between paired ABT-737 (yellow) and vehicle-treated (blue) tumors from LX47 and LX48 tumors at points at or before maximal response. Bottom, an MA plot for the distribution of select HIF-1α responsive genes (e.g., "Elvidge Hypoxia" gene set) as a function of ABT-737 treatment in LX47 and LX48. E, representative H&E and pimonidazole immunohistochemistry of whole LX48 tumor capsules at the point of maximal response to

ABT-737, paired with vehicle counterparts ($n = 3$ tumors per arm); scale bar, 3 mm (top) or 200 μm (bottom).

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Figure 2.

Rapamycin has potent *in vitro* synergy with ABT-737. A, effect of single-agent ABT-737 or rapamycin and fixed dose combinations on cell viability after a 72-hour exposure in Bcl-2 high (NCI-H146 and NCI-H187) and Bcl-2 low (NCI-H82 and NCI-H446) SCLC cell lines; individual data points are shown $\pm$ SD, $n = 6$. B, changes in ABT-737 sensitivity in NCI-H146 and NCI-H187 pretreated with 100 nmol/L rapamycin for 24 (red) or 72 hours (blue) before an overnight (~ 16 hours) exposure to ABT-737; $n = 6$ per data point. IC₅₀ values are shown for control (black) and rapamycin (24 hours, red; 72 hours, blue) pretreatment traces. C, ABT-737 and rapamycin dose combination–response output matrices for the Bcl-2 low SCLC cell line NCI-H82 (top) and the Bcl-2 high SCLC cell line NCI-H146 (bottom). Color gradient of percentage maximal possible response is shown on right. D, fractional effect versus dose plot of ABT-737 and several mTOR(AZD8055; everolimus; rapamycin), PI3K (LY294002; wortmannin), and dual PI3K/mTOR (BEZ235) inhibitors in NCI-H146; data are representative of results obtained from multiple (>3) experiments. E, CI versus fractional effect plot ("synergy plot") for NCI-H146 treated with ABT-737 in combination with compounds described above. Levels of *in vitro* synergy are indicated using cutoffs described by others.

Figure 3.

Combining rapamycin with ABT-737 (R+ABT) provides durable responses in SCLC PDXs that express Bcl-2. Tumor response curves to single-agent ABT-737, rapamycin, and combination in LX47 (A), LX48 (B), and LX33 (C) PDXs; $n = 5$ to 6 mice per arm. Dashed lines, treatment periods (14 days). Statistical comparisons of tumor volumes in combination treatment groups versus all other arms were performed at days 8, 15, and 22; where indicated, *, $P < 0.01$; **, $P < 0.001$. D, effect of treatment arm on platelets counts in whole blood obtained by cardiac puncture in C.B-17 scid mice bearing LX47 hind flank tumors that were treated for 1 week with indicated arms; $n = 5$ per arm. Blood was collected 24 hours after last dose. E, representative paired H&E and pimonidazole immunohistochemistry from whole LX48 tumor capsule sections treated with indicated arms (y-axis) for 7 days; $n = 3$ to 4 tumors were analyzed per arm. Tumors were collected 24 hours after final dose; scale bar, 3 mm. F, combination arm challenge in LX47 previously treated with single-agent ABT-737 or rapamycin. LX47-bearing mice that were initially treated for 14 days with indicated single agents (dashed line) were subsequently treated at day 34; $n = 2$ per previous treatment group. Combination treatments were performed for 14 days (solid orange line) and then tumor volumes were monitored for an additional week. Mice did not receive any treatment between days 15 and 32. Body weights were not significantly different while on combination treatment nor did they differ by previous treatment arm.

Figure 4.

R+ABT differentially affects HIF-1α-regulated genes and the mTOR pathway in LX33 and LX47. A, Western blot for protein expression of select HIF-1α-regulated genes implicated from Fig. 1 in PDX models LX33 and LX47 after 1 week of treatment; $n = 3$ to 4 mice per arm with duplicates reflecting the extent of variability within a group. β-Actin provided for loading control. B, select gene expression fold changes (y-axis) between treatment arms in LX33 and LX47 treated as above; $n = 3$ mice per arm. C, CAT plot shows that a common set of genes are differentially expressed in LX33 and LX47 in response to ABT-737 after 1 week of treatment. D, Western blot for select protein expression in the PI3K/mTOR pathway in PDX models LX33 and LX47 after 1 week of treatment.

Figure 5.

Rapamycin increases levels of BAX protein *in vitro* and *in vivo* in SCLC. A, protein expression of selected Bcl-2 family members in LX33 and LX47 in response to 1 week of treatment with respective arms; $n = 3$ to 4 mice per arm with duplicates reflecting the extent of variability within a group. β-Actin provided for loading control. B, dose-dependent effects of ABT-737 and rapamycin on candidate Bcl-2 family members in LX33 PDXs after 1 week of daily treatment; $n = 3$ to 4 mice per dosing interval arm with duplicates reflecting variability as above; SDHA (complex ii) provided as a loading control for membrane-enriched fractions. C, cell fractionation experiments in NCI-H146 and NCI-H82 exposed to 100 nmol/L rapamycin (+100 nmol/L R) for 24 to 72 hours (1 day–3 days in the figure). Whole cell lysates and vehicle control lanes were exposed to vehicle (DMSO) for 72 hours before collecting protein; 2.5 μg total protein was loaded per lane. D, schematic representation of combined rapamycin and ABT-737 effect in SCLC PDX and cell lines models.