Phosphoinositide 3-kinase/AKT/mTORC1/2 Signaling Determines Sensitivity of Burkitt’s Lymphoma Cells to BH3 mimetics

Lindsay C. Spender and Gareth J. Inman

Abstract

Burkitt’s lymphoma (BL), driven by translocation and overexpression of the c-MYC gene, is an aggressive, highly proliferative lymphoma, and novel therapeutic strategies are required to overcome drug resistance following conventional treatments. The importance of the prosurvival BCL-2 family member BCL-XL in BL cell survival suggests that antagonistic BH3-mimetic compounds may have therapeutic potential. Here, we show that treatment of BL cell lines with ABT-737 induces caspase-3/7 activation and apoptosis with varying potency. Using selective inhibitors, we identify phosphoinositide 3-kinase (PI3K) as a proproliferative/survival pathway in BL cells and investigate the potential of combined pharmacologic inhibition of both the BCL-2 family and PI3K signaling pathway. PI3K/AKT inhibition and ABT-737 treatment induced synergistic caspase activation, augmented BL cell apoptosis, and rendered chemoresistant cells sensitive. Targeting mTORC1/2 with PP242 was also effective, either as a monotherapy or, more generally, in combination with ABT-737. The combined use of a dual specificity PI3K/mTOR inhibitor (PI 103) with ABT-737 proved highly efficacious. PI 103 treatment of BL cells was associated with an increase in BIM/MCL-1 expression ratios and loss of c-MYC expression. Furthermore, blocking c-MYC function using the inhibitor 10058-F4 also induced apoptosis synergistically with ABT-737, suggesting that maintenance of expression of BCL-2 family members and/or c-MYC by the PI3K/AKT/mTOR pathway could contribute to BL cell survival and resistance to ABT-737. The combined use of BH3 mimetics and selective mTORC1/2 inhibitors may therefore be a useful novel therapeutic approach for the treatment of B-cell malignancy, including chemoresistant lymphomas. Mol Cancer Res; 10(3); 347–59. ©2012 AACR.

Introduction

Burkitt’s lymphoma (BL) is an aggressive malignancy derived from germinal center (GC) B cells. Conventional intensive chemotherapeutic regimes are effective in eliminating disease in the majority of cases (1) however, novel treatment strategies are still required to treat patients who relapse with chemoresistant tumors. BLs are characterized by chromosomal translocation events which deregulate the c-MYC proto-oncogene (2), resulting in rapid proliferation of the malignant cells. Although BL cells proliferate rapidly, they are also sensitive to apoptotic stimuli and still maintain many of the proapoptotic and prosurvival signaling pathways that normally regulate their cell of origin. For example, BL cells are acutely sensitive to TGF-β–induced intrinsic apoptosis (3) which occurs via transcriptional control of members of the BCL-2 family of apoptosis regulators (4). BCL-2 family members are frequently deregulated in malignancies of GC origin (reviewed in ref. 5) and can be considered as valid targets for therapeutic intervention in B-cell lymphomas.

The prosurvival members of the BCL-2 family include BCL-2 itself, BCL-XL, BCL-w, MCL-1, and BOO. These proteins are regulated by other BH3-only family members that share only one region of homology with BCL-2 (the BH3 domain). These proapoptotic proteins (BIK, BIM, PUMA, NOXA, BAD, HRK, BID, and BMF) function as naturally occurring inhibitors of the prosurvival family members. Several BCL-2 antagonists, designed to mimic the BH3 domain of the BH3-only proteins (reviewed in ref. 6), have been developed as potential therapeutics and have undergone testing against B-cell malignancies, either as monotherapies, or in combination with other drugs (7–12).

ABSTRACT

Burkitt's lymphoma (BL), driven by translocation and overexpression of the c-MYC gene, is an aggressive, highly proliferative lymphoma, and novel therapeutic strategies are required to overcome drug resistance following conventional treatments. The importance of the prosurvival BCL-2 family member BCL-XL in BL cell survival suggests that antagonistic BH3-mimetic compounds may have therapeutic potential. Here, we show that treatment of BL cell lines with ABT-737 induces caspase-3/7 activation and apoptosis with varying potency. Using selective inhibitors, we identify phosphoinositide 3-kinase (PI3K) as a proproliferative/survival pathway in BL cells and investigate the potential of combined pharmacologic inhibition of both the BCL-2 family and PI3K signaling pathway. PI3K/AKT inhibition and ABT-737 treatment induced synergistic caspase activation, augmented BL cell apoptosis, and rendered chemoresistant cells sensitive. Targeting mTORC1/2 with PP242 was also effective, either as a monotherapy or, more generally, in combination with ABT-737. The combined use of a dual specificity PI3K/mTOR inhibitor (PI 103) with ABT-737 proved highly efficacious. PI 103 treatment of BL cells was associated with an increase in BIM/MCL-1 expression ratios and loss of c-MYC expression. Furthermore, blocking c-MYC function using the inhibitor 10058-F4 also induced apoptosis synergistically with ABT-737, suggesting that maintenance of expression of BCL-2 family members and/or c-MYC by the PI3K/AKT/mTOR pathway could contribute to BL cell survival and resistance to ABT-737. The combined use of BH3 mimetics and selective mTORC1/2 inhibitors may therefore be a useful novel therapeutic approach for the treatment of B-cell malignancy, including chemoresistant lymphomas. Mol Cancer Res; 10(3); 347–59. ©2012 AACR.

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BCL-2 antagonists have shown activity in mouse models of Myc-driven lymphomas (12–14) which suggests that BH3 mimetics may be useful in treating human disease. However, it is currently unknown whether such strategies would be effective against BL. The main targets of the BH3-mimetic ABT-737 (15) are BCL-2, BCL-XL, and potentially BCL-w.
(13, 14). Because BL are derived from GC cells, they lack BCL-2 expression [a characteristic feature of centroblasts and centrocytes (16, 17)] but, given the important role of BCL-XL in BL cell survival (4), we hypothesized that ABT-737 might induce apoptosis of BL cells despite the lack of BCL-2 expression.

ABT-737 is the most selective of several putative BCL-2 inhibitors in that it induces BAX- and BAK-dependent cell death entirely through activation of the mitochondrial apoptosis pathway. The selectivity of ABT-737 for BCL-2 and BCL-XL ensures that it is least effective in cells expressing high MCL-1 levels, which are not as dependent on BCL-2 or BCL-XL for survival. In such cases, the efficacy of ABT-737 can often be enhanced by coadministration of therapeutic agents that "neutralize MCL-1 function" (13) through, for example, the induction of BIM and/or NOXA (18–20).

In this study, we assess the efficacy of ABT-737 as a single agent and identify potential therapeutic targets for synergistic activity with BH3 mimetics in BL cell lines. Cells in the GC are subject to intense selection criteria mediated by signal transduction pathways, some of which impact on BCL-2 family protein expression (reviewed in ref. 5). If flux through such pathways is maintained in BL cells (as in other B-cell malignancies; ref. 21), combined use of BH3 mimetics with inhibitors of proproliferation and/or prosurvival pathways may enhance treatment efficacy or selectivity.

Here, we show that ABT-737 induces apoptosis as a single agent, and that phosphoinositide 3-kinase (PI3K)/AKT/mTOR is required for cell proliferation and/or survival. Inhibition of PI3K augments ABT-737–induced apoptosis. Furthermore, we found that dual PI3K/mTOR inhibitors or active site inhibitors of mTOR (aTORi) are effective either as single agents or synergize with ABT-737 to overcome resistance to ABT-737. These findings suggest that the combined use of BH3 mimetics with mTORC1/2 inhibitors could be a novel and effective therapeutic approach for the management of BL.

Materials and Methods

Cell lines and reagents

Human BL cell lines were a kind gift of Chris Gregory or were obtained from the Institute of Cancer Research cell bank (Ramos). L3055 cell lines overexpressing BCL-XL or expressing BCL-2 (L3055–BCL-XL and L3055–BCL2) were derived by Chris Gregory and have been described previously (22). All lines were EBV negative and mycoplasma negative and were used within 6 months from source or following karyotyping (23). BL cell lines were maintained in RPMI-1640 (Gibco-BRL) supplemented with 5% (v/v) heat-inactivated fetal calf serum, 2 mmol/L/mL glutamine, and antibiotics and were cultured for no more than 25 passages. Cells were treated as required with the BH3 mimic ABT-737 (Selleck Chemicals), the pan-caspase inhibitor zVAD-fmk (Bachem), the pan-PI3K inhibitor, LY-294002 (Calbiochem), the isozyme selective AKT1/AKT2 inhibitor (AKT inhibitor VIII; Merck) the dual PI3K p110α/mTOR inhibitor, PI 103, (Tocris Bioscience), inhibitors of mTOR, rapamycin, and PP242 (Sigma), or the c-MYC inhibitor 10058-F4 (5-[(4-ethylphenyl)methylene]-2-thioxo-4-thiazolidinone; Sigma).

Immunoblotting and antibodies

Cell protein lysates were quantified and equal amounts analyzed by SDS-PAGE. Antibodies used in Western blotting were mouse monoclonals against PARP (BD Biosciences), c-MYC (9E10; Santa Cruz), actin (Sigma), and S6 ribosomal protein (Cell Signaling Technology). Rabbit polyclonal antibodies were used to detect phospho-S6 ribosomal protein (Ser235/235), phospho-4E-BP1 (ser65), 4E-BP1, and phospho-AKT (ser 473), pan-AKT, BCL-2, BCL-XL, BCL-w, BID, PUMA (Cell Signaling Technology), MCL-1, BIK, BAK, BAX (Santa Cruz), and BIM (Chemicon). Secondary horseradish peroxidase–conjugated antibodies were obtained from Dako. Bound immunocomplexes were detected by enhanced chemiluminescence (ECL; Amershams).

Analysis of cell cycle and apoptosis

Cells were fixed, labeled with propidium iodide (PI), and analyzed by flow cytometry (FACS Calibur; BD Biosciences) for DNA content. Histogram plots containing a minimum of 10,000 events were analyzed by CellQuest Pro software (BD Pharmingen) using linear gates to determine the percentage of apoptotic cells with sub-G1 DNA content (M1) or cells in G1 (M2), S phase (M3), or G2–M phases (M4) of the cell cycle. Intracellular caspase-3 activity was assayed by flow cytometry with PhiPhiLux G1D2, (Calbiochem) and PI as recommended by the manufacturer. Caspase 3/7 activity was also analyzed by caspase-Glo (caspase-3/7) assay reagents (Promega). Cell suspensions (50 μL) treated for 24 hours were incubated for 1 hour at room temperature with an equal volume of caspase-Glo substrate. Fluorescence was then analyzed by a Veritas microplate luminometer (Turner Biosystems).

Densitometry and statistical analysis

Western blots were analyzed within the linear range of ECL and the resultant films scanned and analyzed by ImageJ software. Statistical analysis was conducted by paired Student t test. Significant P < 0.05, <0.01, and <0.005 are indicated by 1, 2, or 3 stars, respectively. Potential synergistic effects of drug combinations were determined by CalcuSyn dose effect analysis software (Biosoft) based on the combination index (CI) equation and plot of Chou–Talalay (24), which analyses the doses of drug A or B required to give an effect (fraction affected, Fa) in comparison with the doses of drug A and B used in combination to give the same effect. In our experiments, the ED50, ED75, and ED90 values are compared (i.e., the doses of drug at which 50%, 75%, and 95% maximum caspase activation is achieved). CI values at these points of more than 1 represents antagonism, equal to 1 represents an additive effect, and less than 1 indicates synergy.
Results

**ABT-737 induces apoptosis in BL cells**

Given the critical role for BCL-XL in BL cell survival, we predicted that ABT-737 may induce apoptosis in human BL cells. We assessed its effect on EBV-negative BL cell lines using 3 different assays (Fig. 1). PI staining of BL cells lines treated for 48 hours showed a dose-dependent increase in the percentage of cells with sub-G1 DNA content in several of the lines (Fig. 1A). Induction of apoptosis by ABT-737 was confirmed by increased caspase 3/7 activity (Fig. 1B and Supplementary Fig. S1A), and also by cleavage of PARP (Fig. 1C). ABT-737–induced apoptosis was completely ablated by pretreatment of cells with the pan-caspase inhibitor zVAD-fmk (Supplementary Fig. S1B). We observed clear differences in the sensitivity of individual cell lines to ABT-737, with BL40, BL30, and BL2 being relatively resistant. Analysis of the percent of cleaved PARP (shown above each lane in Fig. 1C) confirmed that BL30 and BL40 lines were largely refractory to ABT-737 treatment.

**BCL-2 family members and sensitivity to ABT-737**

Having identified differential sensitivities of cell lines to ABT-737, we attempted to correlate their responsiveness...
with the relative expression levels of BCL-2 family members in each line (Supplementary Fig. S2A). BL cell lines did not express detectable levels of 2 potential ABT-737 targets, BCL-2 or BCL-w and expression levels of the only other protein targeted by ABT-737, BCL-XL, did not correlate with sensitivity. In B-CLL, reduction of BIM expression renders these cells less sensitive to ABT-737, suggesting that in some lymphomas BIM could be a determinant of sensitivity (25). However, in our cell line panel, there was no correlation between sensitivity and BIM expression. Likewise, we observed no correlation between the levels of MCL-1, PUMA, and BID and the apoptotic response to ABT-737 (Supplementary Fig. S2A). Levels of both BAX and BAK were equivalent in all lines (Supplementary Fig. S2B), therefore the relative resistance of BL30 and BL40 cell lines to ABT-737 does not seem to be due to a “class B” block (26) in apoptosis caused by loss of BAX or BAK (Supplementary Fig. S2B). CA46 BL cells which are genetically deficient in BAX and BAK expression (27; Supplementary Fig. S2C), were unaffected by ABT-737 treatment as assessed by cell counting assays (Supplementary Fig. S2C) and are the only cell line which exhibit a complete lack of caspase activation in response to ABT-737 (Supplementary Fig. S2D). Cells with a class B block in apoptosis are therefore ABT-737 resistant, but BAX/BAK loss does not account for the lack of response in other lines which remain competent for intrinsic apoptosis. The analysis of individual BCL-2 family members in these cell lines therefore cannot predict the outcome of ABT-737 treatment.

Figure 2. PI3K signaling contributes to BL cell proliferation and/or survival. A, BL cells were treated for 2 hours with the pan-PI3K inhibitor LY-294002 (10 μmol/L), lysed, and analyzed by Western blotting for phosphorylation of the PI3K substrate AKT/PKB (ser473). B and C, BL cells treated for 48 hours with LY-294002 (0, 5, and 10 μmol/L) were fixed, stained with PI, and analyzed by flow cytometry and CellQuest Pro for G1 arrest (B), and for cell death (C). D, Ramos and BL2 cells were treated for 2 hours with dimethyl sulfoxide (vehicle) or with 1 or 0.25 μmol/L of the AKT1/2 inhibitor (AKTiVIII). Cell lysates were analyzed by Western blot using phospho-specific and total AKT and S6 ribosomal protein antibodies as indicated. E, BL cells were treated for 48 hours with LY-294002 (5 μmol/L) or AKTiVIII (1 μmol/L), and the amount of apoptosis above background determined by PI. Shown is the mean (± SD) apoptosis induction above background from at least 3 experiments each carried out in triplicate.
are high levels of BCL-2 which is available to complex with BIM. However, all the BL cell lines, consistent with their cell of origin (16, 17), lack BCL-2 expression (Supplementary Fig. S2A) but are nevertheless differentially sensitive to ABT-737. Because BIM can also be sequestered by MCL-1 and BCL-X₇, we assessed the ratios of BIM to MCL-1 and BCL-X₇ in comparison with those of PUMA and BIK. Sensitivity to ABT-737 correlated best with BIM: MCL-1 expression ratios with higher ratios evident in sensitive cells. Ratios of PUMA or BIK to MCL-1 or BCL-X₇ bore no correlation with sensitivity to ABT-737 (Supplementary Fig. S2E). Because BL cell lines are refractory to transfection, we were not able to identify unequivocally which BCL-2 family members conferred sensitivity to ABT-737 using siRNA knockdown approaches however, when we compared sensitivities of the parental human cell line L3055 with BCL-X₇ overexpressing or BCL-2–expressing derivatives, we found that changes in prosurvival factor expression significantly altered the response of cells to ABT-737 (Supplementary Figs. S3A and S3B). Following treatment with ABT-737 (250 nmol/L), BCL-2–expressing cells were highly sensitized to its effects which concurs with studies described with Eμ-myc(bcl-2) lymphomas (12–14). BCL-X₇–overexpressing L3055 cells, however, were more resistant to ABT-737. Taken together, these data suggest that the relative expression levels of prosurvival to proapoptosis BCL2 family members may determine the outcome of ABT-737 monotherapy.

**PI3K signaling contributes to BL cell proliferation and survival**

ABT-737 is often more efficacious when used in conjunction with other agents that impinge on the function of the BCL-2 family. To select potential targets for combination therapy, we considered signaling pathways that might be proproliferative and/or prosurvival. In human B cells, signaling through the PI3K/AKT pathway (reviewed in ref. 5) can promote survival. Constitutive PI3K activity is also reported to be essential for the proliferation of one of the BL cell lines used in this study (28). We therefore first tested whether constitutive PI3K signaling is operative in our BL cell panel.

Basal PI3K signaling measured by serine 473 phosphorylation of AKT/PKB was detectable in all BL lines and could be substantially reduced by treatment with the pan-PI3K inhibitor LY-294002 (Fig. 2A). The effect of blocking PI3K signaling on cell proliferation and apoptosis was assessed by flow cytometry (Figs. 2B and 2C respectively). PI3K inhibition had significant effects on lymphoma cell outgrowth, inducing a G₁ cell-cycle arrest in BL40, BL2, and Ramos (Fig. 2B) and apoptosis in BL30, BL2, and L3055 (Fig. 2C).

To determine whether the effects were mediated through inhibition of AKT, we used a selective inhibitor of AKT1/2, Akt inhibitor VIII (AktIVIII, 1 μmol/L) which decreased AKT activation, [measured by reduced phosphorylation (Fig. 2D)]. Like LY-294002, AktIVIII induced BL cell apoptosis in BL30 and BL2 and L3055 cells (Fig. 2E). PI3K signaling through AKT is therefore critical for maximal BL proliferation and/or survival.

**mTOR inhibitors regulate BL cell proliferation and/or survival as single agents**

AKT has numerous substrates implicated in cell survival pathways, including mTOR. We investigated further whether mTOR might contribute to BL cell survival using selective inhibitors and dual PI3K/mTOR inhibitors. mTOR exists as 2 complexes, mTORC1 and mTORC2. mTORC2 being upstream of AKT and mTORC1 regulating the activity of its downstream effectors p70 S6-kinase/S6 ribosomal protein and 4E-BP1. 4E-BP1

PP242 is an active site, ATP competitive inhibitor of both mTORC1 and mTORC2, and rapamycin is a less effective inhibitor of mTORC1 (29). In agreement with previous studies (30–34), we were able to distinguish rapamycin-sensitive and rapamycin-insensitive effects of mTORC1 signaling. Phosphorylation of both S6 ribosomal protein and 4E-BP1 was inhibited using the active site inhibitor PP242, whereas rapamycin treatment efficiently inhibited phosphorylation of S6 ribosomal protein but had little effect on phosphorylation of 4E-BP1 (Fig. 3A). PI 103 is a dual PI3K/mTOR inhibitor and also efficiently inhibited phosphorylation of both S6 ribosomal protein and 4E-BP1. When we assessed the effect of rapamycin and PP242 in comparison with LY-294002 and PI 103 on BL cell survival, we observed that PP242 and PI 103 induced similar levels of apoptosis as single agents in BL30, BL2, and L3055 (Fig. 3B). Dual inhibition of PI3K and mTOR with PI 103 was the most effective single-agent therapy, inducing caspase-dependent (zVAD-fmk sensitive) cleavage of PARP (Fig. 3C). Rapamycin (2 nmol/L) and LY-294002 (5 μmol/L), used at concentrations that only effectively inhibit S6 ribosomal protein phosphorylation (not phosphorylation of 4E-BP1) generally induced less apoptosis than either PP242 or PI 103, suggesting that the enhanced proapoptotic effects of PP242 and PI 103 may correlate with inhibition of 4E-BP1. 4E-BP1 function. Treatments which had minimal effects on BL40 cell survival instead induced a G₁ cell-cycle arrest (Supplementary Fig. S4).

**PI3K inhibition augments ABT-737–induced apoptosis of BL cells**

Given our observations that BL cell lines have differential sensitivities to inhibitors of either PI3K signaling and/or BCL-X₇ as single agents, we investigated the consequence of combined inhibition of these pathways. When used in conjunction with ABT-737, LY-294002 augmented killing of Ramos cells. LY-294002 treatment alone induced G₁ arrest, but increased the amount of cell death in combination with ABT-737 (Supplementary Fig. S5A). Subsequent ABT-737 titrations, analyzed by PARP cleavage, revealed that LY-294002 potently sensitized Ramos cells to ABT-737–induced death (Supplementary Fig. S5C). Importantly, combination treatment enhanced apoptosis in the relatively chemoresistant cell line BL40 (Supplementary Figs. S5B and S5C). Similar results were obtained in BL2 cells (data not shown) and were confirmed by PI staining in Ramos, BL40, and L3055 (Supplementary Fig. S5B and data not shown).

PI3K/mTOR Inhibition Overcomes Resistance of BL to ABT-737
To establish whether ABT-737 and LY-294002 act synergistically, we carried out dose effect analysis measuring caspase 3/7 activation induced by drug combinations. The data were analyzed by the Chou–Talalay mathematical equation (24) to determine combination indices in which values of more than 1, 1, and less than 1 indicate antagonism, additive effects, and synergism, respectively. ABT-737 and LY-294002 acted synergistically to activate caspase 3/7 in both Ramos (Fig. 4A) and BL40 cells (Fig. 4B). Importantly, synergistic activity was also detected by the combination of ABT-737 and the more selective AKT inhibitor, AKTIVIII (Figs. 4C and D). These data indicate that combining ABT-737 with inhibition of PI3K signaling via AKT has the potential to sensitize ABT-737–resistant BL cells to apoptosis, and augment apoptosis in cells already responsive to ABT-737. Our observations were confirmed by PI staining (Fig. 4E and F), and in BL30 and BL2 cells which show apoptotic responses to AKT inhibition alone (Supplementary Figs. S6A, S6B, S6C, and S6D).

mTORC1/2 inhibitors synergize with ABT-737 to induce BL cell apoptosis

To determine whether the synergy between ABT-737 and PI3K/AKT inhibition could be recapitulated when selectively targeting downstream mTORC1 and mTORC2 complexes, PP242 was used in combination with ABT-737 which resulted in the synergistic induction of caspase3/7 activity (Fig. 5A and B) and increased apoptosis (Fig. 5C and D). In BL30 cells that were killed by PP242 alone, the drug combination of PP242 with ABT-737 had no additional apoptotic effect (data not shown). ABT-737 in combination with PP242 was much more effective than when used in combination with doses of rapamycin which affected only phosphorylation of S6 ribosomal proteins (data not shown). Taken together our data implicate PI3K/AKT/mTORC1/2 signaling as both a survival factor and mediator of resistance to ABT-737 in BL. This conclusion was supported by our observation of a highly synergistic apoptotic effect when combining ABT-737 with the dual PI3K p110α/mTOR inhibitor, PI 103 (Fig. 6A–D) suggesting that concomitant targeting of PI3K/mTORC1/2 along with BCL-XL is optimal for induction of BL cell death.

Because we found that the ratio of basal levels of BIM: MCL-1 correlated best with sensitivity of BL cells to ABT-737 as a single agent (Supplementary Fig. S2A), we examined MCL-1 expression during a time course of PI 103 treatment of Ramos cells. PI 103 treatment caused a decrease in the expression level of MCL-1 (Fig. 6E) resulting in a...

Figure 3. mTOR inhibitors as monotherapies for BL: A, Ramos cells were treated for 2 hours with LY-294002 (LY), the dual PI3K/mTOR inhibitor PI 103 or selective inhibitors of mTOR, rapamycin (Rap), and the active site inhibitor PP242. Equal amounts of cell lysate were analyzed by Western blot. B, BL cell lines were treated for 48 hours with LY-294002 (5 μmol/L), PI 103 (1 μmol/L), rapamycin (2 mmol/L), or PP242 (1.25 μmol/L). Cells were analyzed by PI staining for percent (mean ± SD, n = 3) cell death above background. C, BL2 cells were pretreated with the pan-caspase inhibitor ZVAD-fmk (25 μmol/L) for 1 hour prior to PI 103 (1 μmol/L) addition for the indicated time periods. Cell lysates were analyzed by Western blot.

LY-294002 was also able to render the resistant BCL-XL overexpressing cell line L3055-BCL-XL sensitive to ABT-737 treatment (Supplementary Fig. S5D).

Figure 4. Synergistic effects of PI3K/AKT inhibitors and ABT-737. The combined effect of PI3K/AKT inhibitors and ABT-737 on apoptosis in BL cells was assessed by caspase 3/7 assay (A–D) and by PI staining (E and F). Ramos cells (A and C) or BL40 cells (B and D) were left untreated, or treated with concentrations of ABT-737 (ABT) the presence or absence of LY-294002 (LY) or AKTIVIII in equimolar ratios as indicated. Caspase activity was analyzed by caspase 3/7-glo assay after 24 hours and potential synergism determined by the Chou–Talalay equation for dose effect analysis of drug combinations. CI values of more than 1, 1, and less than 1 represent antagonism, additive effect, and synergism, respectively. E and F, Ramos (E) and BL40 cells (F) were treated with solvent or ABT-737 (500 nmol/L) in the presence or absence of LY-294002 (5 μmol/L) or AKT inhibitor VIII (1 μmol/L). Cells were harvested after 48 hours, and the percent apoptosis (mean ± SD, n = 3) determined by PI staining.
**A** Ramos

- **ABT**
- **LY**
- **Both**

Fa-CI plot

- ED50 0.30
- ED75 0.17
- ED90 0.10

**B** BL40

- **ABT**
- **LY**
- **Both**

Fa-CI plot

- ED50 0.29
- ED75 0.18
- ED90 0.11

**C** Ramos

- **ABT**
- **AKTI VIII**
- **Both**

Fa-CI plot

- ED50 0.34
- ED75 0.19
- ED90 0.10

**D** BL40

- **ABT**
- **AKTI VIII**
- **Both**

Fa-CI plot

- ED50 0.30
- ED75 0.17
- ED90 0.09

**E** Ramos

- **DMSO**
- **ABT-737 (500 nmol/L)**

Apoptotic cells (% sub-G1)

- Vehicle
- LY-294002
- AKTI VIII

**F** BL40

- **DMSO**
- **ABT-737 (500 nmol/L)**

Apoptotic cells (% sub-G1)

- Vehicle
- LY-294002
- AKTI VIII
time-dependent increase in the ratio of BIM/MCL-1 (Fig. 6F) as determined by densitometry analysis of Western blots shown in Fig. 6E. Similar results were observed in 3 independent experiments and following treatment of BL40 cells (data not shown). In addition to an effect on MCL-1 expression, we also observed an effect of PI 103 treatment on the levels of c-MYC. The loss of MCL-1 and c-MYC expression was not due to the generalized loss of proteins caused by cell death, as similar effects were observed in PI 103-treated cells in which apoptosis was blocked with zVAD (Fig. 6G). PI 103 treatment did not significantly affect the level of BCL-XL, also one of the subset of proteins

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Figure 5. Synergistic effects of the mTOR inhibitor PP242 and ABT-737. The combined effect of the active site mTOR inhibitor PP242 and ABT-737 on apoptosis in BL cells was assessed by caspase 3/7 activation assay (A and B), and by PI staining (C and D). Ramos (A) and BL40 cells (B) were left untreated or treated with concentrations of ABT-737 (ABT) in the presence or absence of PP242 in equimolar ratios. Increases in caspase 3/7-glo assay after 24 hours. Potential synergism was determined as described in Fig. 4. Ramos (C) and BL40 cells (D) were pretreated for 15 minutes with vehicle, PP242 (1.25 µmol/L), or LY-294002 (5 µmol/L), followed by addition of ABT-737 (500 nmol/L) or vehicle control. Cells were analyzed by PI staining after 48 hours and the percent apoptotic cells above background (mean ± SD, n = 3) determined.

Figure 6. The dual PI3K/mTOR inhibitor PI 103 increases BIM/MCL-1 ratios and enhances ABT-737 sensitivity. The combined effect of PI 103 and ABT-737 on apoptosis in BL cells was assessed by caspase 3/7 activation assay (A and B) and by PI staining (C and D). Ramos (A) and BL40 cells (B) were left untreated or treated with concentrations of ABT-737 (ABT) in the presence or absence of PI 103 in equimolar ratios. The potential synergistic induction of caspase activity was determined as described in Fig. 4. Ramos (C) and BL40 cells (D) were pretreated for 15 minutes with vehicle, PI 103 (1 µmol/L), or LY-294002 (5 µmol/L), followed by addition of ABT-737 (500 nmol/L) or vehicle control. The percent apoptotic cells above background (mean ± SD, n = 3) was determined by PI staining after 48 hours. E, Ramos cells were treated with PI 103 (1 µmol/L) and cell lysates analyzed by Western blot. F, Western blots [representative blots shown in (E)] were analyzed by densitometry and ImageJ software and the ratio of BIM to MCL-1 protein expression determined over the time course of PI 103 treatment. BIM/MCL-1 ratios determined after 24 hours for untreated versus PI 103-treated cells were 0.78 ± 0.03 and 3.2 ± 1.05, respectively (n = 3). G, Western blot analysis of the indicated proteins in Ramos cells treated with PI 103 (1 µmol/L) in the presence or absence of zVAD-fmk (25 µmol/L).
PI3K/mTOR Inhibition Overcomes Resistance of BL to ABT-737

A Ramos

B BL40

Fa-CI plot

CL

Effect

Fa-CI plot

CI

Effect

C Ramos

D BL40

Apoptotic cells (% above background)

Vehicle LY PI 103

Apoptotic cells (% above background)

Vehicle LY PI 103

E

kDa

Hrs

PI 103

MCL-1

BIMEL

c-MYC

Actin

39-42

23

62

42

0 2 4 8 16 24

F

BIM/MCL-1 ratio

Control PI 103

Time (h)

0 4 8 12 16 20 24

G

kDa

PARP

MCL-1

BIMEL

BCL-XL

c-MYC

pS6 ribosomal protein

S6 ribosomal protein

0 16 24 16 24 16 24

Time (h)
(such as MCL-1 and c-MYC) produced through cap-dependent translation of its mRNA.

To further investigate whether the loss of c-MYC expression could account for apoptosis induction and synergistic effects of PI 103 with ABT-737, we analyzed the effect of inhibition of c-MYC function on BL cells using a small molecule, selective inhibitor of MYC/MAX dimerization, and DNA binding activity, 10084-F3 (35, 36). Inhibition of c-MYC activity induced a dose-dependent increase in apoptosis of all lines tested (Fig. 7A) and, similar to inhibition of the PI3K/AKT/mTOR pathway, synergized with ABT-737 to increase caspase activation (Fig. 7B) and enhance cell death (Fig. 7C).

Discussion

High-dose combinations of cytotoxic drugs can be highly effective in treating BL but novel therapeutic

Figure 7. Inhibition of c-MYC synergizes with ABT-737 to induce BL cell apoptosis. A, cells were treated for 48 hours with a 2-fold dilution series of the Myc inhibitor 10058-F4 (Myc i), fixed, stained with PI, and analyzed by flow cytometry. The mean (± SD, n = 3) increase in the percentage of cells with sub-G1 DNA content above background is shown. B, the combined effect of the MYC inhibitor and ABT-737 on apoptosis in BL cells was assessed by caspase 3/7 assay. BL cells were treated with solvent as a control or treated with concentrations of ABT-737 (ABT) the presence or absence of 10058-F4 in equimolar ratios as indicated. Caspase activity was analyzed by caspase 3/7-glo assay after 24 hours and potential synergism determined by the Chou-Talalay equation for dose effect analysis of drug combinations. CI were determined at ED50, ED75, and ED90. CI values of less than 1 represent synergism. C, the effect of combining ABT-737 (500 nmol/L) with the MYC inhibitor (25 µmol/L) on apoptosis of BL cells was assessed by PI staining and flow cytometry after 48 hours treatment. The drug combination of ABT and LY-294002 was included as a positive control for augmented apoptosis.
strategies may help to tackle the ongoing problem of severe side effects and relapse. In this study, we analyze apoptotic responses of BL cell lines to potential chemotherapeutic agents targeting the BCL-2 family and multiple components of the PI3K/AKT/mTOR signaling cascade. We identify the PI3K/AKT/mTOR signaling as a proproliferative and/or prosurvival pathway in BL cells and report on the synergistic use of BH3 mimetics combined with different pharmacologic inhibitors targeting several effectors within the PI3K/mTOR pathway.

We determined that cell death in EBV-negative BL cells induced by ABT-737 as a single agent, is caspase dependent and that, in general, higher doses of ABT-737 are required to induce apoptosis in BL compared with AML (37) or B-CLL (25). This may be due to the fact that like GC B cells, BL cells lack BCL-2 expression. Several studies in murine Eμ-myc tumor models have indicated that the efficacy of ABT-737 depends on whether cells rely primarily on its specific targets for survival (i.e., Bcl-2 or Bcl-XL). Eμ-myc lines expressing Bcl-2 (12–14), are highly sensitive to ABT-737. Studies in diffuse large B-cell lymphoma (DLBCL) also suggest that “primed” BCL-2–positive cell lines are the most sensitive to ABT-737 (26), whereas Del Gaizo Moore and colleagues, using a technique termed “BH3 profiling” in B-CLL samples, showed that sensitivity to ABT-737 may be determined by the amount of BCL-2 able to sequester the proapoptotic protein BIM (25). In this case, displacement of BCL-2 by ABT-737 releases BIM to activate BAX and induce apoptosis. The fact that BCL-2/BCL-w–negative BL cells are not reliant on 2 of the primary targets of ABT-737 for survival could render them inherently more resistant. This hypothesis is supported by our observation that BCL-2 overexpressing BL cells (L3055-BCL-2) were highly sensitized to ABT-737. Our data also suggest that ABT-737 could act as an effective single-agent therapy in a stratified patient group with aggressive “double-hit” tumors which carry concurrent BCL-2 and c-MYC rearrangements (38, 39).

The BL cells exhibited differential sensitivity to ABT-737. A lack of BAX and/or BAK (as in CA46) renders BL cells completely insensitive to ABT-737, however this "class B" block in apoptosis did not account for resistance in the other relatively insensitive lines. In the absence of reliable, efficient siRNA knockdown procedures for BL cells, the identification of the determinants of this resistance in BL is problematic. However, we have identified active PI3K/mTOR signaling as contributing to the resistant state, such that inhibition of the pathway at various levels increased the effectiveness of ABT-737 treatment at lower doses. PI3K is critical for mature B-cell survival (40) and elevated signaling is also associated with chemoresistance in DLBCL (41). Because many B-cell malignancies retain constitutive signaling through the BCR/Syk/PI3K/AKT pathway [including follicular lymphoma, DLBCL, and B-CLL (reviewed in ref. 42)], our finding that combination treatments using inhibitors of PI3K/mTOR augment apoptosis, may suggest effective therapies for other malignancies of B-cell origin. The EBV-positive status of some BL tumors may be a cofounding factor for the use of ABT-737 because viral genes have a number of effects on expression and function of BCL-2 family members (reviewed in ref. 43) but further studies will be required to address this issue.

Having established that inhibition of the PI3K/AKT pathway sensitizes BL cells to ABT-737, we investigated the possible downstream effectors involved. There are numerous potential mechanisms of AKT-dependent apoptosis suppression (reviewed in ref. 44), however, the use of PP242 (which is a selective active site mTOR inhibitor lacking activity against PI3K/AKT) establishes mTOR activity as a key component for BL survival and chemoresistance. In this study, we used rapamycin at doses which only affected phosphorylation of S6 ribosomal protein without inhibiting phosphorylation of 4EBP-1. Both PP242 and PI 103 (which inhibit both S6 ribosomal protein and 4EBP-1 phosphorylation) were more effective in combination with ABT-737 than rapamycin. Blocking both rapamycin-sensitive and rapamycin-insensitive functions of mTORC1 in this way indicated that inhibition of cap-dependent translation regulated by 4E-BP1/eIF4E may be necessary to overcome resistance to BH3 mimetics.

The hypothesis that 4E-BP1/eIF4E-dependent protein translation is important for maintaining cell survival and chemoresistance is supported by the observation that the expression levels of MCL-1 decrease following PI 103 treatment of BL cells. The effect on MCL-1 concurs with recent studies in DLBCL which showed that phosphorylation of 4EFP-1 is essential for MCL-1 synthesis and that inhibition of mTORC1 activity can overcome MCL-1–mediated resistance to ABT-737 (45). We also found that resistance to ABT-737 correlated with the lowest ratio of BIM to MCL-1 expression. ABT-737 resistance determined by the amount of BIM sequestered by MCL-1 (which cannot be targeted by ABT-737) has been reported in both AML and murine lymphoma models (13, 14, 25). Treatment of BL cells with PI 103 increased the BIM/MCL-1 ratio, which could explain the increased sensitivity of PI 103-treated cells to ABT-737.

PI 103 treatment, however, also decreased expression of c-MYC. The loss of c-MYC protein as a result of mTOR inhibition has been reported previously (46). c-MYC is the oncogenic driver of BL and is bound as a MYC/MAX transcription factor complex to approximately 15% of gene promoters in BL cells (47). Both c-MYC and MCL-1 (48) are regulated at the level of protein stability because their half-life is short. The half-life for c-MYC, for example, is 18 minutes in Ramos cells (49). Recently, proteomic approaches identified c-MYC as the key upstream regulator for the majority of proteins in which expression levels are affected by inhibition of cap-dependent translation (46). The similar synergistic apoptotic effects of either the MYC inhibitor 10058-F4 or active site mTOR inhibitors in combination with ABT-737 implies that continuous synthesis of c-MYC is required for BL survival and chemoresistance. It is possible that the c-MYC inhibitor may also have an indirect effect on the stability of prosurvival factors (MCL-1 or others) through inhibition of c-MYC-mediated...
regulation of genes involved in protein biosynthesis (47). Further studies are required to determine which c-MYC-regulated genes are important in BL cell survival.

Although our data suggests that inhibitors of mTOR or c-MYC could work as effective single agents in BL, they were much more effective treatments when used in combination with ABT-737, across a broader range of cells and at lower doses. Combination therapy may therefore reduce any requirement for patient selection, and lower drug doses may be tolerated better. Reports that PP242 is an effective treatment in combination with ABT-737, but simultaneously shows greater tolerability in models of leukemia (34, 50) are promising. A number of other mTOR inhibitors are currently in clinical trials therefore increasing the likelihood that suitable combination therapy strategies based on our observations could be tested clinically for BL.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Lindsay C. Spender and Gareth J. Inman


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