ARTICLE

Inhibition of 4EBP phosphorylation mediates the cytotoxic effect of mechanistic target of rapamycin kinase inhibitors in aggressive B-cell lymphomas

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ABSTRACT

Mechanistic target of rapamycin (mTOR) complex 1 is a central integrator of nutrient and growth factor inputs that controls cell growth in eukaryotes. The second generation of mTOR kinase inhibitors (TORKi), directly targeting the mTOR catalytic site, are more effective than rapamycin and its analogs in cancer treatment, particularly in inducing apoptosis. However, the mechanism underlying the cytotoxic effect of TORKi remains elusive. Herein, we demonstrate that TORKi-induced apoptosis is predominantly dependent on the loss of mTOR complex 1-mediated 4EBP activation. Knocking out RICTOR, a key component of mTOR complex 2, or inhibiting p70S6K has little effect on TORKi-induced apoptosis. Conversely, increasing the eIF4E:4EBP ratio by either overexpressing eIF4E or knocking out 4EBP1/2 protects lymphoma cells from TORKi-induced cytotoxicity. Furthermore, downregulation of MCL1 expression plays an important role in TORKi-induced apoptosis, whereas BCL-2 overexpression confers resistance to TORKi treatment. We further show that the therapeutic effect of TORKi in aggressive B-cell lymphomas can be predicted by BH3 profiling, and improved by combining it with pro-apoptotic drugs, especially BCL-2 inhibitors, both *in vitro* and *in vivo*. Taken together, the study herein provides mechanistic insight into TORKi cytotoxicity and identified a potential way to optimize its efficacy in the clinical treatment of aggressive B-cell lymphoma.

Introduction

Aggressive B-cell lymphomas are clinically and pathologically heterogeneous entities that consist of diffuse large B-cell lymphoma (DLBCL), Burkitt lymphoma (BL), mantle cell lymphoma (MCL), and rare variants, such as double hit lymphoma (DHL). Rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP)-based chemotherapy regimens have significantly improved the clinical outcome of patients with DLBCL and MCL, whereas intensive chemotherapy was commonly used for BL. DHL responds poorly to R-CHOP treatment and remains challenging in clinical practice. Toxicity and secondary cancer risk due to intensive therapy are also high, particularly in patients treated with high-dose regimens.[1,2] Improvements in therapeutic strategies and target therapy are therefore urgently needed in aggressive B-cell lymphomas. With a central role in cell survival and growth, mechanistic target of rapamycin complex 1 (mTORC1) is one of the most important targets in cancer therapy, given that it is often deregulated in human cancers.[3] The two best characterized targets of mTORC1 are p70S6K1 and 4EBP1.[4] The 4EBP1-eIF4E pathway regulated translation is considered to be critically important in cancer cell survival and proliferation since it has a broad impact on

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protein expression, including many oncogenes.[5-8]

Several mTORC1 inhibitors have been developed and investigated in the treatment of B-cell lymphomas. Rapamycin and its analogs (rapalogs) have been approved by the FDA for the treatment of relapsed MCL. However, many MCL patients respond poorly to rapalog treatment,[9,10] and the efficacy of rapalogs in other B-cell lymphomas remains controversial.[11,12] Rapalogs only block the effect of mTORC1 on low-affinity targets, such as $S6K^{T389}$, but not on high-affinity targets, such as $4EBP1^{Thr37/46}$ or $Grb10^{S150}$,[13,14] and have no effect on the mechanistic target of rapamycin complex 2 (mTORC2).[15,16] Despite the clinical application and multiple active clinical trials of rapalog, this type of mechanistic target of rapamycin (mTOR) inhibitor has been demonstrated to lack cytotoxic effects in lymphoma cells.[17,18] Unlike rapalogs, TORKi function as ATP-competitive inhibitors and block all known mTORC1 targets, as well as mTORC2 activity, by directly targeting the mTOR kinase catalytic site.[13,19,20] Several TORKi have been developed recently and appear to be promising in lymphoma treatment. Many of these studies addressed the effectiveness on the dual inhibition of mTORC1 and mTORC2,[19,21-23] since mTORC2 directly phosphorylates and activates AKT, compromising the inhibitory effect on mTORC1.[24] However, two additional feedback loops, through S6K and Grb10, respectively,[25,26] may eventually lead to the activation of AKT when mTORC1 is inhibited, which makes the crosstalk between mTORC1 and AKT more elusive, especially upon the dual inhibition of mTORC1 and mTORC2 by TORKi.

Because TORKi inhibits both mTORC2 and mTORC1, it is difficult to differentiate which effect plays a major role in the treatment of lymphoma. Although a previous study demonstrated that the block in proliferation of mouse embryonic fibroblasts by TORKi is predominantly through effective inhibition of mTORC1 and independent of mTORC2,[13] it is still important to investigate the significance of mTORC2 inhibition in lymphoma cells, given that AKT signaling is one of the most commonly deregulated oncogenic pathways in B-cell malignancies. In addition, although TORKi is potent in inhibiting target of rapamycin (TOR) kinase, many clinical trials were halted prematurely, with the main problem being that cancer cells responded variably to this class of agent, which is probably because TORKi unequally inhibits the 5'cap-dependent translation of different transcripts.[27] Therefore, fully assessing the effect of TORKi in aggressive B-cell lymphoma and understanding the molecular mechanism will help to optimize the utilization of these drugs in clinical practice. The study herein was designed to: (1) assess the effects of TORKi treatment, especially the cytotoxic effect in aggressive B-cell lymphomas, (2) determine the significance of 4EBP1 pathway inhibition in TORKi-induced cytotoxicity, and (3) identify the molecular basis for resistance to TORKi treatment in aggressive B-cell lymphomas.

Methods

Ectopic gene expression with retrovirus transduction and gene knockout/knockdown with CRISPR-CAS9 system

For retroviral transduction, cell lines were first engineered to express the ecotropic retroviral receptor as described previously.[28] Retroviral constructs were packaged by co-transfecting HEK293T cells with pCL-Eco and PMIP vector carrying GFP, eIF4E, MCL1, BCL-XL, BCL-2, or AKT. CRISPR-Cas9-mediated knockout was delivered by lentiCRISPR v2 vector (#52961, Addgene, Cambridge, MA, USA).[29] For the double knockout experiment, 4EBP1 and 4EBP2 single guide RNAs (sgRNAs) were expressed by lentiCRISPR v2 and a lenti sgRNA expressing vector with a blasticidin resistance gene, respectively. Sequences of sgRNAs are listed in the *Online Supplementary Table S1*.

BH3 profiling

BH3 profiling was performed as previously described.[30] Briefly, lymphoma cells were suspended in dithioerythritol (DTE) buffer at a density of $2.5\text{-}3\times10^6$/ml and then mixed with an equal volume of dye solution containing 4 μM JC-1, 40 μg/ml oligomycin, 20 mM 2-mercaptoethanol, and 0.01% digitonin (w/v). BH3 peptides were dissolved in DTE buffer at a concentration of 160 μM. Equal volumes (15 μL) of cell/dye mix and peptide solution were added to a flat bottom 384 cell plate and incubated at 30°C in a plate reader. Fluorescent signals were detected at excitation of 545 nm and emission of 590 nm every 30 min. The maximum emission value was used for calculating mitochondrial depolarization. Dimethyl sulfoxide (DMSO) and carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) were used as negative and positive controls, respectively, with % depolarization = 1- (sample-FCCP) / (DMSO-FCCP).

Xenograft experiments

Six- to eight-week-old female CB-17/SCID mice (The Jackson Laboratory, Bar Harbor, ME, USA) were subcutaneously inoculated on the flank with Ramos or Mino cells (5×10^6 cells/animal) suspended in 100 μL PBS. Tumor volume was calculated based on the formula $V= L \times S^2 \times 0.5$ (L: the long axis; S: the short axis). When the xenograft reached a volume of ~200 mm^3, mice were randomly assigned to the control, individual or combined treatment groups, with six mice for each group. Torin1 was first dissolved in 100% N-methyl-2-pyrrolidone and subsequently diluted with PEG400 and water at the ratio of 1:2:2, and intraperitoneally injected daily at a dose of 20 mg/kg. ABT-199 was prepared in 60% phosal 50 PG, 30% PEG400 and 10% ethanol, and given daily by gavage at the dose of 20 mg/kg. For the combined treatment, Torin1 was given 4 h before ABT-199. Animals were sacrificed when the control tumor reached ~2000 mm3 or after the loss of more than 10% of body weight. All animal studies were conducted in accordance with the NIH guidelines for animal care. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee at University of Nebraska Medical Center (UNMC).

For cell lines, inhibitors, and antibodies used, and for additional methods including cell proliferation and apoptosis assays, immunoblotting, luciferase assay and RT-PCR, please see the *Online Supplementary Materials* and Methods for details.

Results

TORKi induces cytotoxicity in B-cell lymphoma cells

To examine the effect of TORKi on the proliferation and survival of lymphoma cells, we selected two commonly used TORKi, Torin1 and AZD8055,[13,19] to treat 17 aggressive B-cell lymphoma cell lines. Although these cell lines showed different sensitivity to the treatment, both drugs significantly inhibited cell proliferation in all tested cells, mostly in a dose-dependent manner (Figure 1A). There is no distinct correlation between the different types of lymphoma and the extent of inhibition. However, both drugs induced significant apoptosis in only a few lymphoma cell

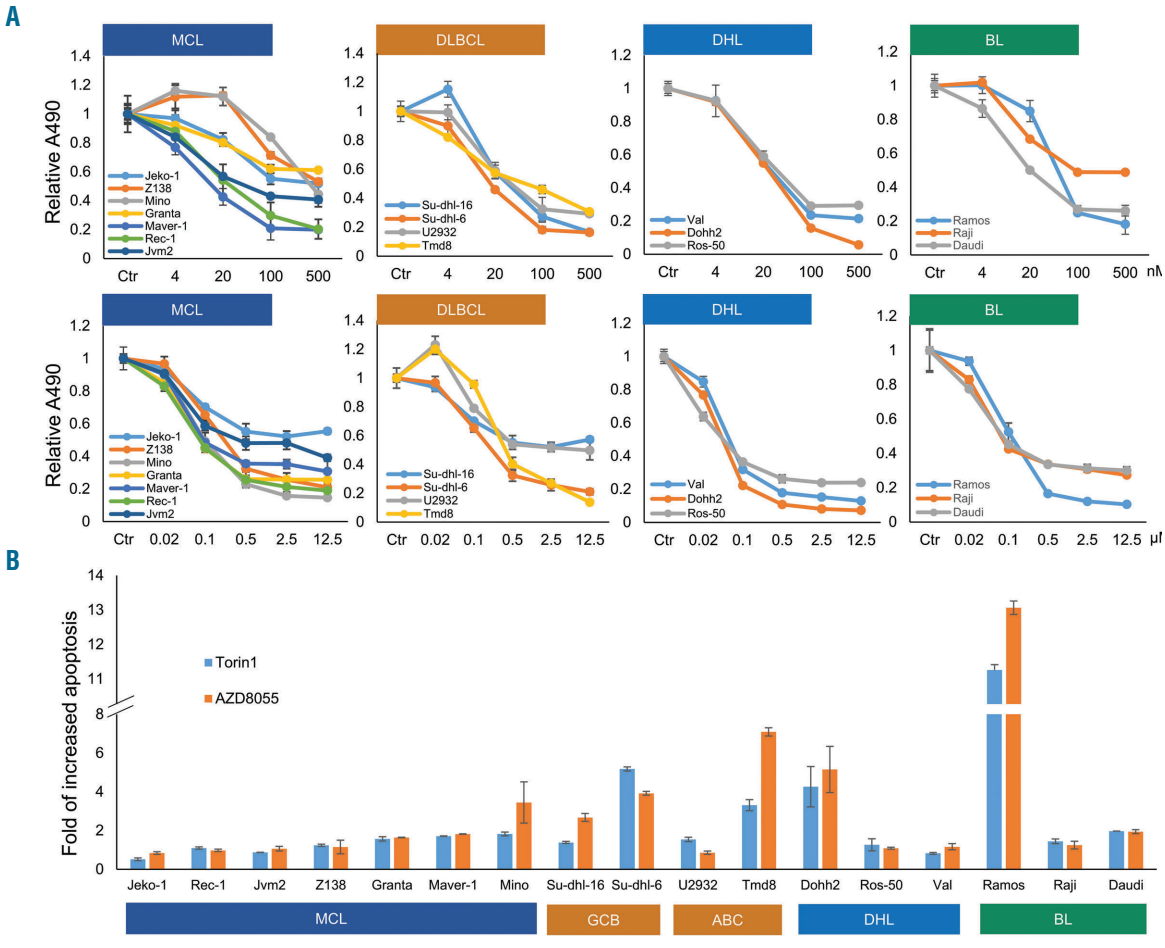

Figure 1. TORKi inhibits cell proliferation and, to a varying extent, induces apoptosis in aggressive B-lymphoma cells. (A) Aggressive B-lymphoma cell lines were treated with TORKi Torin1 (upper panel) and AZD8055 (lower panel) at different doses. Viable cells were determined by MTS assay after 72 h of treatment with each drug. Data shown are the average of three experiments and are presented as mean ± SEM. (B) Apoptosis was quantified by flow cytometry with Annexin V and PI double staining after 48 h of treatment with 250 nM of Torin1 or 5 μM of AZD8055. Relative apoptosis was measured by calculating the fold change to apoptotic fractions of control cells. Data shown are the average of three experiments and are presented as mean ± SEM. MCL: mantle cell lymphoma; DLBCL: diffuse large B-cell lymphoma; DHL: double hit lymphoma; BL: burkitt lymphoma; GCB: germinal center B cell; ABC: activated B cell; Ctr: control.

lines. BL cell line Ramos exhibited the most significant apoptosis upon TORKi treatment, followed by DLBCL lines Tmd8, Su-dhl-6 and DHL line Dohh2; while among MCL lines, increased cell death was only observed in Mino cells (Figure 1B). Prolonged treatment with TORKi (96 h) did not induce significant apoptosis in resistant cell lines either (*Online Supplementary Figure S1A*). Since previous studies have shown that rapalogs induce cell stasis but barely affect cell survival,[17,18] we proceeded to study the significance and mechanism of TORKi-induced apoptosis in lymphoma cells.

TORKi-induced apoptosis is independent of mTORC2 inhibition

We first examined the downstream targets of mTORC1 and mTORC2 as well as the AKT activity in Mino cells upon TORKi treatment. As expected, phosphorylation of RPS6 and 4EBP1 were markedly inhibited upon AZD8055 treatment. Phosphorylation of AKT^{Ser473}, one of the mTORC2 targets, was also inhibited, confirming the effective inhibition of mTORC1 and mTORC2 by TORKi. Phosphorylation of AKT^{Thr308}, one of the targets of PI3K-PDK, was increased upon AZD8055 treatment, suggesting inactivation of the S6K-PI3K negative feedback loop. Surprisingly, phosphorylation of GSK3β, one of the AKT downstream targets, was also increased, suggesting that the net AKT activity was, in fact, elevated (Figure 2A). To avoid unexpected effects secondary to an inadequate amount of AKT protein, we over-expressed AKT in Mino cells, which showed similar effects upon TORKi treatment (Figure 2A).

As one of the most prominent features of TORKi, as compared to rapalogs, is their ability to inhibit both mTORC1 and mTORC2, we further examined whether mTORC2 inhibition plays an important role in TORKi-induced apoptosis. Given that the mTOR pathway crucially regulates protein translation, it would be difficult to evaluate the effects of knocking down one of the mTOR pathway components using post-transcriptional mechanisms, such as shRNA interference, partially due to subsequent alterations in protein synthesis. Therefore, we utilized the CRISPR-Cas9 system to knockout *RICTOR* from genome. Two sensitive cell lines, Ramos and Mino, were chosen for the study. Notably, the knocking out of *RIC-*

TOR had little effect on cell survival in cells without treatment, while TORKi minimally increased apoptosis in Ramos cells (<10%) with *RICTOR* knockout but had virtually no additional effect in Mino cells (Figure 2B-D).

TORKi-induced apoptosis is independent of S6K inhibition

To determine whether S6K inhibition plays a role in TORKi-induced apoptosis, we selected four cell lines, and treated them with either rapalog or TORKi. As expected, temsirolimus, a rapalog, blocked only the S6K pathway, as shown by decreased phosphorylation of S6K target RPS6$^{S235/236}$, whereas TORKi blocked both S6K and 4EBP1 pathways in all tested cells (*Online Supplementary Figure S1B*). In addition, PF4708671, a selective inhibitor of S6K, had no significant impact on apoptosis in both Ramos and Mino cells (*Online Supplementary Figures S1C and S1D*), suggesting TORKi-induced apoptosis is independent of S6K inhibition.

Inhibition of 4EBP-eIF4E pathway plays an important role in TORKi-induced apoptosis

When 4EBP1 binds to eIF4E, it prevents the formation of eIF4F by blocking the recruitment of eIF4G proteins. In fact, eIF4E binding proteins comprise not only 4EBP1 but also 4EBP2 and 4EBP3, all of which are regulated by mTORC1.[8] The ratio of eIF4E/4EBP, rather than their individual protein levels, dictates the 5'cap-dependent mRNA translation efficiency as well as the sensitivity to mTOR inhibition.[8,31,32] To test whether the 4EBP-eIF4E pathway plays an important role in TORKi-induced apoptosis, we first depleted 4EBP1 in lymphoma cells. Ramos and Mino cells were transduced with CRISPR-Cas9 vectors which carry sgRNAs specifically targeting *4EBP1*. After the establishment of stably expressing cells, sgRNA1 exhibited the highest efficiency (Figure 3A) and thus was used in subsequent experiments. For Mino cells, knockout of *4EBP1* almost blocked TORKi-induced apoptosis; the effect is limited in Ramos cells which showed higher sensitivity to TORKi treatment (Figure 3B,C). Since other 4EBPs may act similarly to 4EBP1, and the level of 4EBP3 is very low in leukocytes,[33] we subsequently knocked out *4EBP2* using the CRISPR-Cas9 system. Of the examined sgRNAs, sgRNA2 showed the highest efficiency. Upon treatment, similar results were obtained in both Ramos and Mino *4EBP2* knockout cells, implying that a single 4EBP loss may be insufficient to completely rescue cells from apoptosis because of compensation from other 4EBPs (Figure 3D-F). Therefore, we knocked out both *4EBP1* and *4EBP2* by separate CRISPR-Cas9 constructs in Ramos cells (Figure 3G). Strikingly, the double knockout significantly abolished TORKi-induced apoptosis. Moreover, we found that MCL1 and BCL-XL were substantially upregulated in the double knockout Ramos cells (Figure 3H,I).

Next, we sought to confirm the essential role of the 4EBP-eIF4E pathway in TORKi-induced apoptosis by overexpressing eIF4E in lymphoma cells (Figure 4A). Overexpression of eIF4E in both Ramos and Mino cells significantly attenuated TORKi-induced apoptosis (Figure 4B). We also investigated the level of MCL1, which is remarkably downregulated by TORKi treatment (*Online Supplementary Figure S1A*), and found that eIF4E-overexpressing cells exhibited a higher level of MCL1 than the control cells upon TORKi treatment (Figure 4A), suggesting that increased eIF4E attenuated the TORKi-induced apoptosis, at least partially, through upregulation of MCL1. We also performed similar experiments in Dohh2 cells, one of the DHL cell lines, and revealed similar findings (Figure 4A,B).

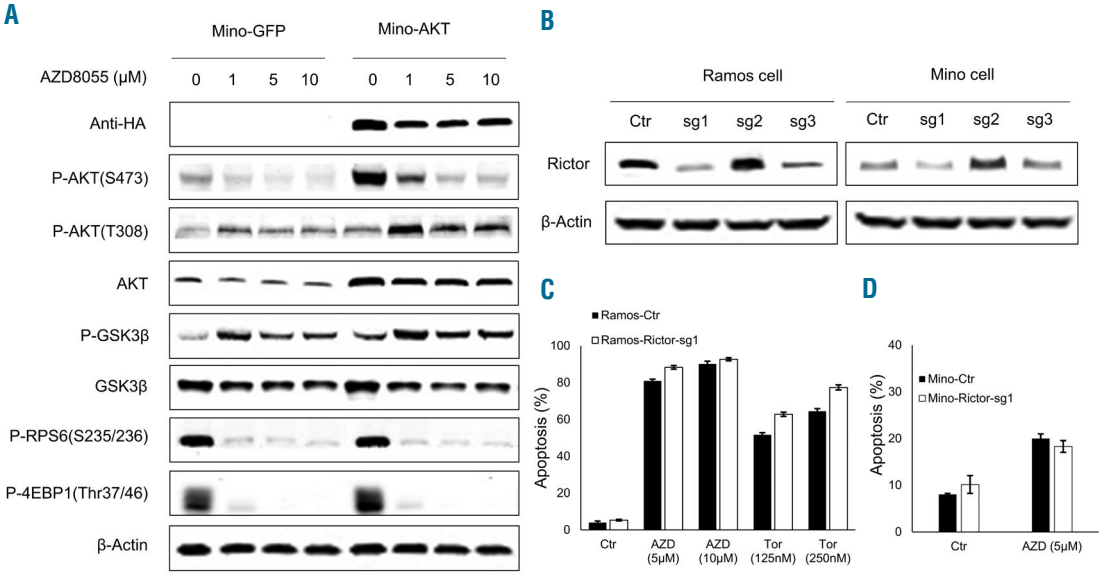

Figure 2. Knocking out Rictor has little effect on TORKi-induced apoptosis in aggressive B-lymphoma cells. (A) Mino cells were transduced with retroviral vectors expressing GFP and AKT. After puromycin selection, cells were treated with AZD8055 for 24 h. AKT and mTORC1 signaling were evaluated. (B) Ramos and Mino cells were transduced with a CRISPR-CAS9 vector that expresses a sgRNA targeting Rictor. After selection for 3 weeks, Rictor protein levels were examined by immunoblotting. (C and D) Cells transduced with Rictor-sgRNA1 were treated with AZD8055 (AZD) or Torin1 (Tor) for 48h. Apoptosis was quantified by flow cytometry with Annexin V and PI double staining. Shown is an average (± SEM) of two independent experiments. Ctr: control.

TORKi treatment downregulates MCL1 and BCL-XL

To further explore the molecular mechanisms of TORKi-induced apoptosis, we examined the effects of TORKi on three important anti-apoptotic proteins in B-cell lymphomas, BCL-2, MCL1 and BCL-XL. As expected, temsirolimus had little effect on the expression of these proteins, whereas TORKi substantially downregulated MCL1 expression, and moderately decreased the BCL-XL level. However, the BCL-2 level was barely affected by TORKi treatment. Interestingly, Ramos, the cell line most sensitive to TORKi treatment, expressed a very low level of BCL-2 (*Online Supplementary Figure S1A*). These findings imply that BCL-2 may play an important role in conferring resistance to mTORC1 inhibition.

To confirm that downregulation of MCL1 and BCL-XL is responsible for TORKi-induced apoptosis, we retrovirally overexpressed MCL1 in Ramos, Mino and Dohh2 cells (Figure 5A-D). Overexpression of MCL1 protein partially rescued cells from TORKi-induced apoptosis in Ramos and was more effective in Mino and Dohh2 cells (Figure 5E-G). Similarly, overexpression of BCL-XL in Ramos cells only partially rescued cells, whereas co-expression of both MCL1 and BCL-XL resulted in a total resistance to TORKi-induced apoptosis. Furthermore, to confirm that high expression of BCL-2 is a major cause for TORKi resistance, we overexpressed BCL-2 in Ramos cells, which completely blocked apoptosis from TORKi treatment (Figure 5A,E).

Prediction of cell sensitivity to TORKi treatment by BH3 profiling

The above findings suggested that reduced expression of MCL1 and BCL-XL secondary to mTORC1 inhibition plays an important role in TORKi-induced apoptosis, whereas elevated BCL-2 expression may correlate with the resistance. An important question is whether the expression of these proteins, especially BCL-2, can be used as a biomarker to predict the susceptibility of lymphoma patients to TORKi treatment. Because apoptosis is controlled by a complicated network consisting of apoptosis activators, sensitizers and anti-apoptotic proteins, quantifying one or a few of the anti-apoptotic proteins alone may be incapable of revealing the most critical proteins the cells rely on for survival. Therefore, we sought to use BH3 profiling, which exposes tumor cells to a series of peptides derived from BH3 proteins and directly determines the important anti-apoptotic proteins for cell survival.[34] Because MCL1 is the main anti-apoptotic protein affected by TORKi treatment, we intended to focus on the effect of its specific binding peptide NOXA. BH3 profiling was performed on Ramos, Mino and Z138 cells, representing cells with high, moderate, and low sensitivity to TORKi treatment, respectively. Ramos cells overexpressing BCL-2 (Ramos-BCL-2), which is highly resistant to TORKI treatment (Figure 5E), were also included as control. Ramos cells clearly demonstrated high mitochondrial

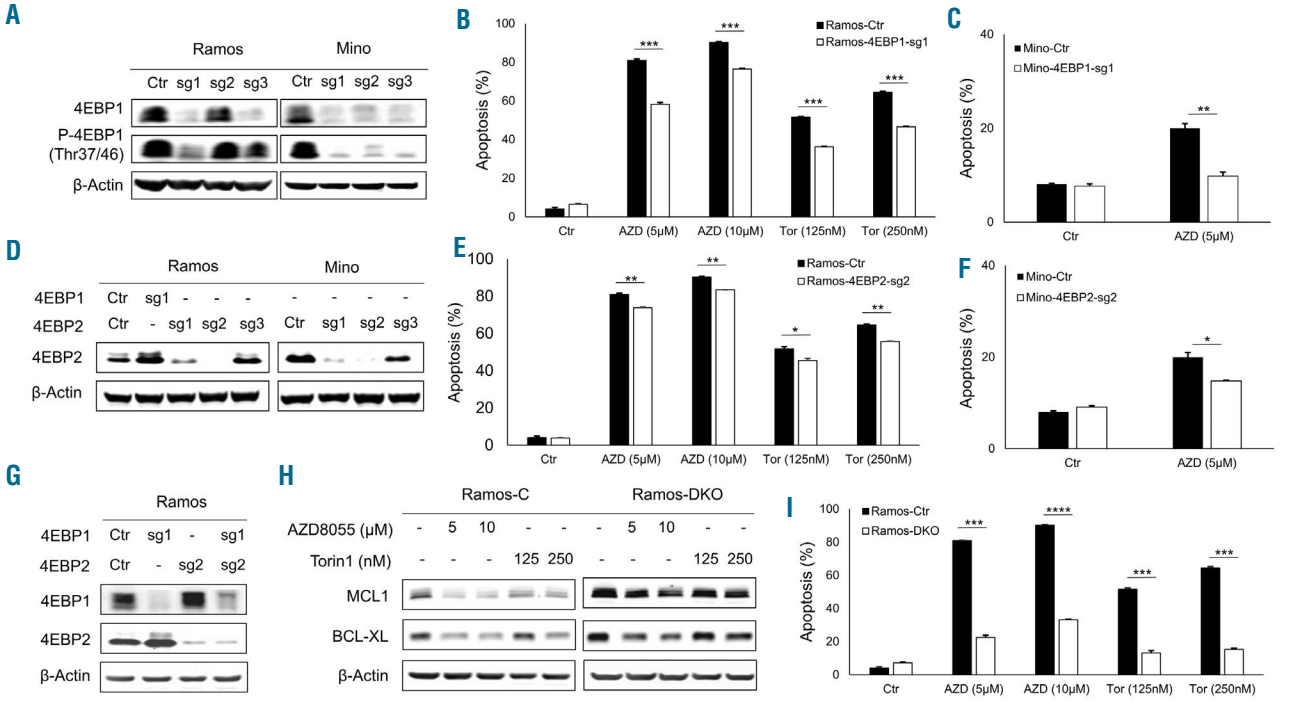

Figure 3. Knocking out of 4EBPs induces resistance to TORKi treatment. (A) Ramos and Mino cells were transduced with CRISPR-CAS9 vectors targeting *4EBP1* and immunoblotted with the indicated antibodies. (B and C) Ramos and Mino cells transduced with 4EBP1-sgRNA1 were treated with AZD8055 (AZD) or Torin1 (Tor) for 48 h, and apoptosis was evaluated using flow cytometry with Annexin V and PI double staining. (D) Cells were transduced with CRISPR-CAS9 vectors targeting *4EBP2* and immunoblotted with the indicated antibodies. (E and F) Ramos and Mino cells transduced with 4EBP2-sgRNA2 were treated with AZD or Tor for 48 h, and apoptosis was evaluated using flow cytometry with Annexin V and PI double staining. (G) Ramos was transduced with CRISPR-CAS9 vectors targeting both 4EPB1 and 4EBP2 and immunoblotted with the indicated antibodies. 48 h after treatment with AZD or Tor, 4EBP1/2 double knockout (Ramos-DKO) and control (Ramos-C) Ramos cells were (H) immunoblotted with antibodies against MCL1 and BCL-XL, and (I) analyzed by flow cytometry with Annexin V and PI double staining to evaluate apoptosis. All data (mean ± SEM) shown are the average of two experiments. *P<0.05; **P<0.01; ***P≤0.001; ****P<0.0001. Ctr: control.

permeabilization triggered by either the NOXA- or BCL-XL-specific binding peptide HRK, whereas Ramos-BCL-2 showed no response to either of the peptides, highlighting the importance of MCL1 and BCL-XL for evading apoptosis in Ramos cells (Figure 6A). Consistent with the sensitivity to TORKi treatment, NOXA induced mild permeabilization in Mino but had no effect on Z138 cells (Figure 6A). We also performed BH3 profiling on a series of TORKi resistant lymphoma cell lines and found that the blocking of MCL1 by the NOXA peptide was unable to induce permeabilization in any of these cells (*Online Supplementary Figure S2*). It should also be noted that blocking BCL-XL by HRK activated the apoptotic pathway in both Mino and Z138 cells, yet they both showed a certain resistance to TORKi treatment, suggesting that decreased MCL1 is the major mechanism for TORKi-induced apoptosis, and a slight decrease of BCL-XL under a high BCL-2 background has little impact on apoptosis.

Optimizing TORKi treatment in aggressive B-cell lymphoma

BH3 profiling not only predicted sensitivity to TORKi treatment, but also provided insights for promoting apoptosis in cancer cells. Based on this analysis, we combined TORKi with the BCL-XL selective inhibitor WEHI-539 to treat wild-type Ramos cells, which exhibited a remarkable synergy in inducing apoptosis (Figure 6B). Notably, inhibition of BCL-2 by ABT-199 or ABT-737 together with TORKi treatment resulted in substantial apoptosis in both Mino and Z138 cells (Figure 6B; *Online Supplementary Figure S3*). This is consistent with the above data demonstrating that BCL-2 overexpression is the major determinant for TORKi resistance. To further explore the necessity of inhibiting BCL-2 in TORKi treatment, we analyzed the mRNA expression of BCL-2 family genes in aggressive B-cell lymphoma cohorts from the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) database. Gene expression profiling data revealed that BCL-2 expression was increased by at least 2-fold in nearly all MCL cases, compared to normal naïve B cells. BCl-2 expression was also increased by at least 2-fold in more than half of DLBCL cases, especially in the activated B-cell (ABC) subtype, and also in a small proportion of BL cases, compared to normal germinal center B cells (Figure 6C). The finding implies that BCL-2 deregulation is a common defect in aggressive B-cell lymphomas, which may induce resistance to TORKi treatment. It is worth noting that only a few cases showed a slight increase in MCL1 levels, but the expression of NOXA (*PMAIP1*) was greatly decreased in almost all of the lymphoma cases, leaving MCL1 free to bind other apoptosis activators or sensitizers. These data suggest that for a large number of aggressive B-cell lymphomas, BCL-2 and MCL1 may collaborate to sustain cell survival. The inhibition of both mTORC1/MCL1 and BCL-2 is therefore likely to provide an effective treatment for these lymphomas. Intriguingly, this strategy was also shown to be very effective in DHL, as the combination of Torin1 and ABT-199 synergistically induced significant apoptosis in Ros-50 and Dohh2 cells (*Online Supplementary Figure S3*).

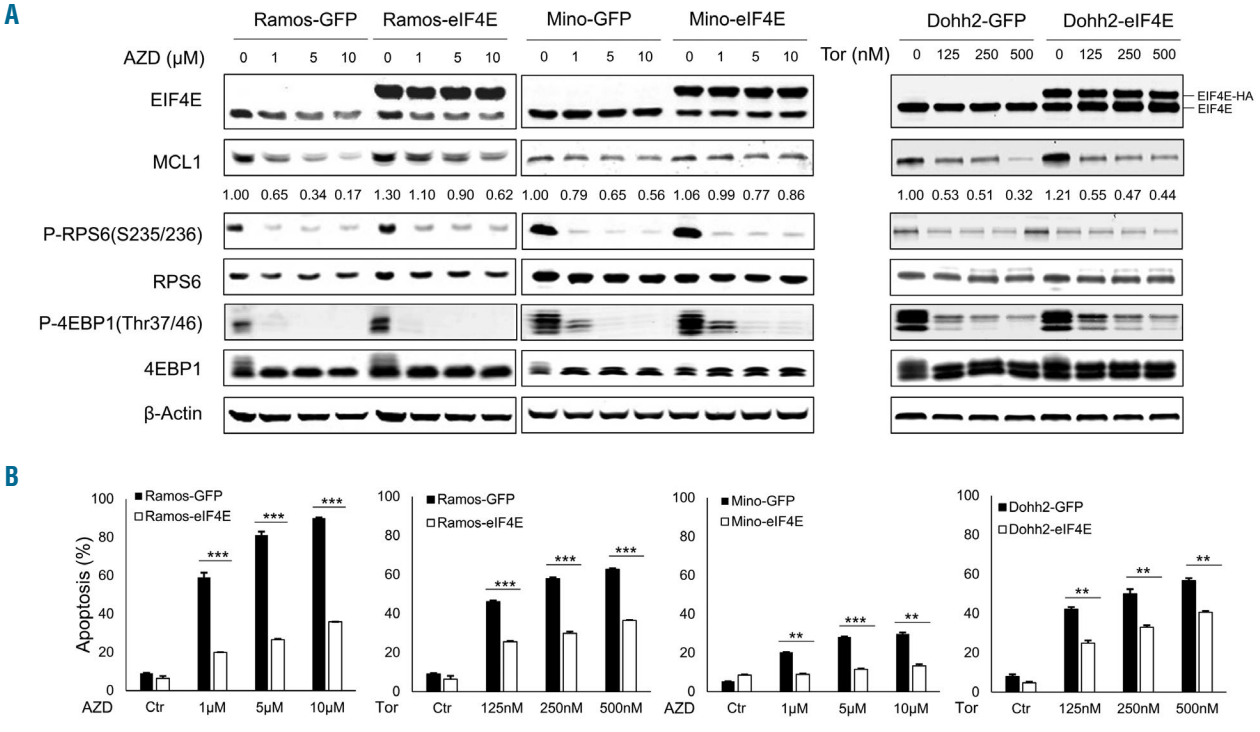

Figure 4. Overexpression of eIF4E rescues lymphoma cells, at least partially, from TORKi-induced apoptosis. (A) Ramos, Mino and Dohh2 cells were transduced with a retroviral vector expressing eIF4E or GFP and immunoblotted by the indicated antibodies. Relative MCL1 expressions were calculated by normalizing individual levels with that of control cells (vector only). (B) Transduced cells were treated with various doses of AZD8055 (AZD) or Torin1 (Tor), and apoptosis was evaluated by Annexin V and PI double staining 48 h after the treatment. Data shown are the average of two experiments and are presented as mean ± SEM. $^{**}P \leq 0.01$; $^{***}P < 0.001$. Ctr: control.

Lastly, we intended to test the efficacy of TORKi treatment in aggressive B-cell lymphomas *in vivo*. First, xenografts of the sensitive cell line Ramos were established and treated with Torin1 daily for 11 days. We found that Torin1 alone is sufficient to suppress tumor growth. The tumor weight at sacrifice was decreased by 60% compared to the placebo group (Figure 7A,B), and an increased number of apoptotic cells were observed in the drug treatment group (Figure 7C,D). Subsequently, we established Mino xenografts and then treated engrafted mice with daily doses of Torin1 and ABT-199, individually or in combination for 14 days. Although each drug alone inhibited tumor growth, the effect was quite limited. In contrast, the combined treatment completely blocked tumor growth, as determined by both tumor volume and weight (Figure 7E,F). Immunostaining for cleaved caspase-3 showed markedly increased apoptosis upon combined treatment, (Ratio of apoptosis: Control: 0.15 ± 0.21%; Torin1 treatment: 0.67 ± 0.26%; ABT-199 treatment: 17.39 ± 2.82%; Torin1 + ABT-199: 69.04 ± 8.59%), indicating that the combination of TORKi and the BCL-2 inhibitor may be of great value in treating aggressive B-cell lymphoma (Figure 7G-J).

Discussion

mTOR complexes are essential for cellular homeostasis as well as controlling cell growth and proliferation. To date, the function and regulation of mTORC1 have been well studied, whereas little is known about mTORC2. One important function of mTORC2 is phosphorylating AGC kinase family members, especially AKT, implicating the regulatory roles of mTORC2 in multiple physiological processes.[35,36] Notably, a recent study using transgenic mice has demonstrated that mTORC2 is required for the homeostasis and function of normal B cells through the regulation of the AKT and NF-κB pathways.[37] However, we found that knockout of Rictor had little impact on lymphoma cell survival, suggesting that mTORC2 becomes dispensable in B-cell lymphomas, most likely due to the constitutive activation of AKT and other oncogenic pathways.[38,39] Furthermore, we showed that AKT^{Ser473} is a direct target of mTORC2, and its phosphorylation was diminished upon TORKi treatment alone. Although this phosphorylation is thought to precede the phosphorylation of AKT^{Thr308} and is required for full AKT activation,[35] our results demonstrated that AKT activity is primarily determined by the phosphorylation of AKT^{Thr308}. This is consistent with previous studies, which demonstrated that the phosphorylation of AKT^{Thr308}, rather than AKT^{Ser473}, is correlated with AKT activity and predicts survival in human non-small cell lung cancer and acute myeloid leukemia.[40,41] It is noteworthy that the highest phosphorylation of AKT^{Thr308} and GSK3β are both present at the low concentration of TORKi treatment. In this condition, phosphorylation of AKT^{S473}, although decreased, was not totally abrogated, supporting the notion that the phosphorylation of AKT^{S473} does enhance the phosphorylation of AKT^{Thr308}. Besides, despite the irrelevance of mTORC2 inhibition in

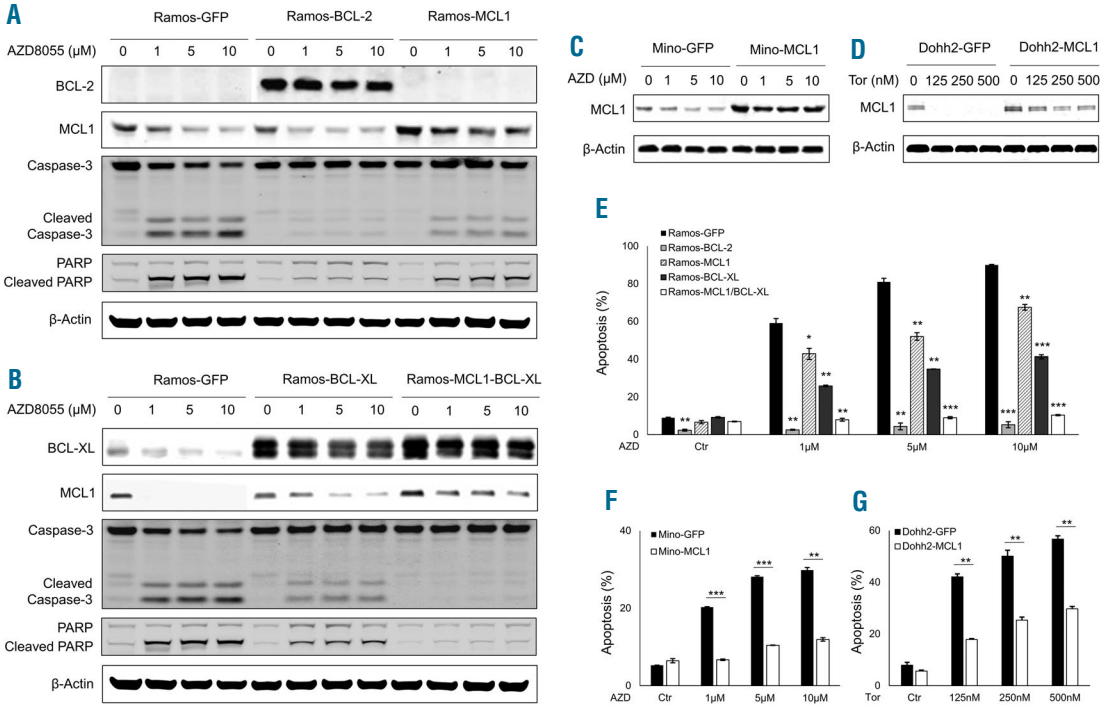

Figure 5. Overexpression of MCL1 and BCL-XL protects lymphoma cells from TORKi-induced apoptosis. (A-D) Exogenous GFP, BCL-2, MCL1 or BCL-XL was overexpressed in lymphoma cells by retrovirus transduction, and the cells were then treated with various doses of AZD8055 for 24 hr. Corresponding protein levels as well as Caspase-3 and PARP were evaluated by immunoblotting. (E-G) Transduced cells were treated with various doses of AZD8055 for 48 h, and apoptosis was evaluated using flow cytometry with Annexin V and PI double staining. Data shown are the average of two experiments and are presented as mean ± SEM. **P*<0.05; ***P*<0.01; ****P*<0.001. AZD: AZD8055; Tor: Torin1; Ctr: control.

TORKi-induced apoptosis, targeting mTORC2 may be still of great value in lymphoma treatment, provided that mTORC2 signaling plays an important part in cell migration and tumor metastasis.[42,43]

The two major substrates of mTORC1 have different roles in cancer development. We found that TORKi-induced apoptosis in lymphoma cells is primarily through the inhibition of 4EBP phosphorylation. However, it does not imply that the S6K pathway is dispensable. In fact, in 4EBP1/2 double knockout cells treated with TORKi, apoptosis is markedly abrogated, yet cell proliferation is still remarkably suppressed (*data not shown*). These data corroborate a previous study which demonstrated that rapalogs, which inhibited S6K alone, have a merely cytostatic effect in B-cell lymphoma cells,[44] and partially explained their limited effect in lymphoma clinical trials, even when combined with rituximab.[9-12] Among the mTORC1-regulated 4EBP proteins, 4EBP1 is the most recognized eIF4E binding protein in mammalian cells. Nevertheless, 4EBP1 knockout only partially rescued the most sensitive lymphoma cells from mTOR inhibition, which implied the importance of 4EBP2 in lymphoma cells. We analyzed the expression of 4EBPs in primary aggressive B-cell lymphomas and found that the abundance of 4EBP2 is higher than, or at least similar to, that of 4EBP1, whereas 4EBP3 is hardly detectable (*data not shown*). As 4EBP1 and 4EBP2 share a conserved eIF4E binding motif and are both regulated by mTORC1, one could compensate for loss of the other.[45,46] Interestingly, a recent study has shown that in Val, a 4EBP1-deficient cell, TORKi treatment increased the amount of 4EBP2 bound to eIF4E, but it seemed to be ineffective in blocking the formation of the initiation complex.[47] One likely explanation is that eIF4E expression largely exceeds that of eIF4G and 4EBPs in Val cells, making 4EBP2 unable to sequester eIF4E from other eIF4F components. This further indicates that the ratio of eIF4E to 4EBPs, rather than the abundance of individual proteins, determines the efficacy of mTOR inhibition.[8,31]

In the study herein, we found that reduced MCL1 expression is a crucial determinant of TORKi sensitivity, which makes using BH3 profiling to predict the sensitivity to mTOR inhibition possible. One of the advantages of BH3 profiling is that it can directly ascertain whether lymphoma cells will undergo apoptosis upon MCL1 blockade by NOXA. Notably, a large proportion of aggressive B-cell lymphomas express relatively high levels of BCL-2, which is the major determinant for TORKi resistance.

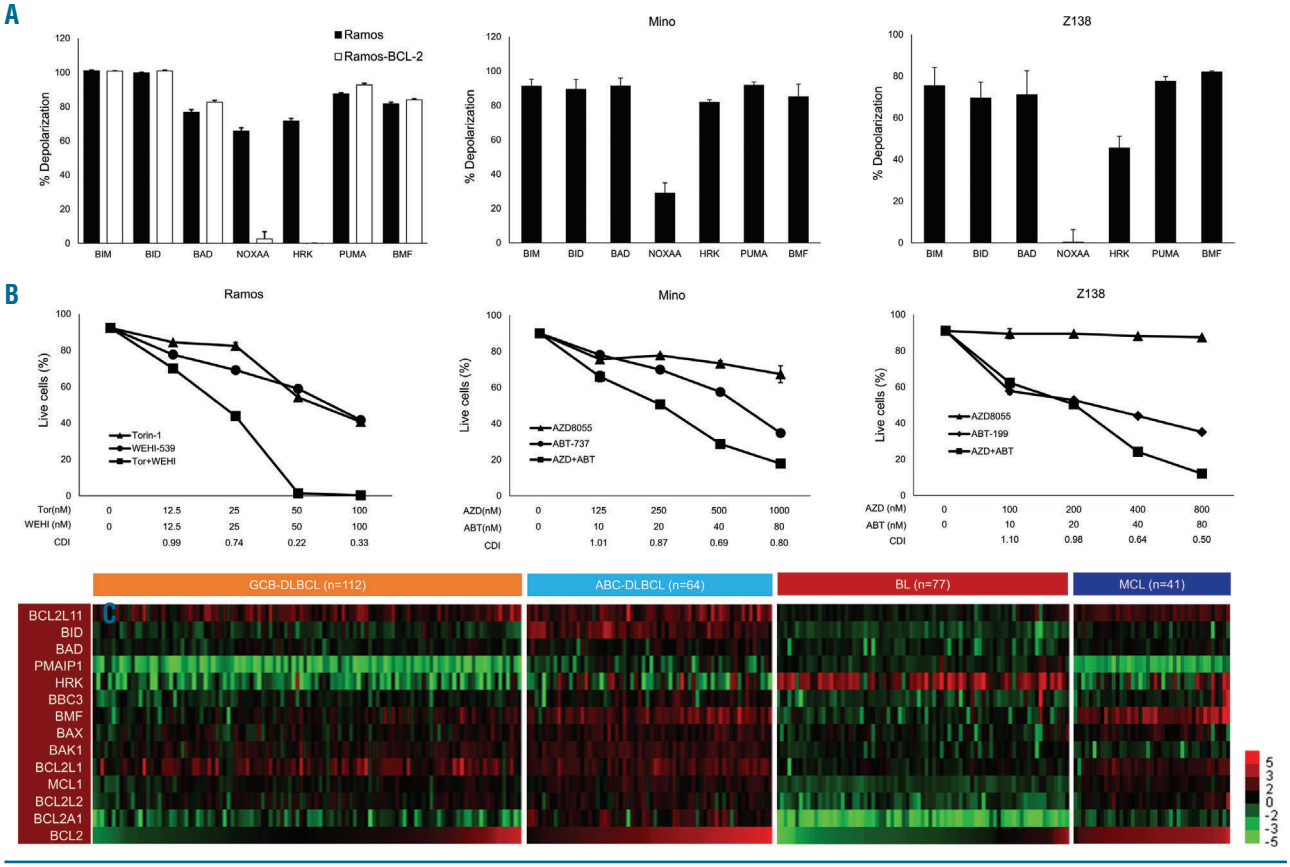

Figure 6. BH3 profiling predicts sensitivity to TORKi treatment. (A) Lymphoma cells were treated with various peptides as indicated in Ramos, Ramos with BCL-2 overexpression, Mino and Z138. Mitochondrial depolarization was determined by JC-1 staining. Results are shown as mean (n =3), compared to solvent control DMSO values; error bar represents standard deviation. (B) Cells were treated with the combination of TORKi with BCL-XL selective inhibitor WEHI-539 or BCL-2/BCL-XL inhibitor ABT-737 or BCL-2 inhibitor ABT-199 for 48 h, and then apoptosis was quantified by flow cytometry. Coefficients of drug interaction (CDI) were calculated based on the inhibitory effect of the individual drugs and combined treatment. Data shown are the average of two experiments and are presented as mean ± SEM. (C) The heatmap illustrates the relative mRNA level (log2 fold change) of BCL-2 family genes with DLBCL and BL compared to that of normal centrocytes and MCL compared to that of normal naïve B cells. GCB-DLBCL: diffuse large B-cell lymphoma germinal center B-cell subtype; ABC DLBCL: diffuse large B-cell lymphoma activated B-cell subtype: BL: burkitt lymphoma; MCL: mantle cell lymphoma; AZD: AZD8055; Tor: Torin1.

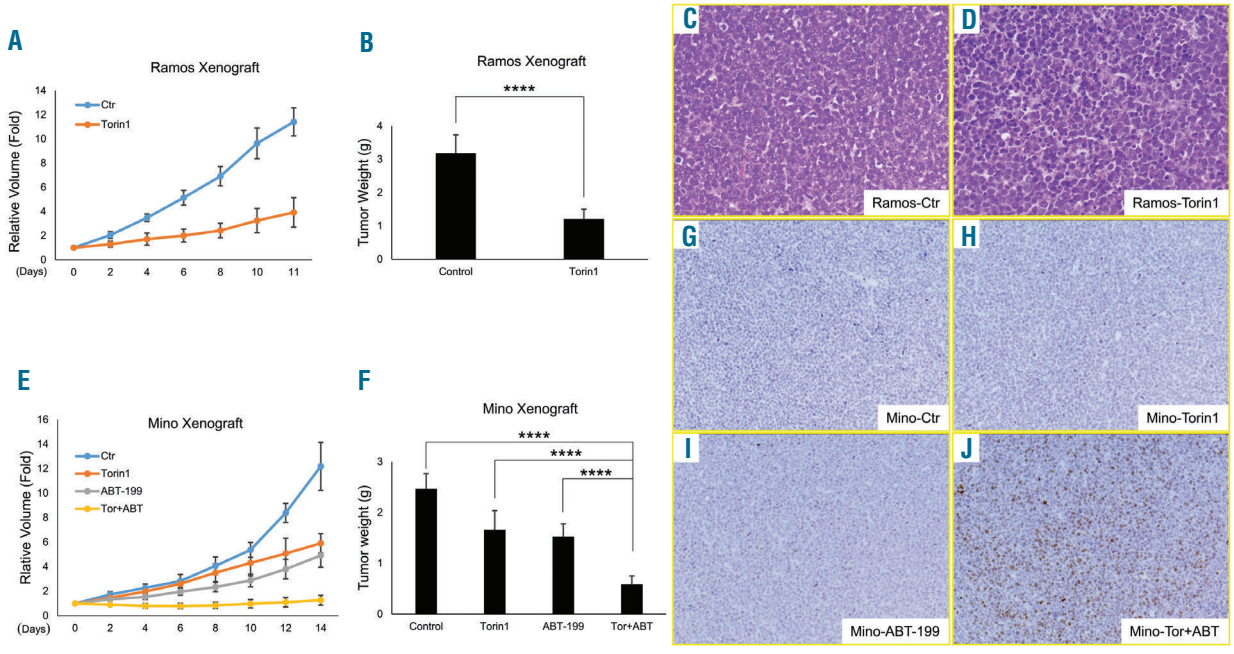

Figure 7. Animals with xenograft lymphomas were treated with Torin1 and/or BCL-2 inhibitor, ABT-199. Ramos and Mino xenografts were established in NOD/SCID mice, and the mice were treated with the indicated drugs. (A and E) Tumor growth was evaluated by the ratio of tumor volume at the indicated time to that at the initiation of drug treatment, results are shown as mean ± SEM (n =6 in each group). (B and F) Tumor weights were obtained at autopsy and compared across different groups of treatment at 11 and 14 days after the initiation of treatment for Ramos and Mino models, respectively. (C and D) H&E staining of the Ramos xenograft tumors. Markedly increased apoptosis was observed in mice treated with Torin1. (G-J) Immunohistochemical staining using antibody against cleaved caspase-3 in Mino xenograft tumors treated with Torin1 (Tor) and ABT-199 (ABT), individually or in combination. ****P<0.0001. Ctr: control.

Combining TORKi with a BCL-2 antagonist, such as ABT-199, will thus likely provide a synergistic anti-tumor effect in aggressive B-cell lymphomas. A previous study has demonstrated that dual PI3K/mTOR inhibitors can overcome resistance to ABT-199 by decreasing MCL1 and BCL-XL in lymphoid malignancies. Our study, from the other aspect, suggested that blockade of BCL-2 is of great value in counteracting resistance to TORKi treatment. Interestingly, the effectiveness of combining TORKi with ABT-199 in DHL cells makes it a promising strategy for this intractable entity. Nevertheless, there are some B-cell lymphomas which do not rely on BCL-2 for survival, including those "unprimed" cases.[34] Therefore, predicting sensitivity to BCL-2 inhibition is also valuable. Unfortunately, due to the lack of a unique BCL-2 binding peptide, it is hard to predict the precise effect of BCL-2 in cancer cells using a peptide-based BH3 profiling method. However, a similar method which measures mitochondrial depolarization following a direct challenge by the BCL-2 inhibitor has been applied in recent studies.[48,49]

In conclusion, we demonstrated that TORKi-induced apoptosis is predominantly dependent on the loss of mTORC1-mediated 4EBP phosphorylation, and subsequent MCL1 downregulation. Overexpression of BCL-2 confers resistance to TORKi treatment, and BH3 profiling is useful in predicting TORKi sensitivity in lymphoma cells. Combined treatment with TORKi and a BCL-2 antagonist exerts significant synergistic anti-tumor effects both *in vitro* and *in vivo*.

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