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

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Supplementary Materials and Methods:

**Cell lines and drugs.** Jeko-1, Mino, Z138, Rec-1, Jvm2, Mauer-1, Ramos, Raji, Daudi, Su-dhl-16, Su-dhl-6, Tmd8, U2932, Dohh2 and ROS-50 cells were maintained in RPMI 1640; Granta and HEK-293T cells were maintained in DMEM; Val cell was maintained in IMDM with 55 µM 2-mercaptoethanol. All media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Torin1, ABT-737 and ABT-199 were from Chemscene (Monmouth Junction, NJ); AZD-8055 was from ChemieTek (Indianapolis, IN); WEHI-539 was from ApexBio (Boston, MA); Temsirolimus was from Sigma-Aldrich (St. Louis, MO).

**Cell proliferation and apoptosis assays.** Viable cell metabolism was determined by using the CellTiter 96 AQueous kit (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, cells were placed at a density of 2-5×10^5 cells/ml and treated with drug or vector for 72 hours. The cells were then incubated with MTS reagent for 2h, and 490 nm absorbance was quantified with a plate reader. Coefficient of drug interaction (CDI) were calculated by the following equation: CDI = AB/ (A × B), where AB is the inhibitory ratio of the combined group to control group; A or B is the ratio of single agent group to control group, respectively. CDI less than 0.7 indicates that two drugs have significant synergy.

Apoptosis were determined by using Annexin-V staining kit (BD Biosciences, San Jose, CA). Cells were treated with drug or vector for 24 or 48 h and then pelleted and washed once with cold phosphate-buffered saline (PBS). The cell pellets were then resuspended in 1X binding buffer at a concentration of 10^6 cells/ml. 100 µl of the suspension was transferred to a culture tube and incubated with Annexin V-FITC and propidium iodide or Annexin V-PE and 7-AAD, 5 µl of each, for 15 min at 25°C, protecting from light. The stained sample were then diluted with 400 µl of 1X binding buffer and analyzed by FACSCalibur flow cytometry (BD Biosciences).

**Immunoblotting and antibodies.** Cell lysates were separated and immunoblotted by using SDS-PAGE (Bio-Rad, Hercules, CA) or Bolt® Bis-Tris system (Life technology, Carlsbad, CA). Blots were scanned with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), and the band intensity was quantified by using Odyssey software v2.0.
Antibodies for immunoblotting in this study were as follows: 4EBP1 (#9644), 4EBP2 (#2845), p-4EBP1^Thr37/46 (#2855), RPS6 (#2217), p-RPS6$^{S235/236}$ (#4858), EIF4E (#9742), BCL-2 (#2870), MCL1 (#5453), p-AKT$^{S473}$ (#4060), p-AKT$^{T308}$ (#13038) and p- GSK3-β (#9336) were from Cell Signaling Biotechnology; BCLX (#556361) and GSK3-β (#610202) were from BD Biosciences (San Jose, CA); AKT (#1080-1) was from Epitomics (Burlingame, CA); Actin (#1616) was from Santa Cruz Biotechnology (Dallas, TX); Rictor (#70374) was from Abcam (Cambridge, MA).

Supplementary Table S1: The sgRNA sequences of CRISPR-CAS9 experiments

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Supplementary Figure Legends:

Supplementary Figure S1. Inhibition of p70S6K has little effect on TORKI-induced apoptosis in aggressive B lymphoma cells. (A) Resistant cells were treated with TORKi for 96h, and the apoptosis was measured. (B) Lymphoma cells were treated with Temsirolimus, AZD8055, or Torin-1 for 24 h, and immunoblotting was performed using the indicated antibodies. (C and D) Ramos and Mino cells were treated with PF4708671, a p70S6K selective inhibitor, and measured with p-RPS6 (S235/236) and apoptosis (48h). All apoptosis was evaluated using flow cytometry with Annexin V and PI double staining. Data shown are average of two experiments and are presented as mean ± SEM.

Supplementary Figure S2. BH3 profiling of TORKI-resistant aggressive lymphoma cells. The assays were performed using 80 μM BH3-only peptides as illustrated in Figure 6.

Supplementary Figure S3. Combination of TORKI with other pro-apoptotic drugs. (A) Mino cells were treated with AZD8055 and ABT-199 (targeting BCL-2) or WEHI-539 (targeting BCL-
XL), individually or in combination. The apoptosis was evaluated using flow cytometry with Annexin V and PI double staining 48h after the treatment. (B) Mino cell were treated with AZD8055 and ABT-737, individually or in combination, and cell viability was measured by MTS assay 72 h after the treatments. (C and D) DHL cells Ros-50 and Dohh2 were treated with Torin1 and ABT-199 for 48 h, individually or in combination. Live cells were evaluated by measuring apoptosis with Annexin V and PI double staining. **: P<0.01; ***: P<0.001. Results are shown as the mean ± SEM of two experiments for apoptosis assay and three experiments for MTS assay.