Cap-Translation Inhibitor, 4EGI-1, Restores Sensitivity to ABT-737 Apoptosis through Cap-Dependent and -Independent Mechanisms in Chronic Lymphocytic Leukemia

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Abstract

**Purpose:** The lymph node microenvironment promotes resistance to chemotherapy in chronic lymphocytic leukemia (CLL), partly through induction of BCL2 family prosurvival proteins. Currently available inhibitors do not target all BCL2 family prosurvival proteins and their effectiveness is also modified by proapoptotic BCL2 homology domain 3 (BH3) only protein expression. The goal of this study was to evaluate synergy between the eIF4E/eIF4G interaction inhibitor, 4EGI-1, and the BH3 mimicetic, ABT-737.

**Experimental Design:** CLL cells were cultured in conditions to mimic the lymph node microenvironment. Protein synthesis and cap-complex formation were determined. Polysome association of mRNAs from BCL2 family survival genes was analyzed by translational profiling. The effects of 4EGI-1 and the BCL2/BCL2L1 antagonist, ABT-737, on CLL cell apoptosis were determined.

**Results:** Protein synthesis was increased approximately 6-fold by stromal cell/CD154 culture in a phosphoinositide 3-kinase (PI3K)–specific manner and was reduced by 4EGI-1. PI3K inhibitors and 4EGI-1 also reduced cap-complex formation but only 4EGI-1 consistently reduced BCL2L1 and BCL2A1 protein levels. 4EGI-1, but not PI3K inhibitors or rapamycin, induced an endoplasmic reticulum stress response including proapoptotic NOXA and the translation inhibitor phosphorylated eIF2α. 4EGI-1 and ABT-737 synergized to cause apoptosis, independent of levels of prosurvival protein expression in individual patients.

**Conclusions:** Overall protein synthesis and cap-complex formation are induced by microenvironment stimuli in CLL. Inhibition of the cap-complex was not sufficient to repress BCL2 family prosurvival expression, but 4EGI-1 inhibited BCL2A1 and BCL2L1 while inducing NOXA through cap-dependent and -independent mechanisms. 4EGI-1 and ABT-737 synergized to produce apoptosis, and these agents may be the basis for a therapeutically useful combination.

Introduction

Defects in apoptosis due to dysregulation of BCL2 family proteins are common in cancers and BCL2 homology domain 3 (BH3) mimic drugs, such as ABT-263, which bind and inhibit antiapoptotic BCL2, and to a lesser extent BCL2L1, are being trialed in lymphoid malignancies (1, 2). ABT-263 was derived from a prior compound, ABT-737, and several causes of resistance to ABT-737 that may limit the clinical use of this class of agents are recognized. For example, increased expression of antiapoptotic proteins BCL2L1 (formerly BCL-XL; refs. 3, 4), BCL2A1 (formerly A1 or BFL1; refs. 4, 5), and MCL1 (5–7) reduce the effectiveness of ABT-737 and extracellular signal–regulated kinase (ERK) signaling pathways also promote resistance to this agent (8).

Circulating chronic lymphocytic leukemia (CLL) cells express more BCL2 than normal B cells and in vitro leukemic cells are highly sensitive to ABT-737 (4, 7), but within lymph nodes leukemic cells express BCL2A1, BCL2L1 (9), and MCL1 (10), which promote resistance to this agent. Induction of these proteins is likely to be due to signals from the lymph node microenvironment (11) and in support of this, CLL cells stimulated in vitro by the T-cell surface molecule, CD154 (9, 12) or through B-cell receptor cross-linking (13) reproduce these expression changes. CLL is
incurable with conventional chemotherapy and robust survival within the lymph node microenvironment is considered to be a cause of treatment failure. Consequently, there is interest in evaluating agents targeting microenvironment-induced signals for treatment of CLL (11).

Translation is increased in transformed cells and cancers (14) and several studies have shown that overexpression of one component of the cap-binding complex, eIF4E, is sufficient to cause cellular transformation (15–17) and transformation of primary embryonic fibroblasts (18) in part by cooperation with c-MYC. Specifically in B cells, overexpression of the cap-binding complex promotes lymphomagenesis, again in cooperation with c-MYC, in eIF4E transgenic mice (19, 20). eIF4E levels are elevated in non-Hodgkin’s lymphoma and are also high in normal germinal centers with little expression in the mantle zone (21). Most cases of clinically aggressive lymphoma show strong expression of eIF4E (21). Therefore, increased expression of components of the translation machinery is sufficient to support lymphomas in experimental systems. Protein translation is a target for treatment in cancer (22) and hematologic malignancies (23), and the eIF4E inhibitor and antiviral agent ribavirin has been trialed in acute myeloid leukemia (24).

There are indications that inhibition of translation may be a useful strategy to induce apoptosis in CLL. Two natural products—homoharringtonine (25) and silvestrol (26)—that exert their effects partly through inhibition of translation cause apoptosis of CLL cells in vitro and in vivo. Homoharringtonine prevented the prosurvival effects of stromal cell culture. However, both these compounds have effects on many aspects of cellular metabolism apart from translation.

We identify increased protein synthesis as being an important feature of CLL cells supported on stromal cells/CD154. We also show that a specific inhibitor of eIF4E/eIF4G interaction, 4EGI-1, causes changes to BCL2 family proteins favoring synergy with a BCL2/BCLL1 antagonist, ABT-737, but part of this effect is independent of cap-dependent translation through induction of an endoplasmic reticulum (ER) stress response. We suggest that this combination of agents will be a useful route to abrogating the prosurvival effects of the microenvironment.

### Translational Relevance

The tissue microenvironment contributes to survival, proliferation, and resistance to conventional chemotherapy in chronic lymphocytic leukemia (CLL), and abrogating the effects of the microenvironment is a goal of therapy. There is interest in using small-molecule BCL2/BCLL1 antagonists for the treatment of cancers, but such agents do not inhibit prosurvival, MCL1 and their effectiveness is also critically dependent on BCL2 homology domain 3 (BH3)—only proapoptotic protein expression. We showed that a culture system mimicking the microenvironment increased protein synthesis and this prompted us to evaluate 4EGI-1, a small-molecule inhibitor of cap-complex formation. This agent repressed prosurvival BCL2L1 and BCL2A1 and induced proapoptotic NOXA, a pattern of activity suggesting useful cooperation with the BH3 mimetic, ABT-737. We showed synergy between 4EGI-1 and a BCL2/BCLL1 antagonist in different individuals across a range of expression levels. Combination of 4EGI-1 with BCL2/BCLL1 antagonists may be highly effective in targeting leukemic cells within the microenvironment.

### Materials and Methods

#### Patient samples

CLL cells were isolated from whole blood using density gradient centrifugation. Patients included those with both early stage and advanced disease. Only patients with white cell count more than 50 x 10^3/L were used in the study. At the time of study, no patient had been treated for 3 months. Local research ethics committee approval was obtained. Patient characteristics are presented in Table 1.

#### Cell culture

CLL cells (3 x 10^6/mL) were cultured in RPMI-1640 medium (Invitrogen) supplemented with FBS (Invitrogen), nonessential amino acids (Invitrogen), penicillin/streptomycin (Invitrogen), and HEPES buffer (Lonza) in a 37°C and 5% CO_2 incubator. The cells were either cultured on tissue culture plastic or cocultured with 80% to 90% confluent and 35 Gy irradiated nontransfected mouse fibroblast cells (NT culture) or human CD154-expressing mouse fibroblast cells (NT culture) or human CD154-expressing mouse fibroblast cells supplemented with rh-IL-4 (10 ng/mL; R&D Systems; CD culture; ref. 27).

The phosphoinositide 3-kinase (PI3K) inhibitors, LY294002 (Calbiochem), PI-103 (Calbiochem), and PIK-294 (Calbiochem) and BH3-mimetics, ABT-737 were used as indicated. Rapamycin was purchased from Sigma. 4EGI-1 (28) and salubrinal (29) from Santa Cruz Biotechnology.

#### Sucrose density gradients

Cycloheximide (100 µg/mL; Sigma) was added before harvesting 36 to 40 x 10^6 cells. Following lysis in 500 µL of polysome extraction buffer [15 mmol/L Tris (pH 7.5), 15 mmol/L MgCl_2, 300 mmol/L NaCl, 1% Triton X-100, 100 mg/mL cycloheximide, 50 mg/mL heparin, 5 mmol/L dithiothreitol, and RNase inhibitors] lysates were centrifuged and the supernatant layered on to a 10% to 50% sucrose gradient [Biocomp Gradient Station (Wolf Laboratories Ltd.)], followed by centrifugation at 38,000 rpm for 2 hours in a Beckman SW40 rotor. mRNA was extracted from each of 12 fractions using phenol/chloroform extraction and lithium chloride precipitation. TaqMan real-time PCR assays were from Applied Biosystems [actin (ACTB) #Hs00664796_m1, RPS6 #Hs00265147_m1, MCL1 #Hs00189665_m1, BCL2A1 #Hs00111011_m1, BCL2L1 #Hs00533304_m1, MTH1 #Hs00265570_m1, RPL13A #Hs00361482_m1, BCL2L2 #Hs00159134_m1, GAPDH #Hs02758991_m1, LAMB1 #Hs00173246_m1, CD44 #Hs00268227_m1, and 18s #Hs02758991_m1]. The relative amount of mRNA in
Table 1. Patient characteristics

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NOTE: Patient gender, clinical stage according to the Binet classification, percentage of cells expressing the CD38 surface marker, immunoglobulin gene mutational status [either unmuted (U) or mutated (M)], V\textsubscript{H} gene segment and cytogenetics, which was obtained either by using a FISH panel or by analysis of metaphase spreads. Blanks under cytogenetics means no abnormalities detected by FISH panel. Abbreviation: ND, not determined.
each fraction of the sucrose gradient was expressed as a fraction of the total amount of mRNA on the gradient.

**Cap-binding assay**

CLL cells (5 × 10⁶) were cultured for 24 hours on CD154/IL-4 in the presence or absence of LY294002 (20 μmol/L). Cells were harvested, washed in 300 μL ice-cold buffer A [50 mmol/L MOPS/KOH, pH 7.2, 50 mmol/L NaCl, 50 mmol/L NaF, 2 mmol/L EGTA, 5 mmol/L EDTA, 7 mmol/L 2-mercaptoethanol, and protease and phosphatase inhibitors (Sigma)], and lysed by the addition of 1.5% (v/v) NP-40 and 1.5% (v/v) Triton X-100. Lysates were centrifuged at 15,000 rpm for 5 minutes at 4°C and the supernatant added to 50 μL of 50% (v/v) m7GTP-Sepharose beads (GE Healthcare). Samples were incubated for 25 minutes at 4°C on an Eppendorf tube shaker, after which the beads were washed 3 times in buffer A and isolated by centrifugation. Bound protein was recovered by boiling the beads for 5 minutes in SDS sample buffer and analyzed by SDS-PAGE.

The following antibodies were used following the manufacturers’ instructions: rabbit anti-elf4G 1:1,000 (Cell Signaling Technology), mouse anti-elf4E 1:1,000 (BD Biosciences), and rabbit anti-4EBP1 1:1,000 (Cell Signaling Technology).

**Western blotting**

CLL cells were harvested and protein extracts made using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (Sigma). Proteins were separated using a 12% SDS-gel, using radioimmunoprecipitation assay (RIPA) buffer 1:2,000. HRP or anti-rabbit-IgG-HRP (Sigma)—were used at mouse-immunoglobulin G (IgG)–horseradish peroxidase were also used at 1:1,000. Secondary antibodies–anti-NOXA phospho-S6 kinase, and phospho-eIF4E were from New England Biolabs and were used at 1:1,000. Anti-NOXA and GAPDH (1:1,000; New England Biolabs). Anti-elf4G and elf4E were from New England Biolabs. Anti-elf4G was a gift from Dr. J. Borst (The Netherlands Cancer Institute, Amsterdam, the Netherlands). Anti-elf4E–BP, phospho-S6 ribosomal protein, phospho-S6 kinase, and phospho-elf4E were from New England Biolabs and were used at 1:1,000. Anti-NOXA (Enzo Life Sciences) and anti-BIM (New England Biolabs) were also used at 1:1,000. Secondary antibodies–anti-mouse-immunoglobulin G (IgG)–horseradish peroxidase (HRP) or anti-rabbit-IgG-HRP (Sigma)—were used at 1:2,000.

**35S methionine incorporation**

Cells (3 × 10⁶/mL) were cultured on plastic or 80% to 90% confluent and 35 Gy irradiated stromal layers for 24 hours. 35S methionine (3 μL of 37 mBq/mL; PerkinElmer) was added to each well and cells cultured for an additional 30 minutes at 37°C. After harvesting, cells were washed in PBS, lysed using Passive Lysis Buffer (Promega), centrifuged (13,000 rpm, 1 minute) to remove cellular debris, and the protein was precipitated using 25% trichloro-acetic acid (Sigma). Lysates were transferred to filter papers using a Millipore vacuum manifold and incorporation of radioactivity measured using a Wallac liquid scintillation counter (PerkinElmer).

**Cell viability**

CLL cells (5 × 10⁶/well of a 96-well microtiter plate) were cultured for 24 hours with 4EGI-1, thapsigargin, or ABT-737. Cells were harvested from the stromal layer and intracellular ATP was determined by incubation with CellTiter-Glo reagent (100 μL; Promega) for 10 minutes in opaque plates before the luminescence intensity was read using a Wallac Victor 1420 Multilabel counter.

**Statistical analysis**

Statistical analyses were conducted using GraphPad Prism version 4.0b (GraphPad Software Inc.). Pharmacologic analysis of drug effects, singly and in combination was conducted with CalcuSyn version 2 (Biosoft; ref. 30).

**Results**

Culture on stromal cells in the presence and absence of CD154 increases protein synthesis and cap-complex formation in a PI3K-dependent manner

35S methionine incorporation was used to determine effects of culture conditions on overall protein synthesis. Stromal cell culture induced a 6-fold increase in 35S methionine incorporation (paired t test; P = 0.006) with a further increment due to the presence of CD154 (Fig. 1A). To determine changes to cap-complex formation under different culture conditions cap-binding assays were conducted. There was little association of elf4G with elf4E in freshly isolated leukemia cells, but following culture on stromal cells alone the amount of elf4E increased as compared with basal conditions, with stromal cell/CD154 culture producing a further increment (Fig. 1B). These results showed that induction of cap-complex formation associated with an increase in overall protein synthesis and both were effects of culture with stromal cells/CD154.

PI3K signaling induced translation in normal and cancer cells and is enhanced by stromal cell contact in CLL (31). We, therefore, determined the role of this pathway in leukemic cells in stromal cell/CD154 culture. The nonisoform-specific PI3K inhibitor, LY294002, reduced protein synthesis by approximately 70% (Fig. 1C; paired t test; P = 0.007) and produced a corresponding reduction in the association of elf4G with elf4E in cap-binding assays (Fig. 1B). A specific PI3Kα inhibitor, PI-103, repressed protein synthesis (P = 0.02), whereas a PI3Kβ inhibitor, PIK-294, had no effect (Fig. 1C). In keeping with this, PI-103 reduced cap-complex formation to subbasal levels, whereas PIK-294 was ineffective (Fig. 1D).
Both rapamycin (P = 0.007), an inhibitor of mTORC1, and the eIF4E/eIF4G interaction inhibitor, 4EGI-1 (P = 0.004), repressed protein synthesis and cap-complex formation.

4EBP1 and S6K are important targets of PI3K/AKT/mTOR in the control of cap-dependent translation. Stromal cell/CD154 culture induced 4EBP1 phosphorylation (Fig. 2A and C) and this was abolished by LY294002, PI-103 and rapamycin, with LY294002 being least effective at the concentrations used. 4EBP1 phosphorylation was not altered by 4EGI-1, as anticipated from its mechanism of action, or by PIK-294, in keeping with ineffectiveness of this agent in reducing 35S methionine incorporation and cap-complex formation (Fig. 2C). S6K seemed to be expressed at low levels in CLL although some induction was seen on stromal cell/CD154 culture (Fig. 2A). Ribosomal protein S6 (rpS6) is a direct target of S6K and its phosphorylation also increased on stromal cell/CD154 culture and was sensitive to PI3K inhibition (Fig. 2B). Therefore, PI3K, which has previously been shown to be required for leukemic cell survival on stromal layers (31), and specifically the PI3Kα isoform, is responsible for 4EBP1 phosphorylation and regulates cap-binding complex formation in CLL.

Stromal cell/CD154 contact increases polysome formation

Highly translated mRNAs are found on heavy polysomes, whereas those that are not being translated are found on lighter polysomes or monosomes. To assess polysome formation, nuclear-poor cell lysates were subjected to sucrose density centrifugation. Polysomes were readily detectable in the human cell line, MCF7 (Supplementary Fig. S1) and in CLL cells increased after culture with stromal cells/CD154 from very low levels (Fig. 3A). Administration of rapamycin and 4EGI-1 reduced polysomes. As a measure of the change in translation efficiency, we compared the area under the polysome component of the trace (fractions 7 to 12) to that under the monosome component (fractions 1 to 6; ref. 32). In stromal cell/CD154 conditions, the polysome:monosome ratio was 0.36, and this fell to 0.1 after administration of rapamycin and 0.15 after 4EGI-1 (Fig. 3A) suggesting an overall reduction in translation efficiency due to these inhibitors.

Phosphorylated eIF4E and 4EBP1 are found in proliferation centers

Proliferation centers in CLL lymph nodes are identified as clusters of prolymphocytes and para-
immunoblasts with a higher fraction of cells expressing the proliferation marker, Ki-67 than in the surrounding tissue. Reasoning that proliferation centers are likely to be sites of increased translation in leukemic cells \textit{in vivo}, we looked for evidence of increased expression of phosphorylated eIF4E and 4EBP1 in CLL lymph node sections. Immunohistochemistry, revealed staining in proliferation centers (Fig. 3B and C) suggesting increased translation in these structures \textit{in vivo}.

Figure 2. Stromal cell/CD154 contact is sufficient to induce 4EBP1 phosphorylation. A, Western blot analysis showing changes in expression of phosphorylated S6K and 4EBP1 on stromal cell/CD154 culture in the presence of LY294002, PI-103, or 4EGI-1. Gray and black arrowheads indicate isoforms of 4EBP1 and GAPDH is a loading control. B, Western blot analysis showing changes in phosphorylation of rpS6 with stromal cell/CD154 culture in the presence and absence of inhibitors, LY294002, PI-103, and 4EGI-1. C, Western blot analyses showing change in 4EBP1 phosphorylation in the presence of the PI3Kα-specific inhibitor, PI-103, and the PI3Kδ inhibitor, PIK-294. The gray arrowhead indicates the predominant isoform in basal conditions and the black arrowhead the isoform induced by stromal cell/CD154 culture. GAPDH is a loading control. Western blot analyses are representative of 4 patients.

Figure 3. Stromal cells/CD154 culture increases polysome formation. A, spectrophotometric traces of fractions taken from sucrose density gradients of nuclear-poor lysates from CLL cells. Traces are from freshly isolated leukemic cells (PB), following 24-hour culture on stromal cell/CD154 and following stromal cell/CD154 culture in the presence of rapamycin or 4EGI-1 immunohistochemistry of proliferation centers stained with (B) anti-phospho-eIF4E and (C) anti-phospho-4EBP1. The magnification is ×40.
Partial dissociation of BCL2A1, BCL2L1, and MCL1 from polysomes following inhibition of cap-dependent translation

mRNAs differ in their sensitivity to inhibition of cap-dependent translation (33). To determine whether inhibition of cap-complex assembly reduced association of specific mRNAs with polysomes, we measured amounts of mRNA in fractions of cell lysates separated on sucrose density gradients. RPS6 mRNA was substantially dissociated from polysomes after administration of LY294002 [82% ± 10% in fractions 7–11 without inhibitor to 32% ± 8% with inhibitor (mean ± range), n = 3] but actin (ACTB) mRNA was relatively resistant to this agent (78% ± 11% without inhibitor to 62% ± 9%; Fig. 4A). To show that these effects were specific, we used EDTA to dissociate the ribosomal subunits and found 35% ± 7% of ACTB mRNA and 33% ± 8% of RPS6 mRNA in fractions 7 to 11 after treatment (Fig. 4A). We, therefore, distinguish mRNAs that are highly sensitive to inhibition of cap-dependent translation (RPS6) from those, such as actin, that are less sensitive (33).

Figure 4. 4EGI-1 partially reduces polysome association and inhibits protein expression of BCL2L1 and BCL2A1. A, nuclear-poor lysates made after 24 hours of stromal cell/CD154 culture in the presence (gray shaded area) and absence (dotted line) of LY294002 or EDTA were separated on sucrose density gradients. Fractions were removed and RPS6 or actin (ACTB) mRNA measured by real-time semi-quantitative PCR. The amount of mRNA in each fraction is presented as a percentage of the total mRNA. Representative of 3 patients. B, nuclear-poor lysates produced from CLL cells after 24 hours of stromal cell/CD154 culture in the presence (gray shaded area) or absence (dotted line) of 4EGI-1 or LY294002 were separated on sucrose density gradients. Fractions were removed and mRNA for BCL2A1, BCL2L1, and MCL1 was measured by real-time semi-quantitative PCR. The amount of mRNA in each fraction is presented as a percentage of the total mRNA (representative of 6 patients). C, Western blot analyses from 2 patients (left hand panels #38 and right hand panels #38) showing changes in protein expression after 24 hours of stromal cell/CD154 culture in the absence and presence of LY294002, PI-103, rapamycin, and 4EGI-1. GAPDH is a loading control. D, densitometry of Western blot analyses of BCL2 family survival proteins (n = 6). The overall amounts of the BCL2 family survival proteins are significantly reduced following administration of 4EGI-1 (P = 0.0013, paired t test). This difference is due to reduction in BCL2A1 (P < 0.0001) and BCL2L1 (P = 0.0086) but not MCL1.
We determined the sensitivity of BCL2 family prosurvival proteins most highly expressed in the lymph node microenvironment—BCL2A1, BCL2L1, and MCL1—to inhibition of cap-dependent translation. Administration of 4EGI-1 (ref. 28; Fig. 4B) caused the amount of BCL2A1 mRNA associating with the polysomal fractions (fractions 7–11) to decrease from a mean of 74% to 50% (n = 3). Similarly, BCL2L1 mRNA decreased from 86% to 60% and MCL1 mRNA from 57% to 44%. To determine whether these effects were dependent on the inhibitor used, we repeated the experiments with LY294002 and similarly found partial dissociation of mRNA from polysomes (Fig. 4B).

To determine the significance of these changes for protein expression, we conducted Western blot analysis for a group of 6 patients (Fig. 4C and Supplementary Fig. S2A). 4EGI-1 repressed both BCL2A1 (P < 0.0001, paired t test) and BCL2L1 (P = 0.0086) expression but not MCL1, whereas LY294002, PI-103, and rapamycin produced little repression of any of these prosurvival proteins (Fig. 4C).

MCL1 protein stability was not diminished following treatment with 4EGI-1 suggesting that there is unlikely to be a major effect of 4EGI-1 on degradation of this protein (Supplementary Fig. S2B).

In summary, LY294002, PI-103 and rapamycin caused partial dissociation of BCL2A1, BCL2L1, and MCL1 mRNAs from polysomes but this was not sufficient to repress protein expression. 4EGI-1 produced a different pattern of protein responses with repression of BCL2A1 and BCL2L1 but not MCL1 (Fig. 4D), whereas causing similar changes to the polysome profiles of BCL2A1, BCL2L1, and MCL1 as LY294002. This suggests that 4EGI-1 has mechanisms of action in addition to inhibition of cap-complex formation.

4EGI-1 induces an ER stress response

Work by others suggests that 4EGI-1 might induce components of an ER stress response (34). ATF4 is such a component and forms part of a protein complex that induces proapoptotic, NOXA (35). 4EGI-1 induced ATF4 and NOXA in myeloma (34). We determined whether 4EGI-1 induced ER stress response proteins in CLL. Western blot analysis showed that ATF4 and CHOP (Fig. 5A) were induced by tunicamycin and thapsigargin, known inducers of ER stress responses, and by 4EGI-1 but not by LY294002, PI-103, and rapamycin (Fig. 5B). MCL1, BCL2A1, and BCL2L1 expression was maintained following thapsigargin treatment (Fig. 5C) suggesting that induction of an ER stress response alone was not sufficient to alter expression of these proteins. Induction of phosphorylated eIF2α is responsible for translation inhibition produced by ER stress responses and 4EGI-1 produced a modest increase in this protein that was not observed with LY294002 or rapamycin (Fig. 5D). To show an effect of phosphorylated eIF2α on BCL2A1, BCL2L1, and MCL1, we used, salubrinal, a specific inhibitor of eIF2α dephosphorylation (ref. 29; Fig. 5E). Salubrinal repressed BCL2L1 and BCL2A1 but not MCL1 suggesting that BCL2L1 and BCL2A1 were more sensitive to the inhibitory effects of phosphorylated eIF2α than MCL1. Overall, neither an ER stress response alone (thapsigargin) nor repression of cap-dependent translation alone (LY294002 or rapamycin) was sufficient to repress BCL2A1 or BCL2L1. However, 4EGI-1, which both repressed cap-dependent translation and induced ER stress response proteins including ATF4, did accomplish these effects.

BH3-only protein, NOXA, is induced by 4EGI-1

4EGI-1 induced NOXA in CLL (n = 6; Fig. 5F) and densitometry showed that the NOXA:MCL1 ratio increased from 0.9 to 5.2 (Fig. 5G; ref. 7). Proapoptotic BIM also binds MCL1 and stromal cell/CD154 culture caused appearance of a slower migrating form of BIM suggesting phosphorylation (36). The addition of 4EGI-1 caused a decrease in expression of the high molecular weight BIM isoform, BIML, but no change to amounts of BIMc (Supplementary Fig. S2C). Overall, 4EGI-1 strongly induced NOXA in CLL cells.

4EGI-1 and ABT-737 synergize to reduce cell viability

4EGI-1 reduced amounts of prosurvival proteins (Fig. 4C) in leukemic cells supported on stromal cells/CD154 and induced proapoptotic NOXA (Fig. 5F), the latter as part of an ER stress response. This suggested that 4EGI-1 might reduce viability of CLL cells cultured in this system and accordingly we determined its effects in a group of unselected patients with CLL (n = 11). 4EGI-1 at 100 μmol/L, the maximum concentration used, reduced viability by approximately 63% (Fig. 6A). To determine the contribution of ER stress response to reducing cell viability, we used thapsigargin (Fig. 5C). This agent achieved a reduction in viability of only approximately 25%, at both its maximum (5 μmol/L) concentration and 50% maximal concentration, suggesting that inhibition of the ER stress response was not sufficient to reproduce the effects of 4EGI-1.

Leukemic cells cultured on stromal cells/CD154 are relatively resistant to the BCL2/BCL2L1 antagonist ABT-737 (4) but by combining ABT-737 with thapsigargin a reduction in viability similar to that of 4EGI-1 alone was achieved. This result suggested that neither induction of ER stress nor inhibition of BCL2/BCL2L1 were alone sufficient to reproduce the effects of 4EGI-1. The combination of 4EGI-1 and ABT-737 produced further enhancement with viability reduced by approximately 90% suggesting that cell survival in the presence of 4EGI-1 was due to residual BCL2/BCL2L1 expression and that 4EGI-1 can overcome resistance to ABT-737.

To formally determine synergy, thapsigargin and 4EGI-1 were used in a fixed ratio with ABT-737 at 4 concentrations. For both thapsigargin/ABT-737 and 4EGI-1/ABT-737, the combination index (CI) was less than 1 (Fig. 6B) indicating synergy (30).

We considered that higher amounts of MCL1 (37) and other BCL2 family prosurvival members induced by stromal cell/CD154 contact might be factors reducing effectiveness.
of combined 4EGI-1 and ABT-737. We, therefore, compared relative expression of BCL2A1, BCL2L1, and MCL1 in 36 patients (Supplementary Fig. S3) showing a range of expression levels. The combination of 4EGI-1 and ABT-737 was effective in patients with either low or high relative BCL2 family prosurvival protein expression, with lack of enhancement being observed in only 1 patient. Our results suggest that specific translation inhibitors synergize with ABT-737 to overcome resistance to apoptosis caused by stromal cell/CD154 culture.

Discussion

CLL cells within the lymph node microenvironment show increased proliferation as compared with those in the peripheral circulation (38) and are also likely to receive signals from T cells. Recent work has shown that in vitro responses to stimulation by the T-cell surface marker, CD154, are associated with clinical outcome (39). Leukemic cells that proliferate within the lymph node microenvironment are a target for therapy, which has prompted work to understand signaling pathways and mechanisms of survival in this context (11) and, in turn, this has required the development of specialized culture systems (40–43).

We showed for the first time that cap-complex formation and overall protein synthesis is induced by contact of leukemic cells with a fibroblast cell layer with a further increment produced by the addition of CD154. We used...
PI3K and mTORC1 inhibition to reduce cap-complex formation but showed that these agents were not effective in repressing BCL2L1, BCL2A1, or MCL1 protein expression. We found relatively little dissociation of ACTB mRNA from polysomes, which is consistent with work from others (33) showing lack of sensitivity of ACTB mRNA to overexpression of eIF4E. Our results suggested that the specific BCL2 family prosurvival gene mRNAs we analyzed behaved in a similar manner to ACTB and were relatively little dissociated from polysomes by PI3K inhibition. Therefore, despite repression of cap-dependent translation, there was continuing expression of BCL2L1, BCL2A1, and MCL1 proteins. BCL2L1 has a 5'-untranslated region (UTR) of 376 bp and contains an internal ribosome entry sequence site (44) providing a mechanism for continuing translation despite repression of cap-dependent translation but MCL1 and BCL2A1 have shorter 5'-UTRs at approximately 50 bp. Further work is needed to elucidate the mechanism for continued expression of MCL1 and BCL2A1 in the presence of inhibitors of cap-dependent translation but the simplest explanation is continuing low-level cap-dependent translation, which is sufficient for MCL1 and BCL2A1 expression. Apoptosis causes inhibition of protein synthesis (33) and speculatively BCL2 family prosurvival protein levels could be relatively resistant to alterations in cap-complex formation to maintain cell survival.

In contrast with PI3K and mTORC1 inhibition, 4EGI-1 repressed protein expression of BCL2L1 and BCL2A1 suggesting mechanisms of action in addition to repression of cap-dependent translation.

Resistance to the effects of small-molecule BH3 mimetics is a clinical problem limiting the effectiveness of these agents. ABT-737 has a similar action to the BH3-only protein, BAD, and is a BCL2 and BCL2L1 antagonist. It has been suggested that ABT-737 can be used as a probe to dissect the BCL2 family protein interactions important for survival (45). Freshly isolated CLL cells are very sensitive to ABT-737, but this was significantly diminished when the leukemic cells were cultured with stromal cells and CD154 (4, 7). The implication was that that freshly isolated leukemic cells were dependent on BCL2 (BCL2L1 not being expressed in these conditions; ref. 46), but on culture with stromal cells/CD154 survival became dependent on MCL1 and BCL2A1 (5, 7). There are, therefore, 2 possible ways to sensitize leukemic cells on stromal cell/CD154 culture to ABT-737: first, repression of BCL2A1 or MCL1, and second, induction of BH3-only proteins, especially NOXA, which binds MCL1 and BCL2A1 (47). Work by others has shown
that knockdown of NOXA enhances resistance to ABT-737 in primary B cells and induction of NOXA by fludarabine synergizes with ABT-737 in CLL (7).

These considerations prompted analysis of NOXA expression in response to 4EGI-1. We showed induction of this protein while amounts of BIM, which binds BCL2, BCL2L1, MCL1, and BCL2A1, remained essentially unchanged, although some repression of the BIMm isoform was observed, there was no change to the more apoptotic BIMg and BIMs. Overall, the 4EGI-1 produced a highly favorable pattern of responses for enhancement of the effects of ABT-737, and we showed synergy between these agents in patients expressing varying amounts of MCL1. Thapsigargin induced NOXA but did not reproduce the effects of 4EGI-1 on cell viability and our overall view is that induction of NOXA by 4EGI-1 is necessary but not sufficient for its effects.

NOXA induction by 4EGI-1 has previously been reported in myeloma cell lines and primary cells (34) and we suggest that this may prove to be a predictable secondary effect of using this agent. NOXA has also been induced by a variety of other agents in association with ER stress (48). Induction of ATF4 in an ER stress response (49) involves skipping of upstream open reading frames mediated by the translation inhibitor, phosphorylated eIF2α. Induction of ATF4 in CLL and myeloma by 4EGI-1 (34) provides indirect evidence for the functional importance of phosphorylated eIF2α. In addition, combining thapsigargin and ABT-737 reduced CLL viability to a similar level to 4EGI-1 (Fig. 6A) suggesting its effects required both inhibition of cap-dependent translation and induction of an ER stress response. ER stress is sufficient to cause CLL apoptosis when cultured on plastic (50) but in stromal cell/CD154 culture thapsigargin did not repress BCL2 family prosurvival proteins (Fig. 5C) or produce major loss of cell viability (Fig. 6A). We speculate that the combination of partial repression of cap-dependent translation together with an ER stress response, that is, translation inhibition by phosphorylated eIF2α and induction of NOXA, was required for the observed effects of 4EGI-1 (Fig. 6C).

In this report, we showed first that cap-dependent translation is induced by a culture system mimicking the lymph node microenvironment, and second that 4EGI-1 reduced cap-complex formation and induced an ER stress response. 4EGI-1 reduced levels of prosurvival BCL2L1 and BCL2A1 and induced NOXA creating a favorable situation for synergy with ABT-737. This combination of agents may be the basis for a therapeutically useful approach to target leukemic cells resistant to conventional chemotherapy in the lymph node microenvironment.

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

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Cap-Translation Inhibitor, 4EGL-1, Restores Sensitivity to ABT-737 Apoptosis through Cap-Dependent and -Independent Mechanisms in Chronic Lymphocytic Leukemia

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