Cap-translation inhibitor, 4EGI-1 restores sensitivity to ABT-737 apoptosis through cap-dependent and independent mechanisms in chronic lymphocytic leukaemia.

Shaun Willimott\textsuperscript{1,2}, Daniel Beck\textsuperscript{1,2}, Matthew J. Ahearne\textsuperscript{1,3}, Victoria C. Adams\textsuperscript{1} and Simon D. Wagner\textsuperscript{1}

\textsuperscript{1}Department of Cancer Studies and Molecular Medicine and MRC Toxicology Unit, University of Leicester, Lancaster Road, Leicester LE19HN, UK and \textsuperscript{3}Department of Haematology, University Hospitals of Leicester, Leicester LE1 5WW

\textsuperscript{2} The first two authors contributed equally to this work.

Address for correspondence: Dr. Simon D. Wagner, MRC Toxicology Unit, Hodgkin Building, University of Leicester, Room 323, Lancaster Road, Leicester LE1 9HN Tel: 01162525584 Fax: 01162525616. Email: sw227@le.ac.uk

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STATEMENT OF TRANSLATIONAL RELEVANCE (145 WORDS)

The tissue microenvironment contributes to survival, proliferation and resistance to conventional chemotherapy in chronic lymphocytic leukaemia, and abrogating the effects of the microenvironment is a goal of therapy. There is interest in using small molecule BCL2/BCL2L1 antagonists for the treatment of cancers but such agents do not inhibit pro-survival, MCL1 and their effectiveness is also critically dependent on BH3-only pro-apoptotic proteins 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 pro-survival BCL2L1 and BCL2A1 and induced pro-apoptotic NOXA a pattern of activity suggesting useful co-operation with the BH3 mimetic, ABT-737. We demonstrated synergy between 4EGI-1 and a BCL2/BCL2L1 antagonist in different individuals across a range of expression levels. Combination of 4EGI-1 with BCL2/BCL2L1 antagonists may be highly effective in targeting leukaemic cells within the microenvironment.
ABSTRACT (250 WORDS)

PURPOSE:
The lymph node microenvironment promotes resistance to chemotherapy in chronic lymphocytic leukaemia, partly through induction of BCL2 family pro-survival proteins. Currently available inhibitors do not target all BCL2 family pro-survival proteins and their effectiveness is also modified by pro-apoptotic BH3 only protein expression. The goal of this study was to evaluate synergy between the eIF4E/eIF4G interaction inhibitor, 4EGI-1, and the BH3 mimetic, 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 analysed 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 ~6-fold by stromal cell/CD154 culture in a PI3Kα 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 ER stress response including pro-apoptotic NOXA and the translation inhibitor phosphorylated eIF2α. 4EGI-1 and ABT-737 synergised to cause apoptosis, independent of levels of pro-survival proteins 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 pro-survival expression but 4EGI-1 inhibited BCL2A1 and BCL2L1 whilst inducing NOXA through cap-dependent and independent mechanisms. 4EGI-1 and ABT-737 synergised 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) mimetic drugs, such as ABT-263, which bind and inhibit anti-apoptotic BCL2, and to a lesser extent BCL2L1, are being trialled 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 utility of this class of agents, are recognised. For example, increased expression of anti-apoptotic proteins BCL2L1 (formerly BCL-XL) (3, 4), BCL2A1 (formerly A1 or BFL1) (4, 5) and MCL1 (5-7) reduce the effectiveness of ABT-737 and ERK signalling pathways also promote resistance to this agent (8).

Circulating chronic lymphocytic leukaemia (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 over-expression 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 co-operation with c-MYC. Specifically in B-cells over-expression 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 centres 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 haematological malignancies (23), and the eIF4E inhibitor and anti-viral agent Ribavirin has been trialed in acute myeloid leukaemia (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 pro-survival 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 favouring synergy with a BCL2/BCL2L1 antagonist, ABT-737, but part of this effect is independent of cap-dependent translation through induction of an ER stress response. We suggest this combination of agents will be a useful route to abrogating the pro-survival effects of 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 >50x10^9/l were used in the study. At the time of study no patient had been treated for three months. Local research ethics committee approval was obtained. Patient characteristics are presented in Table 1.

Cell culture
CLL cells (3x10^6/ml) were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with foetal bovine serum (Invitrogen), non-essential amino acids (Invitrogen, Paisley, UK), Penicillin/Streptomycin (Invitrogen), and HEPES buffer (Lonza, Basel, Switzerland) in a 37°C and 5% CO₂ incubator. The cells were either cultured on tissue culture plastic or co-cultured with 80-90% confluent and 35 Gy irradiated non-transfected mouse fibroblast cells (NT culture) or human CD154 expressing mouse fibroblast cells supplemented with rh-IL4 (10ng/ml) (R&D Systems, Minneapolis, MN, USA) (CD culture) (27).

The PI3K inhibitors, LY294002 (Calbiochem), PI-103 (Calbiochem) and PIK-294 (Calbiochem) and BH3-mimetic, ABT-737 (provided by Abbott) were used as indicated. Rapamycin was purchased from Sigma, St. Louis, MO, USA. 4EGI-1 (28) and salubrinal (29) were from Santa-Cruz Biotechnology, Santa-Cruz, CA, USA.

Sucrose density gradients
Cycloheximide (100µg/ml) (Sigma) was added prior to harvesting 36 to 40x10^6 cells. Following lysis in 500µl of polysome extraction buffer (15mM Tris (pH 7.5), 15mM MgCl₂, 300mM NaCl, 1% Triton X-100, 100µg/ml cycloheximide, 50µg/ml heparin, 5mM DTT and RNase inhibitors). Lysates were centrifuged and the supernatant layered on to a 10 to 50% sucrose gradient (Biocomp Gradient Station (Wolf Laboratories Ltd., York, UK), followed by centrifugation at 38000rpm for 2 hours in a Beckman SW40 rotor. mRNA was extracted from
each of twelve fractions using phenol/chloroform extraction and lithium chloride precipitation. Taqman real time PCR assays were from Applied Biosystems (actin (ACTB) #Hs99999903_m1, RPS6 #Hs04195024_g1 MCL1 #Hs03043898_m1, BCL2A1 #Hs00187845_m1, BCL2L2 #Hs01573809_g1). The relative amount of mRNA in 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 (50x10⁶) were cultured for 24 hours on CD154/IL-4 in the presence or absence of LY294002 (20μM). Cells were harvested, washed in 300μl ice-cold buffer A (50mM MOPS/KOH, pH7.2, 50mM NaCl, 50mM NaF, 2mM EGTA, 5mM EDTA, 7mM 2-mercaptoethanol and protease and phosphatase inhibitors (Sigma)) and lysed by the addition of 1.5% (v/v) NP40 and 1.5% (v/v) Triton X100. Lysates were centrifuged at 15,000 rpm for 5 minutes at 4°C and the supernatant added to 50μl of 50% (v/v) m7GTP-Sepharose beads (GE Healthcare, Bucks, UK). 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-polyacrylamide gel electrophoresis.

The following antibodies were used following the manufacturers’ instructions: rabbit anti-eIF4G 1:1000 (Cell Signaling Technology, Danvers, MA, USA), mouse anti-eIF4E 1:1000 (BD Biosciences, Franklin Lakes, NJ, USA), and rabbit anti-4EBP1 1:1000 (Cell Signaling Technology).

Western blotting
CLL cells were harvested and protein extracts made using RIPA buffer supplemented with protease and phosphatase inhibitors (Sigma). Proteins were separated using a 12% SDS-gel, and blotted onto a PVDF membrane (Sigma). As part of this work we compared expression of BCL2A1, BCL2L1 and MCL1 i.e. the major BCL2 family pro-survival proteins induced by stromal cell/CD154 culture in 36 patients. Densitometry data from westerns was controlled for differences in loading using GAPDH and a pool of cell lysates was included in each gel in order to be able to compare autorads. Membranes were probed with BCL2L1 (1:1000), phospho-AKT (1:1000) and GAPDH (1:1000) (New England Biolabs, Hertfordshire, UK). Anti-BCL2A1 serum was a gift from Dr. J. Borst, The Netherlands Cancer Institute, The Netherlands. Anti-phospho-4E-BP, phospho-S6 ribosomal protein, phospho-S6 kinase and pospho-eIF4E were from New England Biolabs and were used at 1:1000. Anti-NOXA (Enzo Life Sciences, Exeter, UK) and anti-BIM (New England Biolabs) were also used at 1:1000. Secondary antibodies – anti-mouse-IgG-HRP or anti-rabbit-IgG-HRP (Sigma) - were used at 1:2000.

³⁵S Methionine Incorporation
Cells (3x10⁶ /ml) were cultured on plastic or 80-90% confluent and 35Gy irradiated stromal layers for 24 hours. ³⁵S methionine (3 µl of 37MBq/ml) (PerkinElmer, Waltham, MA, USA) 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 (13,000rpm, 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 (5x10⁵ per well of a 96-well microtitre 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 performed using GraphPad Prism version 4.0b (GraphPad Software Inc., La Jolla, CA, USA). Pharmacological analysis of drug effects, singly and in combination was carried out with CalcuSyn version 2 (Biosoft, Cambridge, UK) (30).

RESULTS

Culture on stromal cells in the presence and absence of CD154 increases protein synthesis and cap complex formation in a PI3Kα dependent manner.

³⁵S methionine incorporation was employed 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 (Figure 1A). To determine changes to cap-complex formation under different culture conditions cap-binding assays were carried out. There was little association of eIF4G with eIF4E in freshly isolated leukaemic cells, but following culture on stromal cells alone the amount of eIF4G increased as compared to basal conditions, with stromal cell/CD154 culture producing a further increment (Figure 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 signalling 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 leukaemic cells in stromal cell/CD154 culture. The non-isoform specific PI3K inhibitor, LY294002, reduced protein synthesis by ~70% (Figure 1C) (paired t-test; P=0.007) and produced a
corresponding reduction in the association of eIF4G with eIF4E in cap-binding assays (Figure 1B). A specific PI3Kα inhibitor, PI-103, repressed protein synthesis \((P=0.02)\) whereas a PI3Kδ inhibitor, PIK-294, had no effect (Figure 1C). In keeping with this, PI-103 reduced cap-complex formation to sub-basal levels, whilst PIK-294 was ineffective (Figure 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 (Figure 2A and 2C) 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 \(^{35}\text{S}\) methionine incorporation and cap-complex formation (Figure 2C). S6K appeared to be expressed at low levels in CLL although some induction was seen on stromal cell/CD154 culture (Figure 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 (Figure 2B). Therefore, PI3K, which has previously been shown to be required for leukaemic 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. In order to assess polysome formation nuclear-poor cell lysates were subjected to sucrose density gradient centrifugation. Polysomes were readily detectable in the human cell line, MCF7 (Supplemental Figure 1) and in CLL cells increased after culture with stromal cells/CD154 from very low levels (Figure 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) (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 (Figure 3A) suggesting an overall reduction in translation efficiency due to these inhibitors.

**Phosphorylated eIF4E and 4EBP1 are found in proliferation centres.**

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

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). In order 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 to 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%) (Figure 4A). To demonstrate that these effects were specific we employed 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) (Figure 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).

We determined the sensitivity of BCL2 family pro-survival proteins most highly expressed in the lymph node microenvironment – BCL2A1, BCL2L1 and MCL1 – to inhibition of cap-dependent translation. Administration of 4EGI-1 (28) (Figure 4B) caused the amount of BCL2A1 mRNA associating with the polysomal fractions (fractions 7 to 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%. In order to determine whether these effects were dependent on the inhibitor employed we repeated the experiments with LY294002 and similarly found partial dissociation of mRNA from polysomes (Figure 4B).

To determine the significance of these changes for protein expression we carried out westerns for a group of 6 patients (Figure 4C and Supplemental Figure 2A). 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 pro-survival proteins (Figure 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 (Supplemental Figure 2B). 

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 (Figure 4D) whilst 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 pro-apoptotic, NOXA (35). 4EGI-1 induced ATF4 and NOXA in myeloma (34). We determined whether 4EGI-1 induced ER stress response proteins in CLL. Westerns showed that ATF4 and CHOP (Figure 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 (Figure 5B). MCL1, BCL2A1 and BCL2L1 expression was maintained following thapsigargin treatment (Figure 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 (Figure 5D). To show an effect of phosphorylated eIF2α on BCL2A1, BCL2L1 and MCL1 we employed, salubrinal, a specific inhibitor of eIF2α dephosphorylation (29) (Figure 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) (Figure 5F) and densitometry demonstrated that the NOXA:MCL1 ratio increased from 0.9 to 5.2 (Figure 5G) (7). Pro-apoptotic 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, BIM_el, but no change to amounts of BIM_L (Supplemental Figure 2C). Overall 4EGI-1 strongly induced NOXA in CLL cells.

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

4EGI-1 reduced amounts of pro-survival proteins (Figure 4C) in leukaemic cells supported on stromal cells/CD154 and induced pro-apoptotic NOXA (Figure 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 CLL patients (n=11). 4EGI-1 at 100 µM, the maximum concentration employed, reduced viability by ~63% (Figure 6A). In order to determine the contribution of ER stress response to reducing cell viability we employed thapsigargin (Figure 5C). This agent achieved a reduction in viability of only ~25%, at both its maximum (5 µM) concentration and 50% maximal concentration, suggesting that inhibition of the ER stress response was not sufficient to reproduce the effects of 4EGI-1. Leukaemic 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, but together thapsigargin and ABT-737 produced a similar fall in viability to that observed with 4EGI-1. The combination of 4EGI-1 and ABT-737 produced further enhancement with viability reduced by ~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 employed in a fixed ratio with ABT-737 at four concentrations. For both thapsigargin/ABT-737 and 4EGI-1/ABT-737 the combination index was less than 1 (Figure 6B) indicating synergy (30).

We considered that higher amounts of MCL1 (37) and other BCL2 family pro-survival 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 (Supplemental Figure 3) demonstrating 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 pro-survival protein expression, with lack of enhancement being observed in only one patient. Our results suggest that specific translation inhibitors synergise 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 to those in the peripheral circulation (38) and are also likely to receive signals from T-cells. Recent work has demonstrated that in vitro responses to stimulation by the T-cell surface marker, CD154, are associated with clinical outcome (39). Leukaemic cells that proliferate within the lymph node microenvironment are a target for therapy, which has prompted work to understand signalling pathways and mechanisms of survival in this context (11) and, in turn, this has required the development of specialised culture systems (40-43).
We showed for the first time that cap-complex formation and overall protein synthesis is induced by contact of leukaemic cells with a fibroblast cell layer with a further increment produced by the addition of CD154. We employed PI3K and mTORC1 inhibition to reduce cap-complex formation but demonstrated 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) demonstrating lack of sensitivity of ACTB mRNA to over-expression of eIF4E. Our results suggested that the specific BCL2 family pro-survival gene mRNAs we analysed 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’UTR of 376 bp and contains an IRES site (44) providing a mechanism for continuing translation despite repression of cap-dependent translation but MCL1 and BCL2A1 have shorter 5’UTRs at ~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 pro-survival protein levels could be relatively resistant to alterations in cap-complex formation to maintain cell survival.

By 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 utilised 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 leukaemic cells were cultured with stromal cells and CD154 (4, 7). The implication was that that freshly isolated leukaemic cells were dependent on BCL2 (BCL2L1 not being expressed in these conditions) (46), but on culture with stromal cells/CD154 survival became dependent on MCL1 and BCL2A1 (5, 7). There are, therefore, two possible ways to sensitise leukaemic cells on stromal cell/CD154 culture to ABT-737: firstly repression of BCL2A1 or MCL1 and secondly, 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 synergises with ABT-737 in CLL (7).
These considerations prompted analysis of NOXA expression in response to 4EGI-1. We demonstrated induction of this protein whilst amounts of BIM, which binds BCL2, BCL2L1, MCL1 and BCL2A1, remained essentially unchanged although some repression of the BIM$_{EL}$ isoform was observed there was no change to the more apoptotic BIM$_{L}$ and BIM$_{S}$. Overall the 4EGI-1 produced a highly favourable 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 (Figure 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 pro-survival proteins (Figure 5C) or produce major loss of cell viability (Figure 6A). We speculate that the combination of partial repression of cap-dependent translation together with an ER stress response i.e. translation inhibition by phosphorylated eIF2α and induction of NOXA, was required for the observed effects of 4EGI-1 (Figure 6C).

In this report we showed firstly, that cap-dependent translation is induced by a culture system mimicking the lymph node microenvironment and secondly, that 4EGI-1 reduced cap-complex formation and induced an ER stress response. 4EGI-1 reduced levels of pro-survival BCL2L1 and BCL2A1 and induced NOXA creating a favourable situation for synergy with ABT-737. This combination of agents may be the basis for a therapeutically useful approach to target leukaemic cells resistant to conventional chemotherapy in the lymph node microenvironment.
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CONFLICT OF INTEREST
The author’s declare no conflict of interest.
REFERENCES


**FIGURE LEGENDS**

**Figure 1**

Protein synthesis and cap-complex formation is increased by stromal cell/CD154 contact by a PI3Kα dependent mechanism.

(A) $^{35}$S methionine incorporation. CLL cells were cultured on plastic (PL), stromal cells alone (stoma) or stromal cells with CD154 (stoma/CD154). After 24 hours culture $^{35}$S methionine was added for 30 minutes and the cells harvested and lysed followed by measurement of incorporated radioactivity. Mean±SEM for 3 patients are presented. Protein synthesis is increased significantly by stromal cell contact (paired t-test; $P=0.006$) and stromal cell/CD154 culture ($P=0.017$). (B) Cap-binding assays. Lysates from freshly isolated cells, and cells cultured on stromal cells (NT) or stromal cells with CD154 (CD154) in the presence or absence of LY294002 were mixed with m7-GTP beads. Westerns of material from the washed beads were probed with anti-eIF4E and anti-eIF4G antibodies. Representative of 4 patients. (C) Effects of PI3K inhibitors, LY294002 and PI-103, and 4EGI-1 on $^{35}$S methionine incorporation. Mean±SEM are presented for 3 patients. As compared to stromal cell/CD154 culture without inhibitor protein synthesis is significantly reduced by LY294002 (paired t-test; $P=0.007$), PI-103 ($P=0.02$) and 4EGI-1 ($P=0.004$) (D) Cap-binding assays to show the effects on cap-complex formation of a PI3Kα specific inhibitor, PI-103, a PI3Kδ specific inhibitor, PIK-294, an mTORC1 inhibitor, rapamycin and an inhibitor of the interaction between eIF4E and eIF4G. Representative of 4 patients.

**Figure 2**

Stromal cell/CD154 contact is sufficient to induce 4EBP1 phosphorylation.

(A) Westerns showing changes in expression of phosphorylated S6K and 4EBP1 on stromal cell/CD154 culture in the presence of LY294002, PI-103 or 4EGI-1. Grey and black arrowheads indicate isoforms of 4EBP1 and GAPDH is a loading control. (B) Western showing changes in phosphorylation of ribosomal protein S6 (rpS6) with stromal cell/CD154 culture in the presence and absence of inhibitors, LY294002, PI-103 and 4EGI-1. (C) Westerns showing change in 4EBP1 phosphorylation in the presence of the PI3Kα specific inhibitor, PI-103, and the PI3Kδ inhibitor, PIK-294. The grey 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. Westerns 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 leukaemic cells (PB), following 24 hours culture on stromal cell/CD154 and following stromal cell/CD154 culture in the presence of rapamycin or 4EGI-1. Immunohistochemistry of proliferation centres stained with (B) anti-phospho-eIF4E, and (C) anti-phospho-4EBP1. The magnification is x40.

**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) Westerns from two patients (left hand panels #38 and right hand panels #13, Table 1) 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 westerns 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.

**Figure 5**

**4EGI-1 produces an ER stress response with induction of NOXA.**

Leukaemic cells were either lysed without a period of culture (-) or cultured for 24 hours in the presence of stromal cells/CD154 alone (+) or in the presence of the indicated inhibitor. (A) 4EGI-1 induces ER stress response proteins. Westerns demonstrating the effects of 4EGI-1 (50 and 100 µM), tunicamycin (Tu) (2.5 and 5 µM) and thapsigargin (Tg) (2.5 and 5 µM) on expression of ATF4 and CHOP. (B) ER stress proteins are not induced by PI3K inhibitors, LY294002 (10 and 20 µM), PI-103 (0.2 and 1 µM) and mTORC1 inhibitor, rapamycin (20 and 50 ng/ml). Westerns for ATF4 and CHOP are shown. (C) Westerns showing effects of ER stress inducer thapsigargin (Tg) at 2.5 and 5 µM on BCL2 family proteins NOXA, MCL1,
BCL2L1 and BCL2A1. (-) indicates lysates from cells freshly isolated from patients and (+) after 24 hours culture on stromal cells/CD154. Both sections of the panel are from the same autorad. GAPDH was used as a loading control. (D) Westerns showing phosphorylated eIF2α expression in response to LY294002, rapamycin and 4EGI-1. Total eIF2α is employed as a loading control. (E) BCL2L1, BCL2A1 and MCL1 expression following administration of salubrinal (100 µM). (F) Westerns of three patients (#38, #39, #40, Table 1) demonstrating MCL1 and NOXA expression following stromal cell/CD154 culture in the presence and absence of 4EGI-1. GAPDH is a loading control. (G) Densitometry of westerns was employed to derive values for NOXA/GAPDH (N) and MCL1/GAPDH (M). Normalised expression is shown for freshly isolated cells (-), cells cultured on stromal cells/CD154 (CD154) and cells cultured on stromal cells/CD154 with 4EGI-1 (CD154 + 4EGI-1). Mean±SEM. For each experimental condition the NOXA:MCL1 ratios are presented above the bracket.

Figure 6
4EGI-1 enhances apoptosis due to ABT-737 across a range of different BCL2A1, BCL2L1 and MCL1 expression levels.

(A) Dose-effect curves for ABT-737, 4EGI-1 and thapsigargin alone and in combination (n=11). Viability was determined by luminescent detection of ATP (CellTiter-Glo, Promega) at four different drug concentrations. For 100% doses ABT-737 was employed at 1 µM, 4EGI-1 at 100 µM and thapsigargin at 5 µM. (B) Synergy was determined by combination index-fractional effect analysis (Calcusyn, Biosoft, Cambridge, UK). CI<1 indicates synergy. (C) Diagram showing possible mechanism of action of 4EGI-1. Cap-binding assays show that LY294002, PI-103 and rapamycin repress cap-complex formation in CLL cells on stromal cell/CD154 culture but do not alter BCL2L1, BCL2A1 or MCL1 protein expression. 4EGI-1 inhibits cap-dependent translation and also induces NOXA through an ER stress response to antagonise the effects of MCL1. Inhibition of translation by phosphorylated eIF2α expression, whose expression is maintained by 4EGI-1 in contrast to LY294002 or rapamycin, together with inhibition of cap dependent translation may be necessary for reduction in BCL2A1 and BCL2L1 expression.
Table 1

Patient characteristics. Patient gender, clinical stage according to the Binet classification, percentage of cells expressing the CD38 surface marker, immunoglobulin gene mutational status (either unmutated (U) or mutated (M)), \( V_{H} \) gene segment and cytogenetics, which was obtained either by employing a FISH panel or by analysis of metaphase spreads. ND is not determined. Blanks under cytogenetics means no abnormalities detected by FISH panel.

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Figure 1

A) 35S Methionine incorporation (x10^(-3)cpm)

- PL
- Stroma
- Stroma/CD154

B) m7 GTP pulldown

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C) 35S Methionine incorporation (x10^(-3)cpm)

- LY294002
- PI-103
- PIK-294
- Rapamycin
- 4EGI-1

D) m7 GTP pulldown

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Figure 2
Figure 3
Figure 5

(A) - (B) Western blot analysis showing the expression levels of ATF4, CHOP, and GAPDH under different conditions.

(C) Western blot analysis of NOXA, MCL1, BCL2L1, and BCL2A1 with and without Tg treatment.

(D) Western blot analysis of p-eIF2α and eIF2α with and without LY294002, Rapamycin, and 4EGI-1.

(E) Western blot analysis of BCL2L1, BCL2A1, MCL1, and GAPDH under different conditions.

(F) Western blot analysis of MCL1, NOXA, and GAPDH with #39, #38, and #40 conditions.

(G) Bar graph showing the normalized amount of protein expression with CD154 and 4EGI-1 treatment.

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**Figure 6**

A) Luminescence units (x10^3) vs. dose (% of highest dose)

- ABT-737
- 4EGL-1
- 4EGL-1 + ABT-737
- Tg
- Tg + ABT-737

B) Combination Index

C) Pathway diagram:

- LY294002
- PI-103
- Rapamycin

- Cap-dependent translation
- Cap-dependent translation
- ER stress response
- elf2α phosphorylation
- BCL2L1
- BCL2A1
- NOXA

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Cap-translation inhibitor, 4EGI-1 restores sensitivity to ABT-737 apoptosis through cap-dependent and independent mechanisms in chronic lymphocytic leukaemia.

Shaun Willimott, Daniel Beck, Matthew J Ahearne, et al.

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