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Translation inhibitors induce cell death by multiple mechanisms and Mcl-1 reduction is only a minor contributor

LM Lindqvist*[,1,2], I Vikström[1,2], JM Chambers[3], K McArthur[1,2], M Ann Anderson[1,2,4], KJ Henley[1,2], L Happo[1,2], L Cluse[5], RW Johnstone[5,6], AW Roberts[1,2,4], BT Kile[1,2], BA Croker[1,2], CJ Burns[1,2], MA Rizzacasa[3], A Strasser[1,2] and DCS Huang[1,2]

There is significant interest in treating cancers by blocking protein synthesis, to which hematological malignancies seem particularly sensitive. The translation elongation inhibitor homoharringtonine (Omacetaxine mepesuccinate) is undergoing clinical trials for chronic myeloid leukemia, whereas the translation initiation inhibitor silvestrol has shown promise in mouse models of cancer. Precisely how these compounds induce cell death is unclear, but reduction in Mcl-1, a labile pro-survival Bcl-2 family member, has been proposed to constitute the critical event. Moreover, the contribution of translation inhibitors to neutropenia and lymphopenia has not been precisely defined. Herein, we demonstrate that primary B cells and neutrophils are highly sensitive to translation inhibitors, which trigger the Bax/Bak-mediated apoptotic pathway. However, contrary to expectations, reduction of Mcl-1 did not significantly enhance cytotoxicity of these compounds, suggesting that it does not have a principal role and cautions that strong correlations do not always signify causality. On the other hand, the killing of T lymphocytes was less dependent on Bax and Bak, indicating that translation inhibitors can also induce cell death via alternative mechanisms. Indeed, loss of clonogenic survival proved to be independent of the Bax/Bak-mediated apoptosis altogether. Our findings warn of potential toxicity as these translation inhibitors are cytotoxic to many differentiated non-cycling cells.
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Tight control of protein synthesis is essential for normal cellular function and survival, but unrestrained protein synthesis can promote tumorigenesis. Notably, several translation factors are upregulated in malignancies, such as the RNA helicase eIF4A and the cap-binding protein eIF4E, two members of the eIF4F complex.[1,2] Moreover, eIF4E has transforming activity and cooperates with deregulated Myc expression in lymphomagenesis and mice deficient in the eIF4A inhibitor Pdcd4 also develop lymphoma.[3–6] Translation elongation factors are also upregulated in a range of malignancies.[7] As rapidly cycling cells require increased translation rates, it was postulated that they might be more sensitive to inhibition of protein synthesis.[8,9]

One such inhibitor is homoharringtonine (HHT; Omacetaxine mepesuccinate), which inhibits translation elongation by hindering the peptidyl transferase activity of the ribosome.[10,11] HHT has shown promise in patients with acute myeloid leukemia or chronic myeloid leukemia (CML) and, importantly, has shown activity in imitanib mesylate (Gleevec)-resistant CML.[12,13] Unlike translation elongation inhibitors, which have been studied for decades, cell permeable inhibitors of translation initiation have only recently been developed.[8]

Silvestrol is a translation initiation inhibitor that targets eIF4A.[14,15] It has demonstrated single-agent activity against certain human cancers transplanted into immunocompromised mice and induces remission in the *Eμ-myc* mouse lymphoma model when combined with doxorubicin.[14–17] Although inhibitors of translation elongation, such as HHT, inhibit global protein synthesis, targeting the eIF4F complex has been proposed to be more selective, because the translation of certain mRNAs is thought to be particularly dependent on eIF4F.[18] These eIF4F-dependent mRNAs often have highly structured 5′ untranslated regions and many of them encode proteins involved in controlling cellular proliferation, survival (e.g., Mcl-1) and/or oncogenesis.[19,20] Taken together, these observations have encouraged the development of translation initiation inhibitors as cancer therapeutics.[14,17,21]

Although the mechanisms by which HHT and silvestrol inhibit protein synthesis are well characterized, precisely how they kill cells is unclear. It has been hypothesized that reduction of the anti-apoptotic Bcl-2 family member Mcl-1 constitutes the major, possibly even the sole, driver of cell death.[16,17,22,23] Nevertheless, decreased levels of Bcl-2 have also been reported.[15,21] These pro-survival proteins act to restrain Bax and Bak, the two pro-apoptotic multi-BH domain Bcl-2 family members that are essential for mitochondrial outer membrane permeabilization, an fundamental step in the

[1]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia; [2]Department of Medical Biology, The University of Melbourne, Parkville, Victoria, Australia; [3]School of Chemistry, The Bio21 Institute, The University of Melbourne, Parkville, Victoria, Australia; [4]Departments of Diagnostic Hematology, Clinical Hematology, and Bone Marrow Transplantation, The Royal Melbourne Hospital, Parkville, Victoria, Australia; [5]Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia and [6]Sir Peter MacCallum Department of Oncology, University of Melbourne, Parkville, Victoria, Australia
*Corresponding author: LM Lindqvist, Cell Signalling and Cell Death Division, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria, 3052, Australia. Tel: +61 3 9345 2974; Fax: +61 3 9347 0852; E-mail: lindqvist@wehi.edu.au
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so-called 'Bcl-2 family regulated' (also called 'intrinsic' or 'mitochondrial') apoptotic pathway.[24,25] Once the mitochondrial barrier is breached, cytochrome *c* and other apoptogenic factors are released into the cytosol to activate caspases, thereby driving cellular demolition. Other cell death pathways have also been implicated because translation inhibitors reduce the levels of cyclin D1, c-Myc, XIAP and cFlip.[15,22,23,26] However, most attempts to determine the mechanisms by which translation inhibitors cause cell death are based on observational and correlative data (e.g., reduction of Mcl-1 levels)[16,17,22] and the relative impact of blocking a specific target has not been established.

We, therefore, decided to use genetic tools to determine the *significance* of components of the apoptosis machinery in the cytotoxicity induced by translation inhibition by studying the effects of two promising but divergent inhibitors of protein synthesis: the translation elongation inhibitor HHT and the translation initiation inhibitor silvestrol. The hematopoietic system was our major focus as leukemias and lymphomas appear to be promising targets for these compounds.[12,16,17] We surveyed a wide range of normal and transformed hematopoietic cells to establish the potential indications and determine the likely therapeutic window. In addition to malignant cells, we found that non-transformed B lymphoid cells from many differentiation stages were highly sensitive to translation inhibition. Terminally differentiated non-cycling cells, such as neutrophils, were also sensitive. Unexpectedly, we found that Mcl-1 reduction was not the major contributor to death in a variety of cells and that cell killing did not always occur solely via Bax/Bak-mediated apoptosis. Indeed, we found that long-term clonogenic potential after treatment with protein synthesis inhibitors can be independent of the Bcl-2 regulated pathway altogether. Our studies therefore provide critical information to guide the development and clinical application of these compounds as well as anticipate potential side effects associated with their use.

Results

Many human leukemia-derived cells are highly sensitive to inhibitors of protein synthesis. As cell lines derived from several hematopoietic malignancies have been reported to be sensitive to silvestrol and HHT,[14,15,17] we evaluated a panel of leukemias to determine if some cell types are more sensitive than others. In accord with previous reports,[17,22] we found that chronic lymphoid leukemia (CLL) samples freshly isolated from patients were exquisitely sensitive, with an EC_{50} (concentration at which 50% of the cells are killed within 24 h) in the low nanomolar range when vehicle-treated cells were still healthy (87.7 ± 1.7% viable; Figure 1a). In a larger panel of human leukemia-derived cell lines, we found efficient induction of cell death in many, but not all, of the lines studied (Figure 1b and Supplementary Figure 1a). Of note, K562, a CML-derived cell line appeared insensitive and the T-ALL CCRF-CEM cell line was only moderately sensitive, even after 72 h of continuous treatment. No obvious correlation was evident between the abundance of Bcl-2 family members and the cytotoxicity of translation inhibitors (Supplementary Figure 1b). Interestingly, the majority of leukemia cell lines were > 50% viable at 24 h of treatment, indicating a slower rate of death than CLL, a non-dividing cancer in culture (Supplementary Figure 1c). These observations prompted us to investigate in greater detail whether protein synthesis inhibitors induce death in a wider range of cell types and evaluate the role of the Bax/Bak-mediated apoptotic pathway in mediating the cytotoxicity of these compounds.

Translation inhibitors induce Bax/Bak-mediated apoptosis in diverse cell types. Previous studies suggest that inhibition of translation triggers apoptosis, as indicated by the surface exposure of phosphatidylserine or the presence of other biochemical markers (e.g., caspase activation).[14,16,22] Although these changes are associated with apoptosis, it is unclear whether induction of apoptosis is essential for cell killing by the translation inhibitors and if the Bcl-2 family regulates this death. We took advantage of cells derived from mice lacking one or more key Bcl-2 family member(s) to allow us to ascertain unequivocally whether translation inhibitors kill by impairing Bax/Bak-mediated cell survival.

Death of immortalized mouse embryonic fibroblasts (MEFs) triggered by silvestrol or HHT was impaired when the essential apoptotic cell death mediators Bax and Bak were absent (Figure 2a and Supplementary Figure 2a). Remarkably, loss of Bak alone was sufficient to confer marked

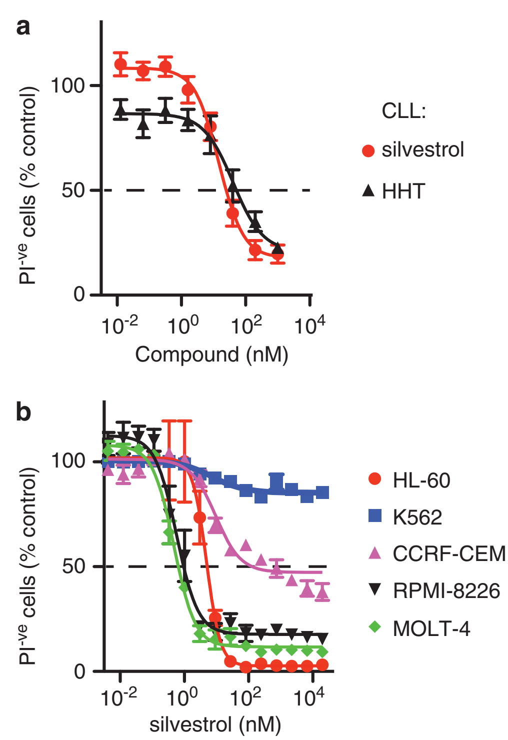

Figure 1 Human CLL cells and many leukemia or lymphoma-derived cell lines are highly sensitive to protein synthesis inhibitors. (**a**) Silvestrol and HHT rapidly kill CLL cells in culture. The survival of freshly isolated CLL cell samples was determined by exclusion of the vital dye PI and assessed by flow cytometry after 24-h treatment with the indicated drugs ($n = 3$ patients). (**b**) Survival of a panel of leukemia or lymphoma-derived cells lines 72 h after exposure to silvestrol. Data represent the mean relative to control: cells treated with the vehicle, DMSO ($n = 4$). Error bars represent the S.E.M. in both graphs

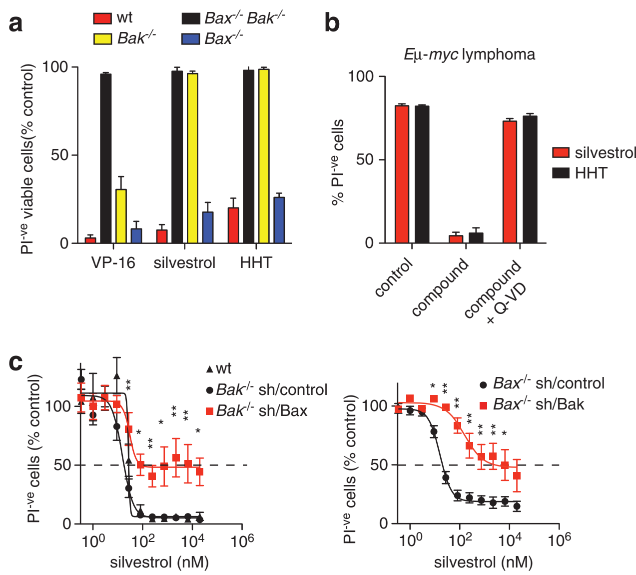

Figure 2 Translation inhibitors induce rapid Bax/Bak-mediated apoptosis. (**a**) Killing of fibroblasts by the translation inhibitors is mediated principally by Bak. The survival (PI^{-ve}) of wild-type (wt) MEFs, ones lacking either Bak, Bax, or both multi-BH domain pro-apoptotic Bcl-2 family members was determined after 24-h exposure to etoposide (VP-16; 34 μM), silvestrol (10 μM) or HHT (10 μM). Data were obtained from two representative cell lines of each genotype, $n=3$ independent experiments. (**b**) Blocking caspases significantly delays killing of *E*μ-*myc* lymphoma cells by translation inhibitors. The survival (PI^{-ve}) of *E*μ-*myc* lymphoma cells ($n=3$ independent lines) treated in culture for 8 h with silvestrol (20 μM) or HHT (20 μM) in the presence or absence of the broad-spectrum caspase inhibitor (Q-VD-OPh) was determined by flow cytometric analysis. (**c**) Bax and Bak are critical for silvestrol-induced killing of *E*μ-*myc* lymphoma cells. The survival (PI^{-ve}) of (left) wt *E*μ-*myc* lymphoma cells, ones lacking Bak or sub-clones also expressing an shRNA to mouse *Bax* (or an irrelevant control hairpin) was determined after 8 h of treatment with silvestrol by flow cytometric analysis. Alternatively (right), *E*μ-*myc* lymphoma cells lacking Bax or sub-clones stably expressing an shRNA to mouse *Bak* (or an irrelevant control hairpin) were examined under identical conditions. Two independent lines of each genotype were studied; $n=5$ independent experiments. Cell survival was normalized to vehicle-treated controls; error bars represent the S.E.M. in all graphs. Statistical analysis was performed using two-way ANOVA (*$P<0.05$, **$P<0.01$)

protection in this setting (Figure 2a). We excluded any potential differences in the ability of the compounds to inhibit protein synthesis in the knockout cells (Supplementary Figure 2b). Of note, Bak was not the sole mediator of cell killing by etoposide (Figure 2a),[25] indicating that translation inhibitors trigger apoptosis by mechanisms distinct from those elicited by DNA damage.

To determine if translation inhibitors also kill hematopoietic cells by a Bak-dependent process, we tested immortalized lymphomas derived from *E*μ-*myc* transgenic mice, a model of human Burkitt's lymphoma.[27] Translation inhibitors induced caspase-dependent killing of these Myc-driven pre-B/B-cell lymphomas (Figure 2b). We found that the *E*μ-*myc* lymphomas were highly sensitive to silvestrol and HHT *in vitro* (Figure 2c and Supplementary Figure S3a), although these agents had been reported to be ineffective as single agents *in vivo* (see Discussion section).[11,14] Although the absence of either Bak (or Bax) alone did not confer resistance, combined deficiency (engineered by RNA interference of one gene plus knock-out of the other) rendered *E*μ-*myc* lymphomas significantly less sensitive (Figure 2c and Supplementary Figures 3a and b). This demonstrates that both pro-apoptotic proteins can have an important role in translation inhibitor-induced cytotoxicity and appear interchangeable in these cells. In summary, our studies formally demonstrate that mitochondrial-mediated apoptosis is a critical mechanism by which the translation inhibitors can kill cells.

Multiple hematopoietic cell types are highly sensitive to translation inhibition, but not all are killed exclusively by Bax/Bak-mediated apoptosis. To determine whether the non-transformed counterparts of *E*μ-*myc* lymphomas are also sensitive to the translation inhibitors, we examined the sensitivity of different B-cell subsets isolated from wild-type mice. Interestingly, pro-B/pre-B, immature B and mature B cells were all highly and comparably sensitive (Figure 3a and Supplementary Table 1), indicating that neither oncogenic transformation nor differentiation stage within the B-cell compartment influenced sensitivity to these compounds. To determine whether the non-transformed B lymphoid cells die by a similar mechanism as *E*μ-*myc* lymphomas, we compared wild-type cells to ones completely lacking Bax and Bak. $Bax^{-/-}Bak^{-/-}$ B cells from all differentiation stages tested were completely resistant to both protein synthesis inhibitors up to 750 nM (Figure 3a).

As normal B cells were sensitive to silvestrol and HHT, we investigated whether these compounds would be cytotoxic to other lymphoid cell types. Like B cells, T cells were sensitive; however, in contrast to B cells, their killing did not rely heavily on Bax and Bak, indicating that other pathways or direct activation of factors downstream must contribute to cytotoxicity in these cells (Figure 3b).

Given the high sensitivity of normal lymphoid cells, we were interested in assessing their impact on non-lymphoid cells. Notably, HHT causes neutropenia,[28] a common side effect of cytotoxic chemotherapy usually ascribed to reduction of neutrophil production. However, we found that purified mouse neutrophils are readily killed *in vitro* by silvestrol and HHT and in a Bax/Bak-dependent manner (Figure 3c). In contrast to lymphoid cells or neutrophils, anuclear platelets were insensitive *in vitro* to both silvestrol and HHT under conditions where the BH3 mimetic compound ABT-737 readily killed platelets[29] by neutralizing the anti-apoptotic $Bcl\text{-}x_L$ (Figure 3d).

In summary, we found that translation inhibitors kill a wide range of normal hematopoietic cell types, even ones that are non-cycling and terminally differentiated, but cell death was not universally dependent on Bax and Bak.

The role of Mcl-1 reduction in cell killing by translation inhibitors. Our data (Figures 2 and 3) strongly indicate that Bax and/or Bak-mediated cell death is a key consequence of protein synthesis inhibition in many cell types. How might compounds that inhibit translation activate these cell death mediators? On exposure to silvestrol or HHT, the levels of pro-survival Mcl-1 decline rapidly (Supplementary Figure 5a) and it has been proposed that this is the key process for inducing apoptosis.[16,17,22,30] In this scenario, translation inhibitors should be particularly cytotoxic to Mcl-1-dependent cells.

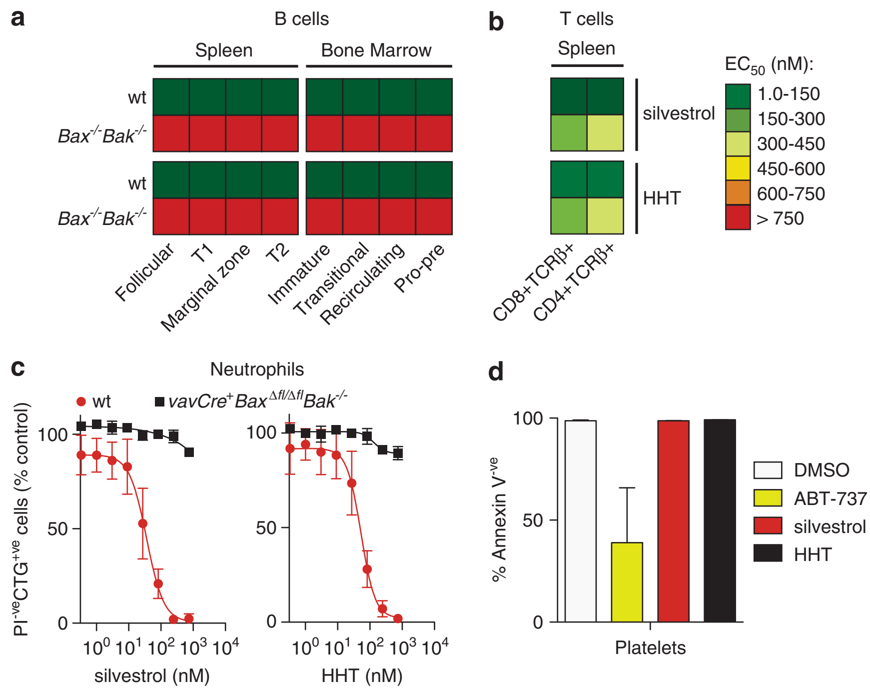

Figure 3 Diverse non-transformed hematopoietic cells are sensitive to translation inhibition in culture, but not all are killed exclusively by Bax/Bak-mediated apoptosis. (**a** and **b**) The translation inhibitors kill many lymphoid cell subsets by Bax/Bak-mediated apoptosis *in vitro*, but the killing of splenic T cells must also involve additional processes. The sensitivity (assessed by PI uptake) of freshly isolated wt or $Bax^{-/-}$ $Bak^{-/-}$ (**a**) B or (**b**) T lymphoid cells after 24 h of treatment with silvestrol or HHT in culture is depicted in the form of heat maps; the mean EC_{50} of cells is represented ($n=3$–6 mice per genotype). See Supplementary Figure 4 for definitions of the B-cell subsets. (**c**) Neutrophils are killed *in vitro* by treatment with translation inhibitors. The survival of neutrophils after 24 h of treatment with silvestrol or HHT was determined by high-content live cell imaging using staining with Cell Tracker Green to image live cells (CTG) and PI to view dead cells ($n=2$–9 mice/ genotype).[49] (**d**) Platelets are insensitive to translation inhibitors *in vitro*. Survival of freshly isolated platelets determined by Annexin V staining (to detect phosphytidyl-serine exposure) followed by flow cytometric analysis after treatment in culture with ABT-737 (1 μM), silvestrol (20 μM) or HHT (20 μM) for 90 min ($n=2$ mice in independent experiments). Cell survival was normalized to vehicle-treated controls; error bars represent the S.D. in all graphs

We initially tested this hypothesis using fibroblasts lacking Bcl-2, Bcl-x_L or Mcl-1 (Figure 4a and Supplementary Figure 5b). Silvestrol and HHT induced cell death completely via Bak-mediated apoptosis in fibroblasts (Figure 2a) and Bcl-x_L and Mcl-1 are the critical guardians of Bak in these cells.[31] We consequently predicted that *Bcl-x*$^{-/-}$ cells would be much more sensitive to Mcl-1 inhibition than their wild-type counterparts because they would be more dependent on Mcl-1 for survival (see schematic in Figure 4a). Although loss of Bcl-x_L increased sensitivity to silvestrol and HHT, the removal of Bcl-2 or Mcl-1 also sensitized fibroblasts to these compounds (Figure 4a). Therefore, although Mcl-1 protein levels are rapidly reduced after protein synthesis inhibition (Supplementary Figure 5a),[17,22,30] its loss does not appear to be the sole or even major trigger of apoptosis in fibroblasts. Furthermore, fibroblasts can survive in the complete absence of Mcl-1 and translation inhibitors still induce death in such Mcl-1-deficient cells (Figure 4a). Our studies suggest that all the pro-survival Bcl-2 family members tested impose critical barriers to death when translation is inhibited.

To extend these studies, we evaluated the role of Mcl-1 in non-transformed B lymphoid cells, which absolutely require this pro-survival protein for their survival.[32] As our previous data suggested that B cells are not killed exclusively via Bak (Figure 2c and Supplementary Figure 3a), we used an alternate strategy to that used above. We hypothesized that if Mcl-1 reduction was an important barrier to cell death induced by translation inhibitors, then B cells with Mcl-1 haplo-insufficiency should be more sensitive than their wild-type counterparts (Supplementary Figure 6a). Indeed, *Mcl-1*$^{+/-}$ B cells were considerably more sensitive than their wild-type counterparts to ABT-737 (Figure 4b), consistent with the notion that Mcl-1 is the critical barrier to the action of this BH3 mimetic compound.[30] In contrast, B cells lacking one allele of *Mcl-1* were not significantly more sensitive to silvestrol or HHT compared with their wt (*Mcl-1*$^{+/+}$) counterparts (Figure 4c and Supplementary Figures 6b and c). Similar to B lymphocytes, loss of one allele of *Mcl-1* did not significantly increase the sensitivity of neutrophils, which also rely heavily on Mcl-1 for their survival (Supplementary Figures 6b and c).[33]

Therefore, contrary to what was predicted,[16,17,22,23] reduction of Mcl-1 protein levels did not have a major role in the apoptotic cell death caused by translation inhibition even in cells that are critically dependent on Mcl-1 for their survival.

Inhibitors of protein synthesis induce multiple anti-replicative effects irrespective of Bax/Bak-mediated apoptosis. As silvestrol has been reported to inhibit proliferation in mantle cell lymphoma,[16] we investigated whether leukemias were similarly affected. The total number of viable cells from all leukemia-derived cell lines tested was substantially reduced after translation inhibition, even when

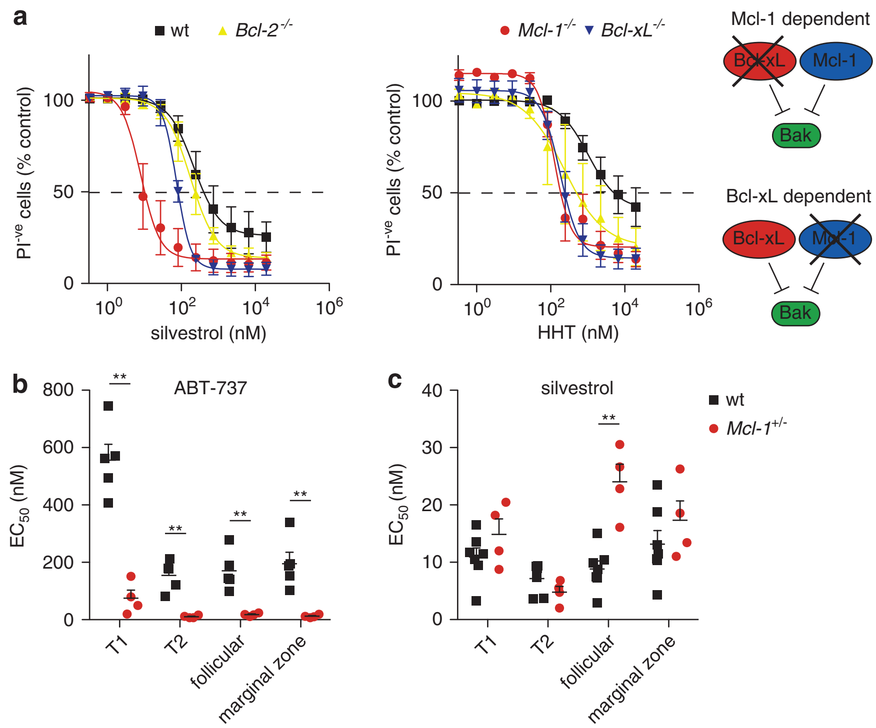

Figure 4 Mcl-1 does not have the major role in apoptosis triggered by protein synthesis inhibition. (**a**) Loss of Mcl-1 and other pro-survival Bcl-2 family members sensitizes fibroblasts to translation inhibitors. The survival of wt MEFs or ones lacking the indicated pro-survival Bcl-2 family members was measured by the exclusion of PI in flow cytometric analysis after treatment in culture with silvestrol (left) or HHT (right) for 24 h ($n=3$–5 independent experiments, 2–5 independently derived lines of each genotype). Far right: schematic illustrating the dependency of knockout MEFs for the other pro-survival Bcl-2 family members after translation inhibition. (**b** and **c**) Reduction of Mcl-1 has only a minor impact on the sensitivity of B lymphoid cells to silvestrol. The survival of wt (*Mcl-1*$^{+/+}$) or *Mcl-1*$^{+/-}$ splenic B cells was assessed by PI staining and flow cytometric analysis after 24-h treatment in culture with (**b**) ABT-737 or (**c**) silvestrol ($n=4-8$ mice per genotype). *P*-values (two-tailed *t*-test) are depicted as follows: $^{**}P<0.01$. See Supplementary Figure 4 for definitions of B-cell subsets. Cell survival was normalized to vehicle (DMSO)-treated controls; error bars represent the S.E.M. in all graphs

cell death was modest (Figure 5a and Supplementary Figure 7a). This suggests that HHT and silvestrol inhibits cell proliferation in addition to inducing cell death. Moreover, the K562 CML-derived cells, which are resistant to silvestrol and HHT-induced apoptosis, lost their ability to replicate after a pulse exposure to these compounds (Figure 5b). In addition, the number of viable cells was considerably reduced in cultures of fibroblasts deficient for Bax and Bak (Figure 5c and Supplementary Figure 7b). This indicates that translation inhibition-induced growth arrest is independent of apoptosis. Therefore, translation inhibitors should have cytostatic activity in cancers with defects in apoptosis pathways.

As there have been conflicting reports regarding the preferential block of specific cell cycle stages induced by translation inhibitor treatment,[16,34,35] we investigated these effects further. In contrast to agents like nocodazole, which block cells at the G_2/M transition, neither silvestrol nor HHT caused *Bax*$^{-/-}$*Bak*$^{-/-}$ cells to accumulate at any specific stage of the cell cycle (Supplementary Figure 7c) when protein synthesis and cell proliferation were inhibited by 95% (Figure 5c and Supplementary Figures 2b and 7b). Thus, silvestrol and HHT do not act on a specific stage of the cell cycle when apoptosis is inhibited.

To investigate if the treated cells can recover their capacity to replicate when Bax/Bak-mediated apoptosis is disabled, we compared the clonogenic potential of wild-type MEFs to ones lacking both Bax and Bak. Intriguingly, colony formation was equally impaired after a 14-h pulse of treatment with silvestrol or HHT (Figures 5d and e), although cell death was only induced in wild-type but not in *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs at this time (Supplementary Figure 8). These results indicate that the loss of clonogenic potential triggered by translation inhibition is independent of Bax/Bak-mediated apoptosis.

Discussion

The role of Mcl-1 reduction and apoptosis in cytotoxicity induced by translation inhibition. The decline in Mcl-1 levels has been proposed to be the major effector of cell death induced by translation inhibitors as Mcl-1 levels drop rapidly after treatment (Supplementary Figure 5a).[16,17,22,30] Inhibition of Mcl-1 by the BH3-only Bik has also been proposed as a mechanism for cell killing.[36] Nonetheless, when using genetic tools to determine the significance of these hypotheses, we found that Mcl-1 did not have the critical role in preventing death by either silvestrol or HHT, even in lymphoid cells and neutrophils that are dependent on Mcl-1 for their normal survival (Figure 4c and Supplementary Figure 6).[32] In fact, we found that all pro-survival Bcl-2 family members could have a role in inhibiting cell death because of translation inhibition (Figure 4a).

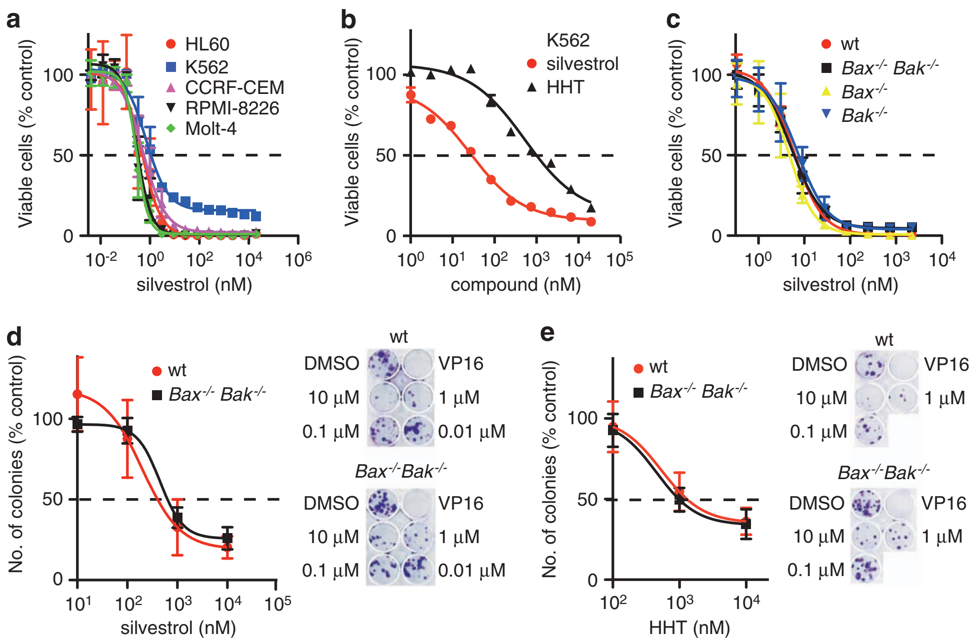

Figure 5 Translation inhibitors reduce cell viability by multiple mechanisms. (**a**) The translation inhibitors impair the proliferation of multiple cell types. The total numbers of viable leukemic cells were determined after 72-h exposure to silvestrol using the CellTiter-Glo assay ($n = 3$ independent experiments). (**b**) The viability of K562 cells is impaired by treatment with protein synthesis inhibitors. K562 cells were treated for 24 h with the indicated concentrations of silvestrol or HHT, then cultured without compound for 3 days before the total numbers of viable cells were measured using the CellTiter-Glo assay ($n = 3$). (**c**) The translation inhibitors impair the proliferation independent of Bax and Bak. The total numbers of viable MEFs were determined after 72-h exposure to silvestrol using the CellTiter-Glo assay ($n = 3$ independent experiments). (**d** and **e**) Long-term (clonogenic) survival of MEFs after treatment with translation inhibitors is independent of Bax and Bak. Clonogenic survival of MEFs of the indicated genotypes was determined after 14 h of treatment with vehicle, etoposide (VP-16, 34 μM) or the indicated concentrations of (**d**) silvestrol or (**e**) HHT, followed by culturing cells without compound for an additional 7 days. Quantification is shown on the left panels and expressed as a percentage of control (vehicle, DMSO)-treated samples ($n = 3$ independent experiments performed in duplicate). Representative stained plates are shown on the right. Data were normalized to vehicle (DMSO)-treated controls and the error bars represent the S.E.M.

Fibroblasts lacking Mcl-1 were more sensitive to silvestrol than HHT when compared with cells lacking other pro-survival Bcl-2 family members (Figure 4a), suggesting that silvestrol can target additional survival protein(s), which are critical for cell survival under these particular circumstances. It is noteworthy that our results do not demonstrate that Mcl-1 is inconsequential for survival of the cell types tested, but instead suggest that other proteins must also be critical when translation is inhibited. Importantly, our results caution that correlations, such as a decrease in Mcl-1 protein levels and cell death, do not always signify causality.

How is apoptosis induced after translation inhibition? The precise mechanism varies between cell types and most likely due to a different balance between the Bcl-2 family members. For instance, Bak is the major driver of translation inhibitor-induced apoptosis in MEFs but not in *Eμ-myc* lymphomas (Figures 2a and c). We therefore caution that generalizations between cell types may not be informative.

Importantly, our data also indicate that Bax/Bak-mediated apoptosis is not always the sole cell death pathway triggered by translation inhibitors. Strikingly, splenic T lymphocytes, which lacked Bax and Bak, underwent plasma membrane permeabilization on treatment with silvestrol or HHT (Figure 3b). Although it is possible that the poorly understood Bax/Bak-related Bok induces cell death under these specific circumstances, this appears unlikely given that cells from Bok-deficient mice respond normally to a broad range of apoptotic stimuli.[37] We propose that either the death receptor pathway is activated or that the Bcl-2 regulated pathway is triggered downstream of Bax and Bak, for example, by reduction of XIAP, an inhibitor of effector caspases.[38] Other possibilities include induction of autophagy-induced cell death, necrosis or necroptosis.

Death of wild-type cells occurs only after near complete inhibition of translation (Figure 4a and Supplementary Figure 2a), and not only when eIF4F-sensitive mRNAs (such as those with 5′TOP sequences or 5′ significant secondary structure such as Mcl-1) are affected. This conclusion is supported by our observations that the translation initiation and elongation inhibitor had similar potencies in almost all cells that we tested. Therefore, we found no benefit of targeting translation initiation over elongation.

The consequences of inhibiting multiple survival pathways. Many chemotherapeutic drugs function by inducing apoptosis and therefore are ineffective against tumors with genetic lesions in specific cell death regulatory genes or have other defects in apoptotic pathways. Remarkably, loss of the mitochondrial apoptotic pathway (by combined loss of Bax and Bak) did not influence the long-term survival and loss of proliferative potential after translation inhibition (Figures 5c–e). Accordingly, the numbers of viable cells were considerably reduced by treatment with HHT or silvestrol, even in the death-resistant K562 line (Figure 5a and Supplementary

Figure 7a). These data indicate that translation inhibitors should at minimum have tumor-static effects even if cell death cannot be induced in a particular cancer.

Interestingly, the ability to replicate was also abrogated after translation-inhibiting compounds were removed in apoptosis-resistant leukemia (e.g., K562; Figure 5b). These results indicate that protein synthesis inhibitors may be effective in many types of chemo-resistant cancers as they can induce apoptosis, inhibit proliferation and impede clonogenic potential even after the inhibitor is removed. On the other hand, the long-term effects on normal replicating cells, such as rapidly dividing progenitor cells in the bone marrow and intestine, would need to be closely monitored.

The sensitivity of differentiated cells and its clinical implications. Translation inhibitors killed patient-derived CLL cells, which are non-dividing in culture, at a faster rate than quickly proliferating leukemia lines (Figure 1 and Supplementary Figure 1).[17,22] Moreover, quiescent mature B cells displayed a similar sensitivity to translation inhibitors as their progenitors, many of which are cycling (Figure 3a and Supplementary Table 1). Therefore, neither proliferation nor differentiation status correlated with translation inhibition-induced cell death. This suggests that these compounds might be effective against tumor types arising from a wide range of differentiation states. Conversely, the sensitivity of normal cells from many differentiation stages raises concerns that protein synthesis inhibition may induce unacceptable toxic side effects. Indeed, although both silvestrol and HHT were highly cytotoxic to *Eμ-myc* lymphoma cells in culture (Figure 2c and Supplementary Figure 3a), they were ineffective as single agents *in vivo* at the doses reported.[11,14] A narrow therapeutic window could explain these results given that *Eμ-myc* lymphoma cells and primary B cells had very similar sensitivity to these two compounds *in vitro* (Supplementary Table 1).

In addition, our data confirm and extend previous reports that treatment with translation inhibitors may put patients at risk of neutropenia (Figure 3c).[28,39] Developing these compounds for combination therapy with other standard-of-care treatments instead of using them as single agents may diminish this risk if they can be efficacious at lower doses. In this regard, inhibitors of protein synthesis have already demonstrated synergistic effects with diverse cytotoxic drugs, including daunorubicin, etoposide, cytarabine and the BH3-mimetic ABT-737 *in vitro*.[40] In fact, both silvestrol and HHT, while ineffective as single agents in the *Eμ-myc* lymphoma mouse model at doses tested *in vivo*, showed efficacy in combination with doxorubicin.[11,14]

In conclusion, although the potential toxicities caused by translation inhibitors need to be closely examined, translation inhibitors have potential as anticancer agents, especially in combination therapy. Their unique ability to inhibit multiple processes required for tumor expansion, some of them independent of apoptosis, suggests that translation inhibitors may be effective in even classically chemo-resistant cancers.

Materials and Methods

Translation inhibitors. HHT was purchased from Enzo Life Sciences (via Sapphire Bioscience Pty. Ltd., Waterloo, Australia), whereas silvestrol was synthetically prepared.[41] Master stocks dissolved in DMSO were stored at $-80\,^{\circ}C$.

Mice. *Bax*$^{-/-}$*Bak*$^{-/-}$ lymphoid cells (Figure 3a) were generated by reconstituting lethally irradiated (2×5.5 Gy, 3 h apart) wild-type recipient mice (C57BL/6-Ly5.1) with fetal liver cells from Ly5.2 *Bax*$^{-/-}$*Bak*$^{-/-}$ embryos (embryonic day 14.5).[42] *Bak*$^{fl/fl}$*Bax*$^{-/-}$ mice were crossed with the *vav-Cre* transgenic mice[43] to generate mice lacking Bax and Bak only in the hematopoietic system (Figure 3c); *Mcl-1*$^{+/-}$ mice have been previously described.[29] All mice were on a C57BL/6 genetic background; either generated on this background, using C57BL/6-derived ES cells, or (in the case of the *Bax*$^{-/-}$ and *Bak*$^{-/-}$ mice) were generated on a mixed C57BL/6 × 129SV background, using 129SV-derived ES cells, but back-crossed onto a C57BL/6 background for at least 10 generations before intercrossing. The Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee approved all animal experiments.

Tissue culture. MEFs were immortalized with SV40 large T antigen and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS; Bovogen, East Keilor, Australia), 50 μM 2-mercaptoethanol (Sigma, Castle Hill, Australia) and 100 μM asparagine (Sigma). MEFs and *Eμ-myc* lymphoma cells have been previously described.[31,44,45] HL-60, K562, CCRF-CEM, RPMI 8226 and MOLT-4 leukemia-derived cell lines (sourced from ATCC, Manassas, VA, USA) were grown in HT-RPMI supplemented with 10% FCS.

***In vitro* culture of CLL samples.** Whole blood from patients with CLL was collected into lithium heparin tubes and the mononuclear layer separated using Ficoll density gradient centrifugation and seeded at 1 million cells/ml in IMDM + 10% FCS as previously described.[46] Viable CLL cells were defined as $CD19^{+}CD5^{+}PI^{-}$ using a FACSCalibur flow cytometer (Becton Dickinson, North Ryde, Australia) after staining with CD19-PE (J3-119; Beckman Coulter, Gladesville, Australia), CD5-FITC (BL1a; Beckman Coulter) and propidium iodide (PI; Sigma). As the CLL cells can lose their surface markers at death, the numbers of viable CLL cells were normalized to the total number of cells in the sample. Samples were obtained from patients with CLL (as defined by World Health Organization classification of hematopoietic neoplasms) after written informed consent as approved by the Melbourne Health Human Research and Ethics Committee, which comprises the Institutional Review Board.

Stable knock-down of *Bax* or *Bak* gene expression in cell lines. *Eμ-myc* lymphoma cells were infected with a retrovirus encoding an shRNA against mouse *Bax* (V2MM_2489; Open Biosystems, via Millennium Science, Surrey Hills, Australia) or, as a negative control, human *IFI-16* (V2HS_63531; Open Biosystems), subcloned into pLMP. shRNAs against *Bak* or the *Renilla luciferase* gene have been previously described.[47,48] Knock-down efficiency was verified by western blotting using antibodies to Bak (Sigma), Bax (21C10; WEHI) and HSP70 (loading control; N6; gift from W Welch).

Cell survival and viability assays for MEFs, *Eμ-myc* lymphoma cells and human leukemia-derived lines. Cells were treated with silvestrol, HHT or VP-16 (etoposide; Mayne Pharma, Salisbury South, Australia) at the indicated concentrations and, in the case of adherent cells, washed in PBS and trypsinized. Cells, washes and culture medium were combined. The cells were then pelleted and resuspended in FACS buffer (KDS BSS/2% FCS/1% sodium azide) containing 10 μg/ml PI. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson). The broad spectrum caspase inhibitor Q-VD-OPh (50 μM; MP Biomedicals, Seven Hills, Australia) was added to cell cultures where indicated. Cell viability and proliferation assays were performed after 72 h of drug treatment using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Alexandria, Australia) following the manufacturer's instructions and data were represented relative to controls.

Survival of primary lymphoid cells. Single-cell suspensions of bone marrow or spleen (0.5 million cells/ml) from 6- to 10-week-old wild-type C57BL/6 or the indicated mutant mice were treated with red cell removal buffer (0.16 M NH_4Cl, 0.13 mM EDTA, 12 mM $NaHCO_3$), washed in medium and then cultured for 24 h in medium (MT-RPMI/10% FCS/50 μM 2-mercaptoethanol) with silvestrol, HHT or vehicle at the indicated concentrations. Ten thousand unlabeled FACS beads were added and resuspended in MT-PBS/2% FCS with the indicated antibodies. After a wash in MT-PBS/2% FCS, cells were resuspended in MT-PBS/2% FCS containing 10 μg/ml PI and subjected to flow cytometric analysis. Antibodies and fluorochromes used for surface staining were as follows: IgD-A680 (11–26C), IgM-PE (331.12), CD21-APC (7G6), B220-56CF (RA3–6B2), CD4-FITC (GK1.5; BD Biosciences, North Ryde, NSW, Australia), CD8a-APC (53-6.7; BD Biosciences), TCRβ-PE (H57–597; BD Biosciences). Cell viability was calculated as the number of PI-negative cells*beads counted/beads added per sample.

Neutrophil survival. Mouse neutrophils were purified from bone marrow on Percoll gradients (52%/68%/78%) and cell survival in culture (in the presence of 10 ng/ml GM-CSF where indicated) was assayed essentially as previously described, using staining with Cell Tracker Green (CTG) (Invitrogen, Mulgrave, Australia) and PI (2 μg/ml).[49] Cell survival (%) was calculated as (live cells ($CTG^{+ve}PI^{-ve}$)/total cells ($CTG^{+ve}PI^{-ve} + CTG^{-ve}PI^{+ve}$)) * 100.

Platelet survival. Whole mouse blood (5 μl) was diluted in 90 μl resuspension buffer (1 mM HEPES pH 7.4, 14 mM NaCl, 0.3 mM KCl, 50 μM $MgCl_2$, 50 μM $NaHCO_3$, 1 mM glucose). After incubation at 37°C with the indicated concentrations of compound, 60 μl was transferred into a 96-well plate, avoiding the sediment of red blood cells, stained with FITC-Annexin V (Invitrogen) to detect dying or dead cells and CD41-APC (eBioMWReg30; eBioscience via Jomar Bioscience, Kensington, Australia) to detect cells of the megakaryocytic lineage. $CD41^{+}$ cells were analyzed using a FACSCalibur (Becton Dickinson). A 90-min time point was chosen because of the exquisitely short half-life of platelets *in vitro*.[50]

Clonogenic cell survival assays. MEFs were seeded at low density (40 cells per well in 12-well dishes). After attachment, cells were treated with compound for 14 h, washed and cultured in fresh medium without compound for another 7 days. The numbers of colonies were determined after staining with Giemsa (Merck, Kilsyth, Australia).

Conflict of Interest

The authors declare no conflict of interest.

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