Down-regulation of Mcl-1 potentiates HDACi-mediated apoptosis in leukemic cells

Satoshi Inoue, Renata Walewska, Martin JS Dyer and Gerald M. Cohen

MRC Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester, LE1 9HN, UK.

Running Title: Mcl-1 downregulation sensitizes to HDACi

Corresponding author: Prof. Gerald M Cohen, MRC Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road Leicester, LE1 9HN, UK

Tel: 44-116-2525601 Fax: 44-116-2525616. email: gmc2@le.ac.uk

Abstract

Mcl-1 is an anti-apoptotic Bcl-2 family member, whose degradation is supposedly required for induction of apoptosis. However, histone deacetylase inhibitors (HDACi) induce apoptosis primarily through the Bak/Mcl-1/Noxa and Bim pathways without decreasing Mcl-1. To investigate this discrepancy, we examined the role of Mcl-1 on HDACi-mediated apoptosis. Inhibition of either Class I or Class II HDAC by selective HDACi caused an upregulation of Mcl-1 mRNA and protein. Down-regulation of Mcl-1 by three structurally unrelated cyclin dependent kinase inhibitors potentiated HDACi-mediated apoptosis in primary chronic lymphocytic leukemic (CLL) cells and K562 cells. Sensitivity to HDACi-induced apoptosis was increased ~10-fold by the cyclin dependent kinase inhibitors. Nanomolar concentrations of HDACi, ~300-fold lower than required to induce apoptosis alone, sensitized cells to TRAIL, emphasizing that the mechanism(s) whereby HDACi induce apoptosis is clearly distinct from those by which they sensitize to TRAIL. Furthermore knockdown of Mcl-1 potentiated HDACi-mediated apoptosis in K562 cells. Thus HDACi-mediated Mcl-1 upregulation plays an important anti-apoptotic regulatory role in limiting the efficacy of HDACi -induced apoptosis, which can be overcome by combination with an agent that down-regulates Mcl-1. Thus a clinical trial in some cancers is warranted using a combination of an HDACi with agents that down-regulate Mcl-1.

Introduction

Chronic lymphocytic leukemia (CLL), one of the most common forms of adult leukemia, is a disease of failed apoptosis and new treatments are needed for aggressive forms of the disease. Induction of apoptosis involves activation of either the intrinsic or extrinsic pathway. [1] Bcl-2 family proteins are important regulators of apoptosis and divide into pro-survival anti-apoptotic proteins, such as Bcl-2, Bcl-x_L, Bfl-1 and Mcl-1, multidomain pro-apoptotic proteins, such as Bax and Bak, and pro-apoptotic BH3-only proteins, including Bad, Bik, Bim, Bmf, Puma and Noxa. [1, 2] In response to apoptotic signals, Bax and Bak oligomerize at mitochondrial membranes leading to cytochrome *c* release and caspase activation. The anti-apoptotic Bcl-2 proteins inhibit cytochrome *c* release through blocking Bax and Bak activation. BH3-only proteins act upstream of Bax and Bak and their activity is tightly controlled by diverse transcriptional and post-translational mechanisms. [3, 4] BH3-only proteins bind selectively to individual anti-apoptotic Bcl-2 family members and neutralize their function. [2]

Increased survival of CLL cells correlates with increased Mcl-1 levels and failure to achieve a complete remission with fludarabine or chlorambucil. [5-7] Mcl-1 plays a critical role in development of the immune system and in early hematopoiesis. [8-10] Mcl-1 has a short half life and its expression is tightly regulated by survival and apoptotic signals. [8] Mcl-1 may be transcriptionally regulated by ERK, JAK/Stat, p38 MAPK, PI3K/Akt, phosphorylation of eIF2α and E2F1. [11-13] Mcl-1 may also be phosphorylated by JNK, GSK-3α and β and cyclin dependent kinase (CDK), and this phosphorylation regulates Mcl-1 turnover and its anti-apoptotic function. [14, 15] Levels of Mcl-1 are

increased by cytokines, such as IL-4 and IFNγ, and by proteasomal inhibitors, which prevents its degradation. [8, 9, 16] Mcl-1 levels are decreased by MAPK inhibitors, tyrosine kinases inhibitors (Gleevec), the Raf inhibitor (BAY43-9006/sorafenib) and CDK inhibitors (roscovitine and flavopiridol). [16] Mcl-1 levels may be decreased by proteasomal degradation [17] or caspase-dependent cleavage. [18] A decrease of Mcl-1 has been proposed to be essential for the induction of apoptosis by diverse apoptotic stimuli including the induction of UV- and cycloheximide-mediated apoptosis. [17, 19-21]

Histone deacetylase inhibitors (HDACi) are a new class of targeted anti-cancer agents. [22, 23] HDACi induce apoptosis primarily by activation of the intrinsic pathway and appears to require *de novo* protein synthesis of BH3-only proteins, such as Bim and Bmf. [23-25] In this regard, we have recently demonstrated that HDACi-mediated apoptosis in CLL and lymphoma cells was accompanied by a functionally important upregulation of Bim and Noxa. [26] Although HDACi alone may be clinically useful, they will most likely be of value in combination with other anti-tumor agents. We have shown that CLL cells, which are inherently resistant to TNF-related apoptosis-inducing ligand (TRAIL), are sensitized by prior treatment with an HDACi that preferentially inhibits HDAC Class I but not Class II. [27, 28] HDACi also potentiate the activity of several anti-cancer drugs that act primarily through the intrinsic pathway. [23] However, the precise mechanisms whereby HDACi potentiate the activation of the "intrinsic pathway" have not been elucidated.

We now demonstrate that exposure of cells including CLL cells to HDACi, which induce apoptosis, results in the upregulation of Mcl-1, which is functionally important and exerts an anti-apoptotic effect on HDACi-mediated apoptosis.

Method and Materials

Lymphocyte purification, cell lines and culture. CLL cells, obtained with patient consent and local ethical committee approval, were cultured (4-5 x 10^6 cells ml^{-1}) as described. [27] Jurkat T cells (clone E6-1), K562, a human myelogenous leukemic cell line and H1299, a lung cancer cell line, were cultured in RPMI 1640 medium supplemented with 10 % FBS and 5% Glutamax™.

Reagents. Media and serum were from Life Technologies, Inc (Paisley, UK). Unless otherwise stated, antibodies to Bcl-2 family proteins and other proteins were as previously described. [26, 28]The HDACi, LBH589, was kindly provided by Dr. P. Atadja (Novartis Pharmaceuticals Corporation, East Hanover, NJ). Depsipeptide was kindly provided by Dr. E. Sausville (National Cancer Institute, Bethesda, MD). MG132, 2-naphthylvinyl ketone (2-NVK), MS-275, Bisindolylmaleimide IX (Bis 9), Bay11-7082, Nutlin-3a and the CDK inhibitors, NU6102, SU5612 and roscovitine, were from Calbiochem (La Jolla, CA). MC1568 and MC1575, two specific class II HDACi, were kindly provided by Dr. A. Mai (Università degli Studi di Roma, Rome, Italy). [28] The anti-Fas Ab (CH-11) was from Upstate (Lake Placid, NY) and the rabbit anti-Bim Ab was from Chemicon (Temecula, CA). Other chemicals were from Sigma (Poole, United Kingdom). Z-VAD.fmk was from MP Biomedicals and Obiogene (Irvine, CA). Recombinant His-TRAIL was prepared as previously described. [27]

Quantification of apoptosis and western blot analysis. Apoptosis in CLL cells and all cell lines was assessed by an increase in phosphatidylserine (PS) externalization as

previously described. [27] EC_{50} values were determined by non-linear regression analysis using Graph PAD Prism (GraphPAD software Inc, San Diego, CA).

Real-time RT-PCR. Samples for real-time RT-PCR analysis were prepared as described. [26] Real time amplification reactions were performed with 900 pmol forward or reverse primers, designed using the Primer Express software v2.0 (Applied Biosystems, Warrington, UK). The sequences for β-actin were as previously described [26] and those for Mcl-1 were forward, GAGGCTGGGATGGGTTTGT and reverse, AAAGCCAGCAGCACATTCT. Statistical significance of the data was assessed by an unpaired two tailed t-test on log 2 transformed data.

Small RNA Interference (siRNA) of Mcl-1. K562 cells ($1.5x10^5$ cells) were transfected with small interfering RNA (siRNA) oligonucleotides (Ambion, Austin, TX) at a final concentration of 25 nM (a) and 75 nM (a') using HyPerFect transfection reagent (3 μl in serum free RPMI medium), essentially as recommended by the manufacturer (Qiagen, Crawley, West Sussex, UK). Briefly, after 6 h transfection, RPMI, 10%FCS, 1 x glutamax were added followed by incubation for another 42 h. Cells were exposed to LBH589 as described in the legend to Figure 5. H1299 cells were seeded at $1.5x10^6$ cells per well in 6-well plates and incubated for 24 h. Cells were transfected with siRNA oligonucleotides (Ambion) at a final concentration of 20 nM using Effectene (Qiagen). After 24 h, cells were exposed for a further 12 h in the presence or absence of an HDACi. The sequences used for RNAi were as follows; Mcl-1a sense, 5'-(GAAACGCGGUAAUCGGACUtt)-3'; Mcl-1a antisense, 5'-(AGUCCGAUUACCGCGUUUCtt)-3'; Mcl-1a' sense, 5'-(GUAUCACAGACGUUCUCGUtt)-3'; Mcl-1 a' antisense,

5'-(ACGAGAACGUCUGUGAUACtt)-3'. Untargetted oligos were from Ambion (ID #4611).

Results

Induction of apoptosis by diverse chemicals but not by HDACi and MG132 is accompanied by a decrease of Mcl-1

To investigate whether a decrease in Mcl-1 was a common feature accompanying apoptosis, Jurkat cells were exposed to diverse agents including, cycloheximide (a protein synthesis inhibitor), MG132 (a proteasomal inhibitor), LBH589 (a HDACi), etoposide (a DNA topoisomerase II inhibitor), Bis IX (a PKC inhibitor), 2-naphthylvinyl ketone (2-NVK) (a Jak3 inhibitor), [29] cyclosporin A (a calcineurin inhibitor), roscovitine (a CDK inhibitor) and anti-Fas Ab (CH-11). A decrease in Mcl-1 accompanied the induction of apoptosis by all these agents except LBH589 and MG132 (Figure 1A). LBH589 and MG132, and to a lesser extent roscovitine and 2-NVK, but not the other agents, caused a marked induction of Noxa, whereas only LBH589 and MG132 caused a marked increase in Bim (Figure 1A). Using K562 cells to examine whether these effects were cell type specific, a clear decrease in Mcl-1 was observed after exposure to Actinomycin D (a transcriptional inhibitor), Bay11-7082 (an IκB phosphorylation inhibitor) and 2-NVK but not after MG132 or both HDACi, LBH589 and TSA, despite induction of marked apoptosis (39-65 %) (Figure 1B). Trichostatin A (TSA) and LBH589, caused an increase in Noxa and Bim (Figure 1B lanes 4 and 5), as in our earlier study. [26]

To examine if similar changes also occurred in CLL cells, freshly isolated cells

from four patients were exposed to different apoptosis inducers, including prednisilone, used to treat CLL, and nutlin-3a, an MDM2 antagonist that induces apoptosis in CLL cells. [30] Induction of apoptosis by all these chemicals except LBH589, TSA and MG132 was accompanied by a loss of Mcl-1, although some inter-individual variation was observed (Figure 1C and Supplementary Figure 1). Except with cycloheximide, this decrease of Mcl-1 was accompanied by an immunoreactive band of ~30 kDa (Figure 1C, marked with an asterisk), which most likely was due to caspase mediated cleavage of Mcl-1. [18, 31] This cleavage product was also observed in cells exposed to MG132, LBH589 and TSA, but in these cases no clear loss of Mcl-1 was observed (Figure 1C lanes 3, 4, 12 and 13). Increases in Noxa were observed following exposure of CLL cells to MG132, LBH589 and TSA (Figure 1C and Supplementary Figure 1). LBH589 and TSA increased Bim_{EL} in some patients in agreement with our previous observations, although greater variation was observed. [26] Prednisolone also caused an increase in Bim levels (Figure 1C lanes 9 and 18 and Supplementary Figure 1). No changes in the levels of Bcl-2, Bax and Bak accompanied the induction of apoptosis in CLL cells by all 8 chemicals (data not shown).

Taken together these results demonstrated that although a decrease of Mcl-1 was a common feature of apoptosis in CLL, Jurkat and K562 cells induced by variety of different chemical agents, it was not an absolute requirement, as no decrease was observed in MG132 and HDACi-induced apoptosis (Figure 1). Whilst inhibition of the proteasome was responsible for the accumulation of Mcl-1 following MG132, this did not appear to be the primary explanation for Mcl-1 accumulation following exposure to

HDACi, as HDACi resulted in only a small accumulation if any of ubiquitinated proteins (Supplementary Figure 2A). Rather our data suggested that despite extensive induction of apoptosis, HDACi may even cause accumulation of Mcl-1 by a mechanism other than proteasomal inhibition. To test this possibility, we assessed the ability of HDACi to induce a transcriptional upregulation of Mcl-1 and also measured Mcl-1 levels in cells exposed to HDACi in the presence of the caspase inhibitor, z-VAD.fmk.

HDACi causes a transcriptional upregulation of Mcl-1 mRNA

LBH589 caused a time dependent increase in Mcl-1 mRNA levels in Jurkat and K562 cells, which was largely abrogated by Actinomycin D (Figure 2A and B). To investigate whether inhibition of class I or class II HDACs was required for Mcl-1 upregulation, we used MS-275 and MC1568, which are specific for class I and II, respectively, whereas LBH589 inhibits both classes. [28] MS-275 (25 μM) also caused an increase in Mcl-1 mRNA similar to LBH589 (10 μM), whereas MC1568 resulted in a smaller increase (Figure 2B). Thus inhibition of class I, and to a lesser extent class II HDAC, results in a transcriptional upregulation of Mcl-1.

Prior to examining effects of these different HDACi on Mcl-1 protein levels, we confirmed their efficacy to inhibit HDACs. LBH589 induced hyperacetylation of histone H3, H4 and tubulin, confirming it inhibited both class I and II HDACs (Figure 2C lane 2). [28, 32] MS-275 induced hyperacetylation of histone H3 and –H4 but not of tubulin, confirming it specifically inhibited HDAC class I (Figure 2C lanes 3 and 4), whereas MC1568 and MC1575 induced hyperacetylation of tubulin but not of histones H3 and –H4, confirming they specifically inhibited HDAC class II (Figure 2C lanes 5 and 6). [28]

As inhibition of HDAC6, a Class II HDAC, has been reported to cause an accumulation of protein aggregates due to inhibition of proteasomal activity [33], we examined further the ability of the HDACi to inhibit the proteasome. In agreement with this study, both HDAC Class II inhibitors caused a small accumulation of ubiquitinated proteins (Supplementary Figure 2B), suggesting that Mcl-1 accumulation may be partially due to proteasomal inhibition. Next we examined the effects of the HDACi on levels of Mcl-1 in the presence of z-VAD.fmk, to exclude possible effects of caspase cleavage of Mcl-1. All four HDACi resulted in an increase in Mcl-1 compared to the untreated cells, which contained z-VAD.fmk but no HDACi (Figure 2C compare lanes 2-6 with lane 1). As these results suggested that increases in Mcl-1 protein levels were more readily seen in the presence of a caspase inhibitor, we examined this possibility in both Jurkat and CLL cells. In both Jurkat and CLL cells, LBH589 alone induced a small increase in Mcl-1 levels but this increase was markedly enhanced in the presence of z-VAD.fmk (Figure 2D and E). Taken together our data demonstrated that inhibition of either HDAC class I or class II resulted in an increase in Mcl-1 mRNA and protein.

CDK inhibitors decrease Mcl-1 and potentiate HDACi-mediated apoptosis.

To ascertain if this increase in Mcl-1 was functionally important, we used CDK inhibitors, which decrease Mcl-1 in some cells. [34, 35] Non-toxic concentrations of 3 CDK inhibitors, roscovitine, SU9516 and NU6102, all caused a marked decrease in Mcl-1 without affecting Noxa (Figure 3A compare lanes 3-5 with lane1). LBH589 (250 nM and10 μM) caused an induction of Noxa (Figure 3A lanes 8 and 12). Combination of the CDK inhibitors with LBH589 (250 nM) resulted in a marked sensitization of

LBH589-induced apoptosis assessed by PS externalization, caspase-3 processing and PARP cleavage (Figure 3A compare lane 8 with lanes 9-11). A higher LBH589 concentration (10 μM) alone for 24 h induced apoptosis (Figure 3A lane 12). Thus concentrations of CDK inhibitors, which decreased Mcl-1, potentiated K562 cells to LBH589. The EC_{50} for LBH589 to induce apoptosis was 1728 nM and this was reduced to 146, 148 and 181 nM in the presence of roscovitine, SU9516 and NU6102, respectively (Figure 3B). The similarity of the concentration response of the 3 CDK inhibitors to sensitize cells to LBH589 suggested that the mechanism of sensitization was similar (Figure 3B). Low concentrations of LBH589 (~25 nM), which neither induced apoptosis nor decreased Mcl-1, markedly potentiated TRAIL-induced apoptosis in K562 cells, with an EC_{50} = 6 nM (Figure 3B). Thus the mechanism whereby LBH589 potentiated TRAIL-induced apoptosis differed from how CDK inhibitors potentiated LBH589-induced apoptosis.

Similarly all 3 CDK inhibitors potentiated some but not all CLL cells to LBH589-induced apoptosis, as assessed both by PS externalization (Figure 3C and Supplementary Figure 3) and by caspase-3 activation and PARP cleavage (Figure 3D compare lane 8 with lanes 9-11). LBH589 was ~10-fold more potent at inducing apoptosis in the presence of the CDK inhibitors (Figure 3C). Low LBH589 concentrations (~25 nM), which alone did not induce apoptosis, sensitized CLL cells to TRAIL, assessed by PS externalization (Figure 3C), processing of caspase-3 and PARP cleavage (Figure 3D lanes 2 and 6-7).

Mcl-1 knockdown potentiates HDACi-mediated apoptosis

To assess the specific contribution of Mcl-1 to the sensitization, we utilized two sets of siRNA (#a and #a') of Mcl-1 in K562 cells both because of their high transfection efficiency and their low toxicity to HyperFect transfection reagent. Both sets of Mcl-1 siRNA reduced Mcl-1 without affecting other Bcl-2 family proteins (Figure 4B and data not shown). Knockdown of Mcl-1 by siRNA alone, which did not induce apoptosis (Figures 4A lanes 1-3), potentiated apoptosis induced by intermediate (250 nM) but not low (25 nM) LBH589 concentrations assessed by PS externalization (Figure 4A lanes 4-9) and enhanced processing of caspase-3 and cleavage of PARP (Figure 4B lanes 4-6).

To investigate whether Mcl-1 levels were important for the induction of apoptosis by LBH589 alone, we examined the effect of Mcl-1 siRNA on the induction of apoptosis by a high concentration of LBH589 (10 μM). This concentration alone did not induce apoptosis in K562 cells when exposed for only 15 h (Supplementary Figure 4) but it did induce apoptosis if exposed for 24 h (Figure 1B). Both sets of siRNA for Mcl-1 potentiated LBH589-induced apoptosis (Supplementary Figure 4) demonstrating a critical anti-apoptotic role for Mcl-1 in inhibiting LBH589-induced apoptosis. To test whether this effect was cell type or HDACi specific, we examined the effects of Mcl-1 siRNA on sub-toxic concentrations of three HDACi in H1299 cells. Mcl-1 siRNA caused a specific reduction of Mcl-1 without affecting other Bcl-2 family proteins and resulted in a marked potentiation of HDACi-induced apoptosis (Supplementary Figure 5 and data not shown).Thus levels of Mcl-1 are also key regulators of HDACi-induced apoptosis in H1299 cells, supporting the suggestion that HDACi-mediated Mcl-1 upregulation exerts an important anti-apoptotic effect on HDACi-induced apoptosis.

Discussion

Several reports have proposed that a decrease of Mcl-1 is essential for the induction of apoptosis by diverse apoptotic stimuli. [17, 19-21] However, no such decrease was observed following apoptosis induced by either HDACi or MG132 in Jurkat, K562 and CLL cells and in some cases an increase in Mcl-1 was observed, which was particularly evident in the presence of a caspase inhibitor (Figures 1-3). Thus prevention of caspase mediated cleavage of Mcl-1 greatly facilitated the visualization of HDACi-mediated increase in Mcl-1 (Figure 2), which is compatible with the known ability of HDACi to modulate transcription. [22] It is also possible that HDACi-mediated proteasomal inhibition may also have made a minor contribution to the observed increase in Mcl-1, in agreement with a recent suggestion. [36] Our finding that Mcl-1 was not decreased in the presence of HDACi was also surprising in view of recent work demonstrating that Noxa binding to Mcl-1 could neutralize its anti-apoptotic function and also enhance Mcl-1 degradation. [21, 37] Thus HDACi-induced elevation of Noxa observed in both our present and previous study [26] would have been predicted to result in Mcl-1 degradation. Our findings of elevated Mcl-1 levels in the presence of high levels of Noxa (Figures 1 and 3) raise the possibility of additional levels of control in some cell types, such as CLL cells, including limiting activities of MULE the proposed ubiquitin E3 ligases for Mcl-1. [38]

The upregulated Mcl-1 was functionally important as marked potentiation to HDACi-induced apoptosis was observed following inhibition of the upregulation by either CDK inhibitors or Mcl-1 siRNA (Figures 3-4 and Supplementary Figures 4-5).

How did the combination of an HDACi and the CDK inhibitor induce apoptosis despite LBH589 (250 nM) resulting in increased Mcl-1 levels (Figures 3-4)? Down-regulation of Mcl-1alone did not induce apoptosis (Figure 4), although it was required for potentiation of HDACi-mediated apoptosis. The most likely explanation for this potentiation was the HDACi-mediated increased levels of Bim and Noxa, which neutralize Mcl-1 and activate Bax/Bak, together with CDK inhibitor-mediated decreased levels of Mcl-1. Other studies have also indicated that down-regulation of Mcl-1 may be a promising strategy to sensitize to apoptosis by many agents, including the novel Bcl-2 antagonist, ABT-737. [16, 39, 40] The HDACi-mediated down-regulation of Bcl-x_L may also contribute to HDACi-mediated apoptosis.

CDK inhibitors act as potent transcriptional repressors resulting in the inhibition of synthesis of short-lived anti-apoptotic proteins, such as Mcl-1, and their rapid proteasomal degradation. [41] CDK inhibitors down-regulate Mcl-1 through disruption of RNA polymerase II function and enhanced binding of E2F1 to the Mcl-1 promoter. [12] Transcriptional regulation of Mcl-1 is complex and may be affected by many signaling pathways as discussed in the Introduction. It is not known how these various pathways may be modified by HDACi to upregulate Mcl-1 transcription. HDACi-mediated increases in Mcl-1, Noxa and Bim are induced in a p53-independent manner, as K562 cells are p53 negative.

Relatively high concentrations of LBH589 alone were required to induce apoptosis in K562 cells, with an $EC_{50} \cong 1700$ nM (Figure 3B). Induction of apoptosis by HDACi alone appears to be mediated primarily by induction of BH3-only proteins, in a

cell type specific manner. [23, 26] In H1299 cells, HDACi did not cause an increase in either Bim or Noxa (data not shown) suggesting they may regulate an as yet uncharacterized proapoptotic protein(s). In the presence of a CDK inhibitor, LBH589 was ~10-fold more potent at inducing apoptosis compared to LBH589 alone (Figure 3B) and this potentiation was mediated primarily by decreasing Mcl-1 levels. Approximately 300-fold lower concentrations of LBH589 were required to sensitize K562 and CLL cells to TRAIL than to induce apoptosis alone, most likely by increased formation of the death inducing signaling complex (DISC). [27] In the present study, low nanomolar concentrations of LBH589, which sensitized to TRAIL-induced apoptosis in K562 and CLL cells, did not cause a decrease in Mcl-1 levels (Figure 3A and D compare lanes 1 and 6). Thus these results demonstrate that the mechanism of HDACi-sensitization to TRAIL is Mcl-1 independent and clearly different from that described in other studies. [42-44]

In summary, our results highlight that the combination of an HDACi with either a CDK inhibitor or with TRAIL will enable the induction of apoptosis at 10-300-fold lower HDACi doses, thus potentially circumventing much if not all of the inherent toxicity, such as cardiotoxicity, associated with higher HDACi doses. Thus our studies provide a mechanistic basis to support the use of a combination of an HDACi either with agents that decrease Mcl-1, such as CDK inhibitors, or with TRAIL in a clinical trial in certain hematological malignancies, such as CLL.

Figure legends

Figure 1. A decrease in Mcl-1 does not accompany MG132- and HDACi-induced apoptosis in Jurkat, K562 and CLL cells.

(A) Jurkat cells were exposed for 8 h to cycloheximide (CHX 1 μM), MG132 (1 μM), LBH589 (10 μM), etoposide (10 μM), bisindoylmaleimide IX (Bis IX 1 μM), 2-naphthylvinyl ketone (2-NVK 10 μM), cyclosporin A (10 μM), roscovitine (10 μM) and anti-Fas Ab (CH-11 50 ng/ml). (B) K562 cells were exposed for 24 h to Actinomycin D (Act D 5 μg/ml), MG132 (2 μM), Trichostatin A (TSA 25 μM), LBH589 (10 μM), Bay11-7082 (5 μM) and 2-naphthylvinyl ketone (2-NVK 25 μM). (C) CLL cells from 2 patients were exposed for 20 h to cycloheximide (CHX 10 μM), MG132 (1 μM), LBH589 (10 μM), 2-naphthylvinyl ketone (2-NVK 2.5 μM), cyclosporin A (10 μM), roscovitine (10 μM), Nutlin-3a (10 μM) and prednisolone (25 μM). The asterisk (*) indicates the probable caspase-mediated Mcl-1 cleavage product. Apoptosis was assessed by PS (PS) externalization as described in Method and Materials and cells were analyzed by western blotting using tubulin as a loading control.

Figure 2 LBH589 causes a transcriptional upregulation of Mcl-1 mRNA

(A) Jurkat cells were exposed to LBH589 (10 μM) for 1 or 2 h either alone or in the presence of Actinomycin D (Act D, 5 μg/ml). (B) K562 cells were exposed to LBH589 (250 nM and 10 μM), MS-275 (25 μM) and MC1568 (10 μM) for 2 or 4 h either alone or with Actinomycin D (Act D, 5 μg/ml). Expression of Mcl-1 and β-actin was analyzed by real-time RT-PCR and the results expressed as the fold change in Mcl-1 expression

normalized to the expression of β-actin. (C) K562 cells were exposed to LBH589 (10 μM), MS-275 (10 or 25 μM), MC1568 (10 μM), MC1575 (10 μM) or MG132 (2 μM) for 20 h. z-VAD.fmk (25 μM) was present in all incubations including the control cells. Cells were analyzed by western blotting. * $p<0.05$. (D) Jurkat cells were exposed to LBH589 (LBH) (10 μM) for 8 h either alone or in the presence of z-VAD.fmk (25 μM). (E) CLL cells from patients #3 and 4 were exposed to the indicated concentration of LBH589 for 20 h either alone or in the presence of z-VAD.fmk (75 μM). In (D and E), apoptosis was assessed by phosphatidylserine (PS) externalization and Mcl-1 was assessed by western blotting using tubulin as a loading control.

Figure 3 CDK inhibitors decrease Mcl-1 levels and potentiate LBH589-mediated apoptosis

(A and B) K562 cells were exposed to LBH589 for 24 h either alone or in the presence of roscovitine (Ro, 10 μM), NU6102 (N, 25 μM) or SU9516 (S, 5 μM). K562 cells were also exposed to LBH589 for 24 h prior to exposure to TRAIL (TR, 200 ng/ml) for a further 4 h. (C and D) CLL cells were exposed to LBH589 for 20 h either alone or in the presence of roscovitine (5 μM), NU6102 (25 μM) or SU9516 (5 μM). CLL cells were also exposed to LBH589 for 20 h prior to exposure to TRAIL (200 ng/ml) for a further 4 h. K562 and CLL cells were then analyzed for the induction of apoptosis by PS externalization (A-D) or for effects on the indicated proteins by western blotting (A and D). Ci and Cii depict data from 2 individual patients.

Figure 4 Mcl-1 siRNA potentiate LBH589-mediated apoptosis

K562 cells were transfected for 48 h with either two sets of siRNA for Mcl-1 (a and a') or with a control untargeted sequence (-). Cells were then exposed to LBH589 (either 25 or 250 nM) for 24 h. Cells were analyzed for the induction of apoptosis by PS externalization (A and B) or for effects on the indicated proteins by western blotting (B).

Acknowledgements

We thank Ms Joan Riley and Dr. Shu Dong Zhang for validation of the primers for the real time RT-PCR and statistical analysis, respectively. We thank Mr Roger Snowden for sharing data. This work was supported primarily by Medical Research Council and some support from the Leukemia Research Fund.

References

1 Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004; **116**: 205-219.

2 Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 2007; **26**: 1324-1337.

3 Huang DC, Strasser A. BH3-Only proteins-essential initiators of apoptotic cell death. *Cell* 2000; **103**: 839-842.

4 Labi V, Erlacher M, Kiessling S, Villunger A. BH3-only proteins in cell death initiation, malignant disease and anticancer therapy. *Cell Death Differ* 2006; **13**: 1325-1338.

5 Kitada S, Andersen J, Akar S, Zapata JM, Takayama S, Krajewski S*, et al.* Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. *Blood* 1998; **91**: 3379-3389.

6 Pedersen IM, Kitada S, Leoni LM, Zapata JM, Karras JG, Tsukada N*, et al.* Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. *Blood* 2002; **100**: 1795-1801.

7 Petlickovski A, Laurenti L, Li X, Marietti S, Chiusolo P, Sica S*, et al.* Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells. *Blood* 2005; **105**: 4820-4827.

8 Craig RW. MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis. *Leukemia* 2002; **16**: 444-454.

9 Michels J, Johnson PW, Packham G. Mcl-1. *Int J Biochem Cell Biol* 2005; **37**: 267-271.

10 Opferman JT, Iwasaki H, Ong CC, Suh H, Mizuno S, Akashi K*, et al.* Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* 2005; **307**: 1101-1104.

11 Fritsch RM, Schneider G, Saur D, Scheibel M, Schmid RM. Translational repression of MCL-1 couples stress-induced eIF2 alpha phosphorylation to mitochondrial apoptosis initiation. *J Biol Chem* 2007; **282**: 22551-22562.

12 Rosato RR, Almenara JA, Kolla SS, Maggio SC, Coe S, Gimenez MS*, et al.* Mechanism and functional role of XIAP and Mcl-1 down-regulation in

flavopiridol/vorinostat antileukemic interactions. *Mol Cancer Ther* 2007; **6**: 692-702.

13 Wang JM, Chao JR, Chen W, Kuo ML, Yen JJ, Yang-Yen HF. The antiapoptotic gene mcl-1 is up-regulated by the phosphatidylinositol 3-kinase/Akt signaling pathway through a transcription factor complex containing CREB. *Mol Cell Biol* 1999; **19**: 6195-6206.

14 Inoshita S, Takeda K, Hatai T, Terada Y, Sano M, Hata J*, et al.* Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress. *J Biol Chem* 2002; **277**: 43730-43734.

15 Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell* 2006; **21**: 749-760.

16 Mandelin AM, 2nd, Pope RM. Myeloid cell leukemia-1 as a therapeutic target. *Expert Opin Ther Targets* 2007; **11**: 363-373.

17 Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F*, et al.* Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev* 2003; **17**: 1475-1486.

18 Snowden RT, Sun XM, Dyer MJ, Cohen GM. Bisindolylmaleimide IX is a potent inducer of apoptosis in chronic lymphocytic leukaemic cells and activates cleavage of Mcl-1. *Leukemia* 2003; **17**: 1981-1989.

19 Adams KW, Cooper GM. Rapid turnover of mcl-1 couples translation to cell survival and apoptosis. *J Biol Chem* 2007; **282**: 6192-6200.

20 Cuconati A, Mukherjee C, Perez D, White E. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. *Genes Dev* 2003; **17**: 2922-2932.

21 Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI*, et al.* Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* 2005; **19**: 1294-1305.

22 Marks PA, Richon VM, Miller T, Kelly WK. Histone deacetylase inhibitors. *Adv Cancer Res* 2004; **91**: 137-168.

23 Rosato RR, Grant S. Histone deacetylase inhibitors: insights into mechanisms of lethality. *Expert Opin Ther Targets* 2005; **9**: 809-824.

24 Zhang Y, Adachi M, Kawamura R, Imai K. Bmf is a possible mediator in histone

deacetylase inhibitors FK228 and CBHA-induced apoptosis. *Cell Death Differ* 2006; **13**: 129-140.

25 Zhao Y, Tan J, Zhuang L, Jiang X, Liu ET, Yu Q. Inhibitors of histone deacetylases target the Rb-E2F1 pathway for apoptosis induction through activation of proapoptotic protein Bim. *Proc Natl Acad Sci U S A* 2005; **102**: 16090-16095.

26 Inoue S, Riley J, Gant TW, Dyer MJ, Cohen GM. Apoptosis induced by histone deacetylase inhibitors in leukemic cells is mediated by Bim and Noxa. *Leukemia* 2007; **21**: 1773-1782.

27 Inoue S, MacFarlane M, Harper N, Wheat LM, Dyer MJ, Cohen GM. Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies. *Cell Death Differ* 2004; **11**: S193-206.

28 Inoue S, Mai A, Dyer MJ, Cohen GM. Inhibition of histone deacetylase class I but not class II is critical for the sensitization of leukemic cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Cancer Res* 2006; **66**: 6785-6792.

29 Brown GR, Bamford AM, Bowyer J, James DS, Rankine N, Tang E*, et al.* Naphthyl ketones: a new class of Janus kinase 3 inhibitors. *Bioorg Med Chem Lett* 2000; **10**: 575-579.

30 Coll-Mulet L, Iglesias-Serret D, Santidrian AF, Cosialls AM, de Frias M, Castano E*, et al.* MDM2 antagonists activate p53 and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells. *Blood* 2006; **107**: 4109-4114.

31 Michels J, O'Neill JW, Dallman CL, Mouzakiti A, Habens F, Brimmell M*, et al.* Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage. *Oncogene* 2004; **23**: 4818-4827.

32 Qian DZ, Kato Y, Shabbeer S, Wei Y, Verheul HM, Salumbides B*, et al.* Targeting tumor angiogenesis with histone deacetylase inhibitors: the hydroxamic acid derivative LBH589. *Clin Cancer Res* 2006; **12**: 634-642.

33 Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell* 2003; **115**: 727-738.

34 Chen R, Keating MJ, Gandhi V, Plunkett W. Transcription inhibition by flavopiridol: mechanism of chronic lymphocytic leukemia cell death. *Blood* 2005; **106**: 2513-2519.

35 Kitada S, Zapata JM, Andreeff M, Reed JC. Protein kinase inhibitors flavopiridol and 7-hydroxy-staurosporine down-regulate antiapoptosis proteins in B-cell chronic lymphocytic leukemia. *Blood* 2000; **96**: 393-397.

36 Strasser A, Puthalakath H. Fold up or perish: unfolded protein response and chemotherapy. *Cell Death Differ* 2007, in press.

37 Czabotar PE, Lee EF, van Delft MF, Day CL, Smith BJ, Huang DC*, et al.* Structural insights into the degradation of Mcl-1 induced by BH3 domains. *Proc Natl Acad Sci U S A* 2007; **104**: 6217-6222.

38 Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell* 2005; **121**: 1085-1095.

39 Dai Y, Grant S. Targeting multiple arms of the apoptotic regulatory machinery. *Cancer Res* 2007; **67**: 2908-2911.

40 van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE*, et al.* The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* 2006; **10**: 389-399.

41 Shapiro GI. Cyclin-dependent kinase pathways as targets for cancer treatment. *J Clin Oncol* 2006; **24**: 1770-1783.

42 Han J, Goldstein LA, Gastman BR, Rabinowich H. Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis. *J Biol Chem* 2006; **281**: 10153-10163.

43 Ricci MS, Kim SH, Ogi K, Plastaras JP, Ling J, Wang W*, et al.* Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell* 2007; **12**: 66-80.

44 Taniai M, Grambihler A, Higuchi H, Werneburg N, Bronk SF, Farrugia DJ*, et al.* Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. *Cancer Res* 2004; **64**: 3517-3524.

Figure 1

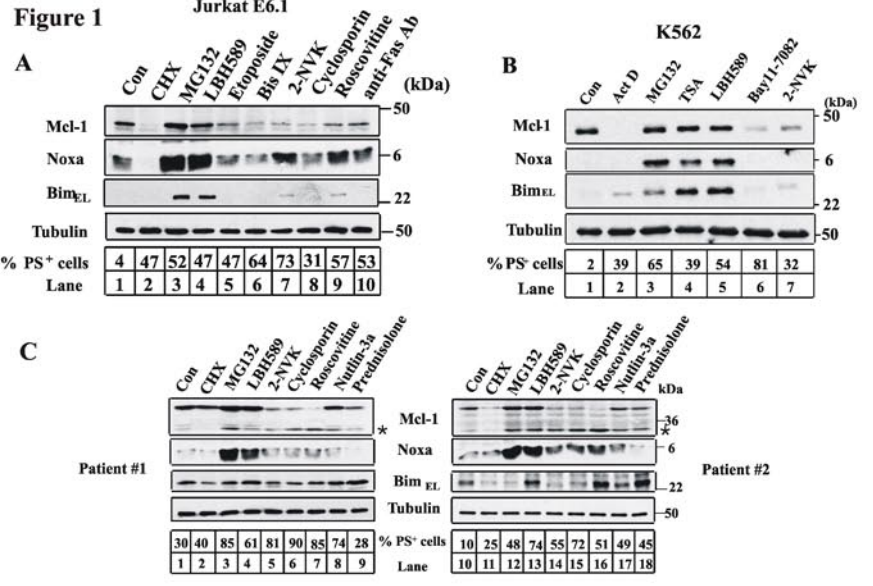
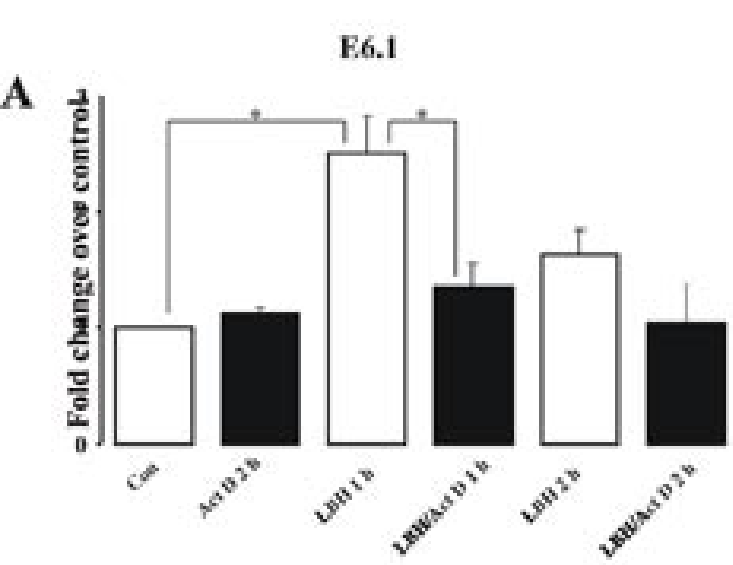
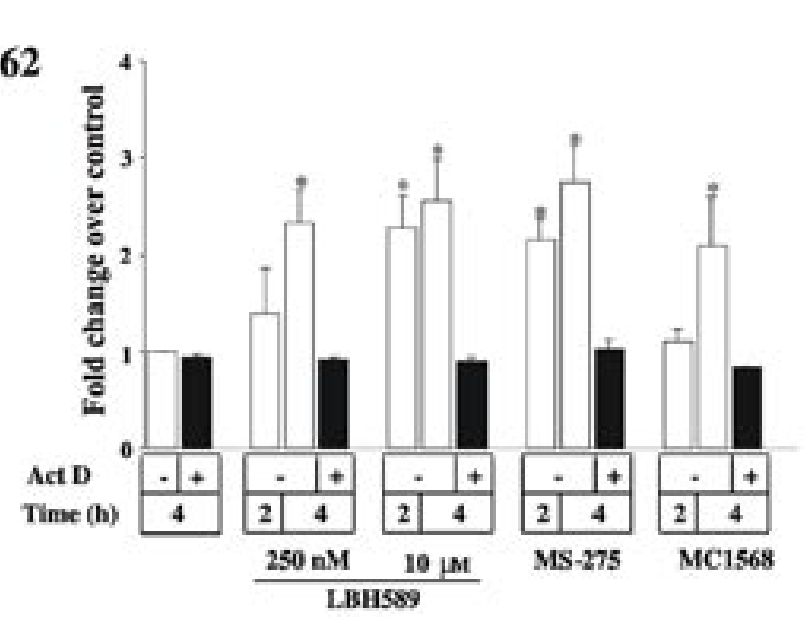
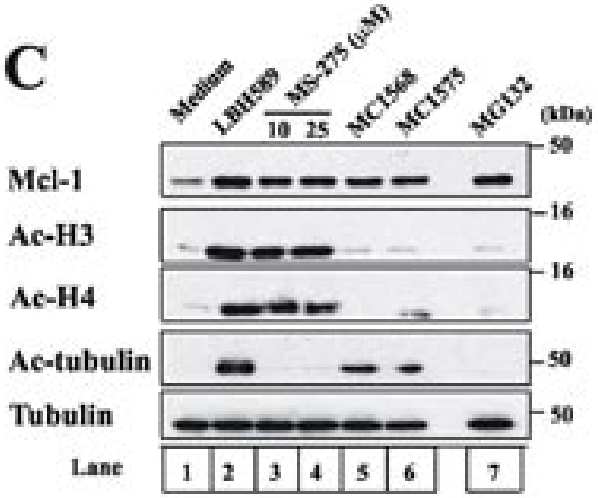
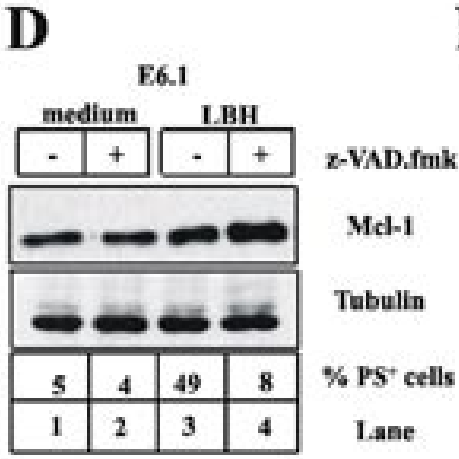

Jurkat E6.1
A
Con
CHX
MG132
LBH589
Etoposide
Bis IX
2-NVK
Cyclosporin
Roscovitine
anti-Fas Ab
(kDa)
Mcl-1
Noxa
Bim EL
Tubulin
50
6
22
50
% PS+ cells
4 47 52 47 47 64 73 31 57 53
Lane
1 2 3 4 5 6 7 8 9 10
K562
B
Con
Act D
MG132
TSA
LBH589
Bay11-7082
2-NVK
(kDa)
Mcl-1
Noxa
Bim EL
Tubulin
50
6
22
50
% PS- cells
2 39 65 39 54 81 32
Lane
1 2 3 4 5 6 7
C
Con
CHX
MG132
LBH589
2-NVK
Cyclosporin
Roscovitine
Nutlin-3a
Prednisolone
Patient #1
Mcl-1
Noxa
Bim EL
Tubulin
% PS+ cells
30 40 85 61 81 90 85 74 28
Lane
1 2 3 4 5 6 7 8 9
kDa
36
6
22
50
Patient #2
% PS+ cells
10 25 48 74 55 72 51 49 45
Lane
10 11 12 13 14 15 16 17 18

Figure 2


A
E6.1
Fold change over control
Con
Act D 2 h
LBH 1 h
LBH/Act D 1 h
LBH 2 h
LBH/Act D 2 h


B
K562
Fold change over control
Act D
Time (h)
250 nM
10 μM
LBH589
MS-275
MC1568


C
Medium
LBH589
MS-275 (μM)
10 25
MC1568
MC1575
MG132
(kDa)
Mcl-1
Ac-H3
Ac-H4
Ac-tubulin
Tubulin
Lane
K562 + z-VAD.fmk


D
E6.1
medium
LBH
z-VAD.fmk
Mcl-1
Tubulin
% PS+ cells
Lane

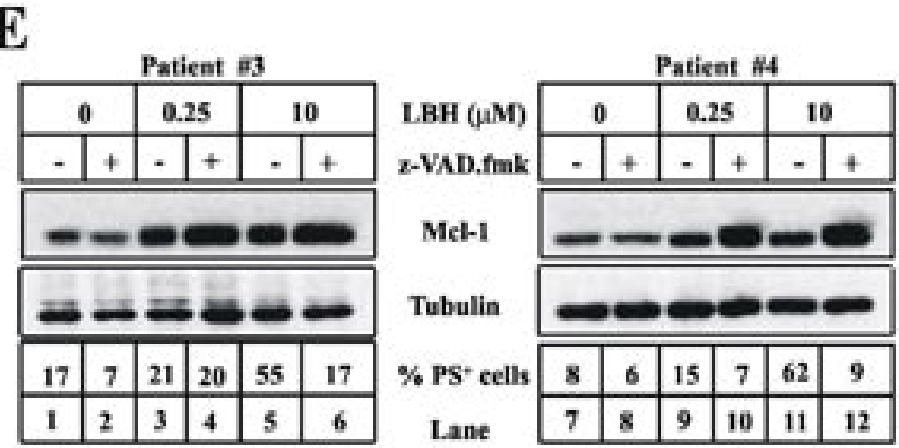
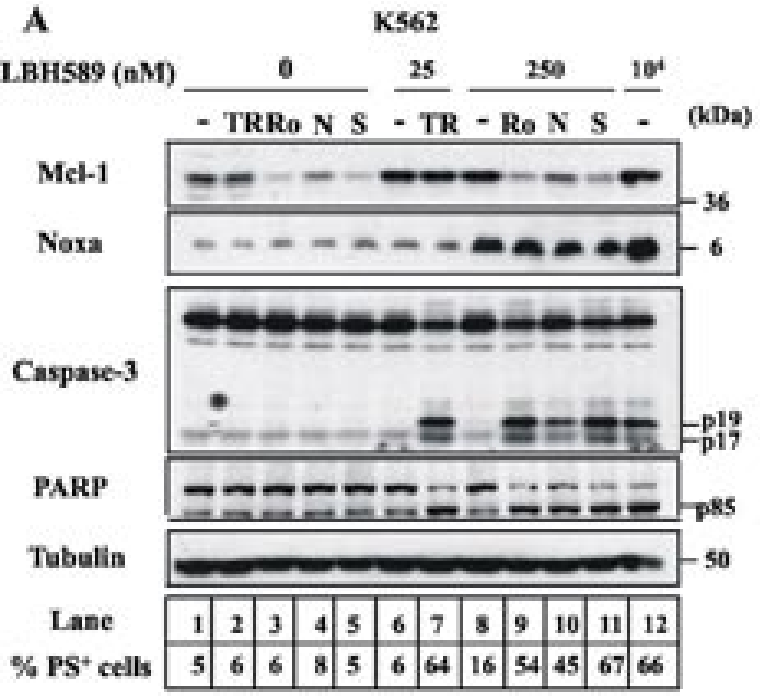
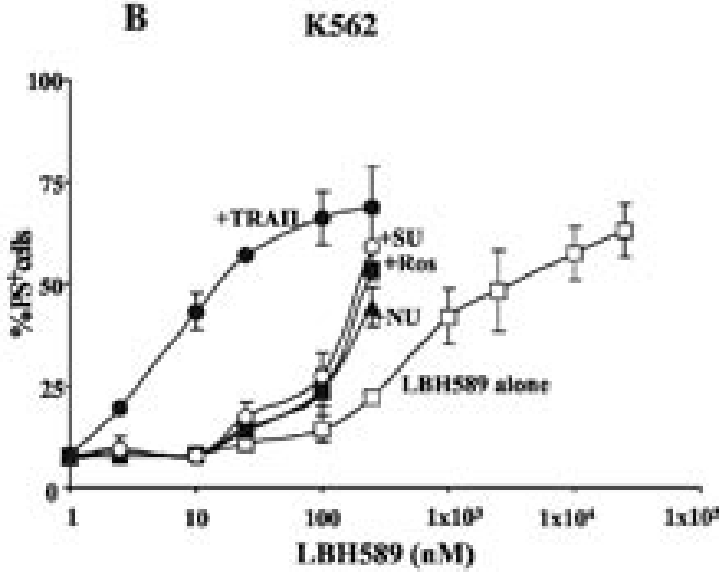
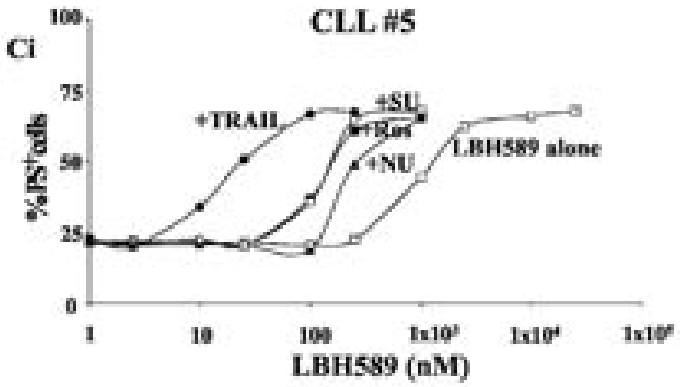
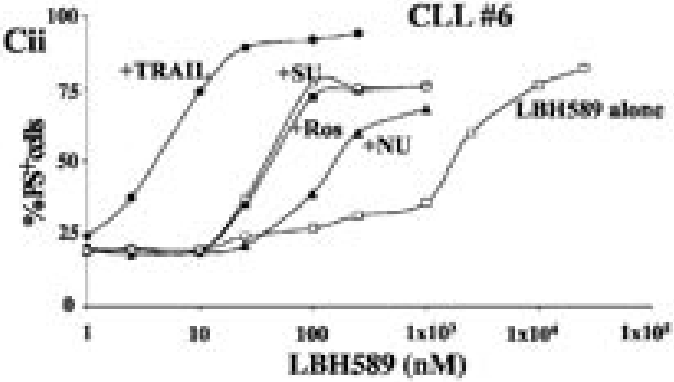

E
Patient #3
Patient #4
LBH (μM)
z-VAD.fmk
Mcl-1
Tubulin
% PS+ cells
Lane

Figure 3


A
K562
LBH589 (nM)
0
25
250
10⁴
- TR Ro N S - TR - Ro N S -
(kDa)
Mcl-1
Noxa
Caspase-3
PARP
Tubulin
36
6
p19
p17
p85
50
Lane
1 2 3 4 5 6 7 8 9 10 11 12
% PS⁺ cells
5 6 6 8 5 6 64 16 54 45 67 66


B
K562
%PS⁺cells
+TRAIL
+SU
+Ros
+NU
LBH589 alone
LBH589 (nM)


Ci
CLL #5
%PS⁺cells
+TRAIL
+SU
+Ros
+NU
LBH589 alone
LBH589 (nM)


Cii
CLL #6
%PS⁺cells
+TRAIL
+SU
+Ros
+NU
LBH589 alone
LBH589 (nM)

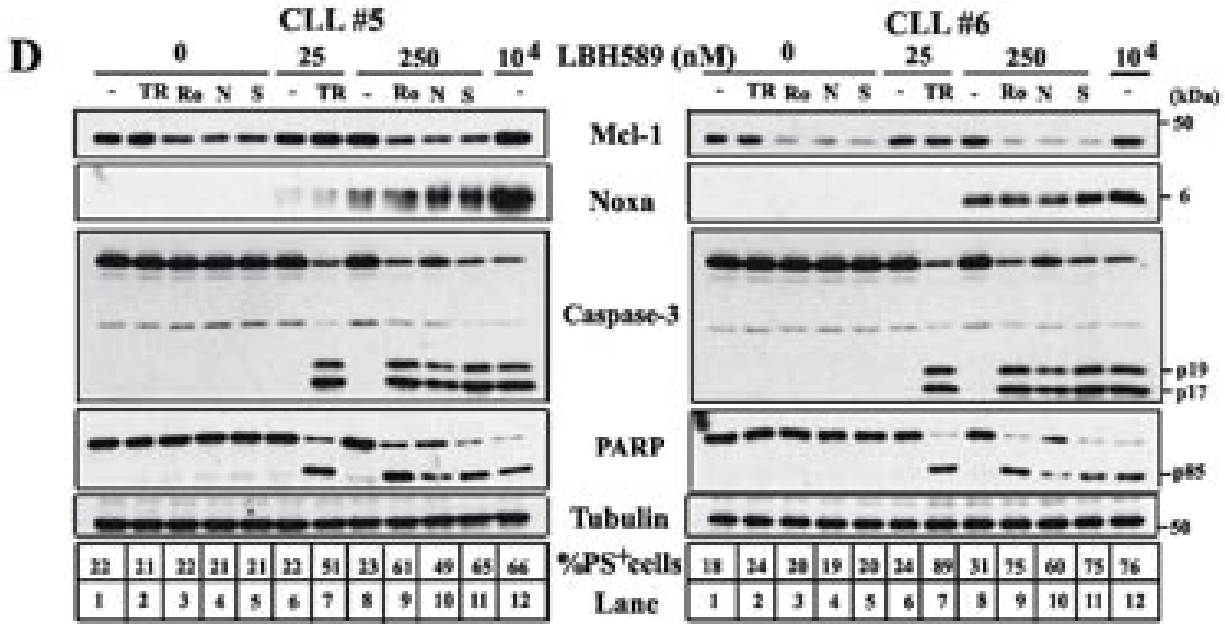
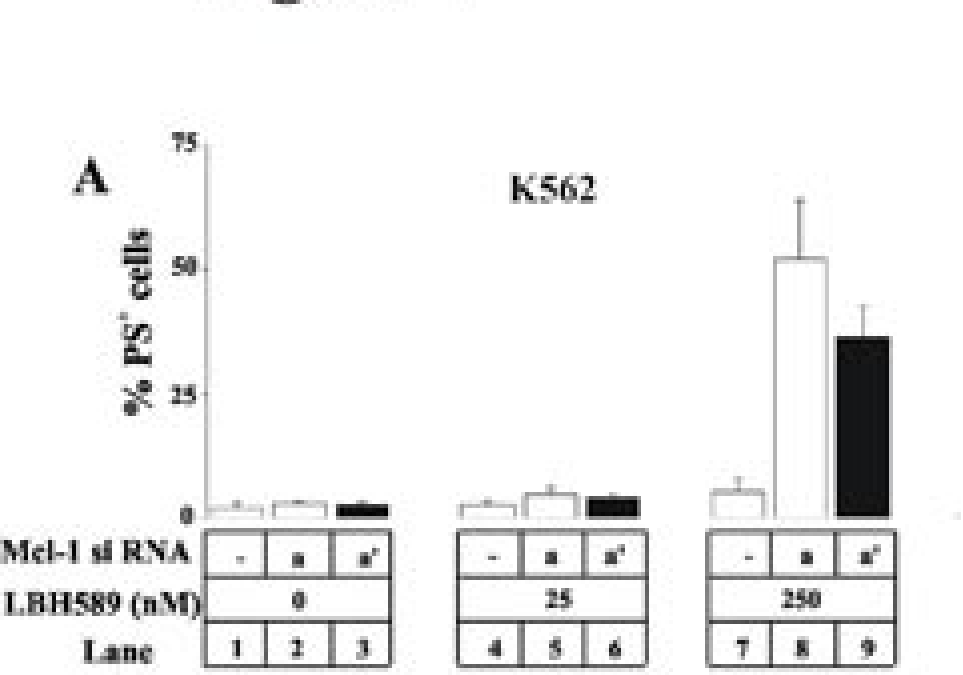
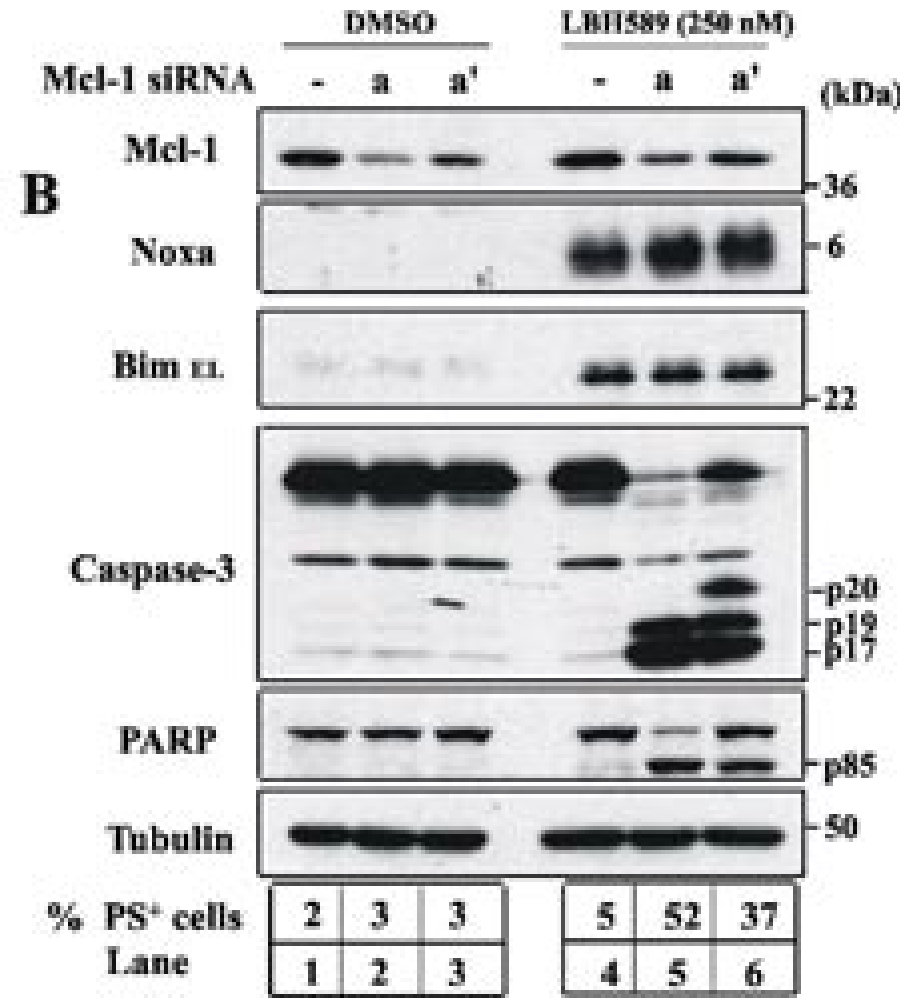

D
CLL #5
CLL #6
LBH589 (nM)
0
25
250
10⁴
- TR Ro N S - TR - Ro N S -
(kDa)
Mcl-1
Noxa
Caspase-3
PARP
Tubulin
50
6
p19
p17
p85
50
%PS⁺cells
22 21 22 28 21 32 51 23 61 49 65 66
18 24 28 19 20 24 89 31 75 60 75 76
Lane
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4


A
K562
% PS+ cells
75
50
25
0
Mcl-1 si RNA
LBH589 (nM)
Lane
-
a
a'
0
25
250
1
2
3
4
5
6
7
8
9


B
DMSO
LBH589 (250 nM)
Mcl-1 siRNA
-
a
a'
(kDa)
Mcl-1
36
Noxa
6
Bim EL
22
Caspase-3
p20
p19
p17
PARP
p85
Tubulin
50
% PS+ cells
2
3
3
5
52
37
Lane
1
2
3
4
5
6