Apoptosis induced by histone deacetylase inhibitors in leukemic cells is mediated by Bim and Noxa.

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Running Title: HDACi-mediated apoptosis by Bim and Noxa

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Abstract

Several histone deacetylase inhibitors (HDACi), which have recently entered early clinical trials, exert their anti-cancer activity in part through the induction of apoptosis although the precise mechanism of this induction is not known. Induction of apoptosis by structurally diverse HDACi in primary cells from patients with chronic lymphocytic leukemia (CLL) and different leukemic cell lines was mediated by the Bcl-2 regulated intrinsic pathway and demonstrated a requirement for *de novo* protein synthesis. A marked time dependent induction of the pro-apoptotic BH3-only proteins, Bim, Noxa and Bmf was observed, which preceded the induction of apoptosis. A key role for both Bim and Noxa was proposed in HDACi-mediated apoptosis based on our findings that siRNA for Bim and Noxa but not Bmf largely prevented the HDACi-induced loss in mitochondrial membrane potential, caspase processing and phosphatidylserine externalization. Noxa, induced by HDACi, in CLL cells and tumor cell lines, bound extensively to Mcl-1, a major anti-apoptotic Bcl-2 family member present in CLL cells. Our data strongly suggests that HDACi induce apoptosis primarily through inactivation of anti-apoptotic Bcl-2 family members by increases in Bim and Noxa and highlights these increases as a potential clinical target for CLL/lymphoma therapy.

Key words: Histone deacetylase inhibitors, CLL, apoptosis, Bim, Noxa, Mcl-1

Introduction

Histone deacetylase inhibitors (HDACi) are a relatively new class of targeted anti-cancer agents that exert anti-tumor activity both *in vivo* and *in vitro* by their ability to induce growth arrest, differentiation and apoptosis.[1-3] The acetylation status of core histones, which is normally regulated by the balance between the opposing activities of histone acetyltransferases and histone deacetylases (HDACs), is frequently altered in many cancers and is further disrupted by HDACi.[1-3] Based on their chemical structure, four types of HDACi have been characterized including short chain fatty acids (valproic acid), hydroxamic acids (suberoylanilide hydroxamic acid [SAHA]), trichostatin A and LBH589), synthetic benzamide derivatives (MS-275) and cyclic tetrapeptides (depsipeptide).[1-3]

Induction of apoptosis occurs by two main pathways: triggering of cell surface death receptors (the extrinsic pathway) or by perturbation of mitochondria (the intrinsic or Bcl-2-family-regulated pathway), with caspase-8 and -9 being the apical caspases, respectively.[4] Members of the Bcl-2 family are critical regulators of apoptosis and comprise pro-survival anti-apoptotic members, such as Bcl-2, Bcl-x_L and Mcl-1, multidomain pro-apoptotic members, such as Bax and Bak, and pro-apoptotic BH3-only proteins, including Bad, Bik, Bim, Bmf, Puma and Noxa.[4, 5] On receipt of a death signal, Bax and Bak can form oligomers in mitochondrial membranes leading to release of cytochrome *c* and caspase activation, whereas anti-apoptotic Bcl-2 members prevent this release by blocking activation of Bax and Bak. BH3-only proteins are critical for cell death initiation, they act upstream of Bax and Bak and their pro-apoptotic activity is tightly controlled by diverse transcriptional and post-translational mechanisms.[4, 6-8] Activation of different BH3-only proteins by multiple stimuli including cytokine withdrawal and many chemotherapeutic agents result in their binding into the hydrophobic groove of pro-survival Bcl-2 family members. Although initially such interactions were considered promiscuous, it has been realized recently that certain BH3-only proteins selectively bind to different functional subclasses of anti-apoptotic Bcl-2 family members.[9, 10] Thus Bim and Puma bind tightly to all pro-survival proteins, Bad binds tightly to Bcl-2, Bcl-X_L and Bcl-w but very weakly to Bfl-1/A1 and not to Mcl-1, whereas Noxa only binds to Bfl-1/A1 and Mcl-1.[7, 9, 10] Thus, pro-survival proteins can be divided into two groups, one comprising Bcl-2, Bcl-X_L and Bcl-w and the other containing Mcl-1 and Bfl-1/A1 and

importantly efficient apoptosis and effective therapy requires neutralisation of both sets of pro-survival proteins.[7, 9, 10]

The precise mechanism whereby HDACi induce apoptosis is not known, although most studies suggest they act by the intrinsic pathway [3, 11, 12] compatible with reported HDACi-mediated increases in Bim and Bmf.[13-17] Furthermore such increases in BH3-only proteins may be important in HDACi-induced apoptosis as early studies demonstrated that HDACi-induced apoptosis was inhibited by cycloheximide.[18, 19] Reactive oxygen species (ROS) may be involved in induction of apoptosis by SAHA and MS-275 but it is unclear if this applies to other HDACi and whether the increase in ROS precedes or is a consequence of apoptosis.[11, 20, 21] Depsipeptide has been proposed to induced apoptosis via the extrinsic pathway in primary cells from patients with chronic lymphocytic leukemia (CLL) and MS-275 induced a late generation of ROS that did not precede the commitment to apoptosis in CLL cells.[21, 22]

Although several HDACi have entered Phase I and II clinical trials, they will likely be of most value in combination with other anti-tumor agents including agents that induce death receptor-induced apoptosis.[2, 3] We have shown that CLL cells, which are inherently resistant to TNF-related apoptosis-inducing ligand (TRAIL),[23] are sensitized by prior treatment with an HDACi that preferentially inhibits HDAC Class I but not Class II [14, 24] and this sensitization occurs almost exclusively through the death receptor TRAIL-R1 but not TRAIL–R2.[25-27]

In this study we further investigate the mechanism whereby HDACi induce apoptosis in various leukemic cell lines and in freshly isolated CLL cells, with particular emphasis on the role of BH3-only proteins. HDACi induce apoptosis by *de novo* protein synthesis of the BH3-only proteins, Noxa, Bim and Bmf and activation of the intrinsic pathway. We report an HDACi-mediated increase in Noxa that is critically important in HDACi-induced apoptosis in leukemic cells.

Materials and Methods

Lymphocyte purification, cell lines and culture. CLL cells, obtained with patient consent and local ethical committee approval, were purified and cultured (4 x 10^6 cells ml^{-1}) as described.[14, 23] Jurkat T cells (clone E6-1), Z138, a human mantle cell lymphoma cell line,[28] and K562, a human myelogenous leukemic cell line, were cultured in RPMI 1640 medium supplemented with 10 % FBS and 5% Glutamax™.[14, 23] K562 cells were from ATCC.

Reagents. Media and serum were from Life Technologies, Inc (Paisley, UK). Antibodies were sourced as follows: rabbit anti-Puma Ab was from Cell Signalling Technology (Beverly, MA), rabbit anti-Bmf (Ct) and anti-acetylated tubulin mAb (clone 6-11B-1) were from Sigma (Poole, UK). A second rabbit anti-Puma Ab (NT) was from ProSci (Poway, CA). The antibody to poly(ADP-ribose) polymerase (PARP) (clone C2-10) was from Alexis Corp. (Nottingham, UK). The rabbit anti-acetylated -H3 and -H4 Abs, rabbit anti-Bak (NT) Ab and rabbit anti-Bax (NT) Ab were from Upstate Biotechnology (Lake Placid, NY). The rabbit anti-p21 (H164) mAb, mouse anti-p53 (clone DO-1) mAb and rabbit anti-Mcl-1 Ab (sc-19) were from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-Bid Ab and mouse anti-Bcl-2 mAb were from BioSource (Camarillo, CA) and DAKO (Cambridge, UK), respectively. The rabbit Bim Ab and mouse anti-Noxa mAb were from Merck Biosciences (Nottingham, UK). The rabbit Bcl-x_L Ab, mouse anti-Bad mAb and mouse anti-Bik mAb were from BD Biosciences (San Diego, CA). LBH589 was kindly provided by Dr. P. Atadja (Novartis Pharmaceuticals Corporation, East Hanover, NJ). SAHA was kindly provided by Dr. R. Schultz (NCI, MD). MS-275 and sodium valproate were from Calbiochem (La Jolla, CA).

Quantification of apoptosis and western blot analysis. Apoptosis was quantified either by loss in mitochondrial membrane potential ($\Delta\Psi m$) or by phosphatidylserine (PS) externalization in the presence of propidium iodide as described.[14, 23] Samples for western blot analysis were prepared and acetylated histones, caspases and cleaved PARP detected as previously described.[14]

Measurement of ROS production. Cells were exposed for 30 min at 37^0C to the nonfluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (20 μM - DCFDA)

(Molecular Probes, Eugene, OR) in phenol red free RPMI medium. After washing the medium, cells were exposed to a series of HDACi for 60 min at 37^0C and oxidation to the highly fluorescent 2′,7′-dichlorofluorescein was measured by flow cytometry using a BD FACSCalibur™ System (Becton Dickinson, Oxford, UK).

Real-time RT-PCR

Total RNA was isolated using the RNeasy kit (Qiagen, West Sussex, UK) and 50 ng reversed-transcribed using standard techniques with Superscript III (Invitrogen, Paiseley, UK). The resulting cDNA was used for the real time amplification reaction with a SYBR green PCR master mix (Applied Biosystems, Warrington, UK). Primers were designed using the Primer Express software v2.0 (Applied Biosystems, Warrington, UK) and the sequences used were as follows;
Noxa forward, 5'-GCTCCAGCAGAGCTGGAAGT-3', reverse, 5'-AAGTTTCTGCCGGAAGTTCAGT-3'; β-actin forward, 5'-CAGCTCACCATGGATGATGATATC-3', reverse, 5'-AAGCCGGCCTTGCACAT-3'. These were used at 900 pmol (forward) and 300 pmol (reverse) in a 25 µl reaction volume and were checked for linear amplification characteristics at the these concentrations across a range of cDNA concentrations prior to use in assay. Reactions were carried out using an ABI PRISM7000 RT-PCR machine. The expression level of the Noxa gene was determined by normalization to β-actin in all samples. Statistical significance of the data was assessed by an unpaired two tailed t-test on log 2 transformed data.

Small RNA Interference (siRNA) of Bim, Bmf, Noxa and Bak. Jurkat cells were electroporated with siRNA oligonucleotides using a Nucleofector system (Amaxa). Cells ($2x10^6$ cells) were suspended in annealed siRNA oligonucleotides (0.25 nmol) in solution V (100 µl) and electroporated using program C16. Cells were transferred to pre-warmed medium (0.9 ml) in 12-well plates for 24 h and then pre-warmed medium (1 ml) was added and cells were exposed to LBH589 for 8 h. The sequences used for RNA interference (RNAi) for Bim and Bmf were validated by previous studies.[15, 29] The sense strand sequences used were as follows:-
Bim a sense, 5′-(AGCAACCUUCUGAUGUAAGtt)-3′;
Bim a' sense, 5′-(GACCGAGAAGGUAGACAAUtt)-3′;

Bmf b sense, 5′-(AAUCGUGUGUGGUGGCAGAtt)-3′;

Bmf b' sense, 5′-(CAAGGUGUCAUGCUGCCUUtt)-3′;

Noxa c and c' oligos were predesigned by Ambion (ID: 5926 and 144356, respectively); Bak and untargeted siRNA oligos were predesigned by Ambion (ID: 120201 and 4635, respectively).

Immunoprecipitation of Mcl-1

After treatment, the cells were washed twice with ice-cold PBS and solubilized in lysis buffer (20 mM Tris-HCl (pH 8), 150 mM NaCl and 1% CHAPS supplemented with a cocktail of protease inhibitors) for 30 min on ice. The lysates were spun at 13,000 g for 20 min at 4^0C. Cell lysates were pre-cleared with Protein G Sepharose beads (Amersham Pharmacia Biotech AP, Uppsala, Sweden) for 1 h at 4^0C. Protein concentration was determined by the Bradford assay (Biorad, Herts, UK). Protein G Sepharose beads (100 µl) were washed 3 times with PBS, 0.05% Tween 20 and then incubated at 4^0C for 1 h using a daisy wheel with 2 µg of mouse IgG or mouse anti-Mcl-1 mAb (BD Biosciences). The labelled beads were washed three times with 0.2 M triethanolamine (pH 8.2) followed by incubation, using a daisy wheel, with cross-linker (0.2 M ethanolamine (pH 8.2), 20 mM dimethyl pimelinediimidate (Fluka Biochemika, Switzerland)) at room temperature for 30 min. The cross-linking reactions were terminated by incubation with 20 mM Tris-HCl (pH 8) for 15 min at room temperature. The cross-linked beads were washed three times with PBS and 0.05% Tween 20 and incubated on a daisy wheel overnight at 4^0C with 0.6 mg of pre-cleared lysate. The samples were eluted by SDS-sample buffer without 2-mercaptoethanol.

Densitometric analysis

Blots were scanned on a Bioimaging Systems machine (Syngene, Cambridge, UK). The density of the bands was measured using the GeneSnap and Gene tool software (Syngene, Cambridge, UK).

Results

HDACi-induced apoptosis requires *de novo* protein synthesis and occurs by activation of the intrinsic pathway

The HDACi, LBH589 and SAHA, induced apoptosis in both K562 and Z138 cells, as assessed by both an increase in cells with phosphatidylserine (PS) externalization and a decreased mitochondrial membrane potential ($\Delta\Psi m$) (Figure 1a lanes 4, 7, 13 and 16). Pre-treatment with the broad spectrum caspase inhibitor, z-VAD.fmk (25 μM), prevented the HDACi-mediated increase in PS externalization but not the loss in $\Delta\Psi m$ (Figure 1a lanes 5, 8, 14 and 17), whereas pre-treatment with cycloheximide blocked both (Figure 1a lanes 6, 9, 15 and 18). Thus these results suggested that HDACi-induced apoptosis required both *de novo* protein synthesis and occurred by activation of the intrinsic pathway as the loss of $\Delta\Psi m$ occurred prior to caspase activation. To test this hypothesis, we examined the processing of caspase-3 and -9 and the cleavage of the caspase-3/-7 substrate, poly(ADP-ribose) polymerase (PARP). LBH589 induced a time dependent loss of $\Delta\Psi m$ and increase in PS externalization in both K562 and Z138 cells with $\Delta\Psi m$ loss clearly preceding increases in PS externalization in K562 cells (Figure 1b lanes 2-7). A time-dependent HDACi-mediated processing of caspase-3 and -9 as well as PARP was observed, which was prevented by cycloheximide (Figure 1b lanes 9 and 15). In contrast z-VAD.fmk (25 μM) was not as efficient as cycloheximide at inhibiting caspase processing, its effects being primarily to significantly inhibit the processing of caspase-3 to its processed and catalytically active p17/19 forms and to almost completely prevent PARP cleavage (Figure 1b lanes 8 and 14). Similar effects were observed with z-VAD.fmk in Jurkat cells (Figure 1b lanes 16-19). Cycloheximide (1 μM) alone induces apoptosis in Jurkat cells so precluding its use in conjunction with LBH589. Taken together our data strongly suggested that *de novo* protein synthesis was required for HDACi-mediated apoptosis and caspase processing and that HDACi-induced apoptosis occurred by the intrinsic pathway.

Generation of reactive oxygen species (ROS) is not a general mechanism for HDACi-mediated apoptosis.

As ROS generation has been implicated in HDACi-induced apoptosis,[11, 20, 30] we wished to investigate if this was a general mechanism. Using hydrogen peroxide as a

positive control, an increase in ROS was observed within 1 h in K562, Z138 and Jurkat E6.1 cells (Supplemental Figure 1a) and this increase was almost totally inhibited by pre-treatment with diphenyleneiodinium chloride (5 μM) (data not shown). Similarly sodium valproate (2.5 mM) and MS-275 (250 μM) induced an increase in ROS within 1 h that was blocked by diphenyleneiodinium chloride, whereas no increase in ROS was observed following incubation for up to 8 h with LBH589 (10 nM - 50 μM) and SAHA (250 nM - 250 μM) (Supplemental Figure 1a and data not shown). These concentrations of SAHA, LBH589 and MS-275 all induce apoptosis in these cell lines (Figure 1 and data not shown). These results do not support a general role for ROS at the early stages of HDACi-induced apoptosis, although they may be important in apoptosis induced by certain specific HDACi in some cell types.

HDACi induce an increase in Bim, Bmf and Noxa

LBH589 induced a time dependent accumulation of both acetylated tubulin and acetylated histones in K562, Z138 and Jurkat cells (Figure 2a) compatible with it being an inhibitor of Class I and II HDACs in agreement with previous observations.[31] LBH589 caused a time dependent increase in p21 expression in all three cell lines, which was p53 independent (K562 cells are p53 null) and was completely prevented by pre-treatment with cycloheximide but not z-VAD.fmk (Figure 2a). HDACi resulted in accumulation of acetylated proteins resulting in *de novo* synthesis of proteins, such as p21, which was completely blocked by cycloheximide. In addition this experiment shows that this concentration of cycloheximide was sufficient to inhibit *de novo* protein synthesis in these cell lines.

Based on previous reports, we suspected that HDACi modulated apoptosis by affecting Bcl-2 family members.[15, 17, 32] Examination of the effects of LBH589 on the expression of multi-domain anti-apoptotic and pro-apoptotic proteins revealed no major alterations in the levels of Bax, Bak or Bcl-2 (Figure 2b). A time dependent decrease in Bcl-X_L was observed being most marked in K562 and Z138 cells (Figure 2b), compatible with HDACi ability to down regulate expression of many genes, including Bcl-2.[1, 2, 33] The effects on Mcl-1 appeared somewhat more complex. A marked loss of Mcl-1 was observed in the presence of cycloheximide in Z138 cells

(Figure 2b lane 15) and to a lesser extent in K562 cells (Figure 2b lanes 6 and 9) compatible with the known short half life of Mcl-1 due to proteasomal degradation.[34] A time-dependent uncharacterized immunoreactive protein of ~32 kDa appeared that was inhibited by cycloheximide but not by z-VAD.fmk (Figure 2b compare lanes 6, 9 and 15 with 5, 8 and 14). In Jurkat cells, a caspase-dependent cleavage product of Mcl-1 of ~ 28 kDa was observed that was inhibited by z-VAD.fmk (Figure 2b lanes 18-19). Although no significant changes were observed in Bad or Bik in the three cell lines, marked changes were noted in Noxa and Bim (Figure 2c). Incubation with LBH589 resulted in a loss of Bid and Puma at later times that was prevented by z-VAD.fmk (Figure 2c compare lanes 7 and 8, 13 and 14 and 18 and 19), raising the possibility that Puma may be a caspase substrate as previously observed for Bid. LBH589 also caused a marked induction of Bmf in Jurkat cells, but not in K562 or Z138 cells, which did not express detectable levels of the protein (Figure 2c). A rapid time-dependent increase in Noxa was observed in all three cell lines after exposure to LBH589 (Figure 2c lanes 2, 11 and 17), which was totally abrogated by cycloheximide (Figure 2c lanes 6, 9 and 15) but not by z-VAD.fmk. Similarly LBH589 induced a time dependent increase in Bim_{EL} that was inhibited by cycloheximide but not z-VAD.fmk (Figure 2c).

To confirm these increases in Noxa and Bim were observed with other HDACi, we examined the effects of MS-275 and SAHA using LBH589 as a positive control. Both MS-275 and SAHA induced a marked increase in Bim_{EL} and Noxa that was inhibited by cycloheximide (Figure 2d). MS-275 and SAHA also caused a decrease in Bcl- X_L in K562 and Z138 cells (Figure 2d). A decrease in Mcl-1 was clearly observed in both K562 and Z138 cells following exposure to cycloheximide for 24 and 16 h, respectively (Figure 2d) consistent with its known short half-life.

HDACi-mediated increases in Noxa and Bim but not Bmf are functionally active

As Bmf was only detected in Jurkat cells, we chose these cells to investigate the functional importance of the increase in Bim, Bmf and Noxa in HDACi-induced apoptosis. Exposure of Jurkat cells to LBH589 again caused an up-regulation of Bim, Bmf and Noxa (Figure 3a compare lanes 1 and 2). We then examined the effects of two siRNAs for Bim, Bmf and Noxa on this HDACi-mediated up-regulation of the BH3-only proteins. Only one siRNA for Bim (a′) and one for Bmf (b′) caused a

specific reduction in Bim or Bmf, respectively without affecting the other BH3-only proteins (Figure 3a lanes 2, 4 and 6). Both siRNAs for Noxa (c and c′) caused a decrease in Noxa without affecting Bim or Bmf (Figure 3a lanes 2, 7 and 8). Combinations of the different siRNAs for Bim, Bmf and Noxa, using only the active siRNAs (a′ and b′), also gave the desired specificity of knockdown of different BH3-only proteins (Figure 3a lanes 9-15). Another siRNA for Noxa (ID: 144355, Ambion) showed similar results (data not shown).

Next we examined the effects of these siRNAs on LBH589-induced apoptosis in Jurkat cells. LBH589 induced apoptosis as assessed by an increase in PS externalization and loss of ΔΨm as well as processing of caspase-9 and -3 to their p37/35 and p19/17 large fragments, respectively (Figure 3b lane 2). Knockdown with the active siRNA for Bim (a′) resulted in a partial protection from LBH589-induced apoptosis, assessed by PS externalization, ΔΨm and processing of caspase-9 and -3, whereas no protection was afforded by transfection of the inactive siRNA for Bim (a) (Figure 3b compare lanes 4 and 3). Neither the active nor inactive siRNA for Bmf afforded any protection against LBH589-induced apoptosis (Figure 3b lanes 5 and 6), whereas both siRNAs for Noxa caused a partial protection (Figure 3b lanes 7 and 8). Significantly the protection afforded by both the siRNAs for Bim and Noxa involved preservation of cells with high ΔΨm and a decrease in PS externalization together with a decreased processing of both caspase-9 and caspase-3 to their large subunits (Figure 3b lanes 4, 7 and 8). These results demonstrated that knockdown of Bim or Noxa conferred a marked protection against LBH589-induced apoptosis and strongly supported the hypothesis that this HDACi-mediated apoptosis resulted from an induction of Bim and Noxa upstream of mitochondria followed by loss of ΔΨm and formation of the Apaf-1 apoptosome and activation of caspase-9 followed by activation of effector caspases. No marked protection was provided by any combination of two individual siRNAs over the effective siRNA alone (Figure 3b lanes 9-13). However, the combination of all three siRNAs may have conferred some added protection (Figure 3b lanes 14-15).

As Jurkat cells have undetectable levels of Bax expression (Figure 2b),[35] we assumed that HDACi-induced apoptosis was mediated by Bak. To test this hypothesis,

HDACi-induced apoptosis was examined in Jurkat cells with decreased Bak. Firstly we confirmed that siRNA for Bak resulted in a marked reduction in cellular Bak (Figure 3c compare lanes 2 and 4) without affecting either endogenous levels or HDACi-induced increased levels of Noxa or Bim_{EL} (Figure 3c lanes 2-4). Knockdown of Bak caused a marked protection against HDACi-induced apoptosis (Figure 3c compare lanes 2 and 4). Similarly knockdown of Noxa, used as a positive control, again abrogated LBH589-induced apoptosis (Figure 3c compare lanes 2 and 3).

HDACi causes a transcriptional up-regulation of Noxa mRNA

As we had observed that the increase in Noxa was functionally important, we wished to ascertain whether the increase in Noxa was due to transcriptional upregulation. Using real time RT-PCR we determined that LBH589 caused a time dependent increase in Noxa mRNA levels which was statistically significant at 2 h (Figure 3d). The HDACi-mediated increase in Noxa mRNA levels was abrogated by the transcriptional inhibitor, Actinomycin D (Figure 3d). Additionally exposure of Jurkat cells to Act D blocked LBH589 mediated increases in protein levels of Noxa (Supplemental Figure 2). Taken together these data demonstrate that LBH589 causes increased Noxa by elevated gene transcription.

Noxa immunoprecipitates with endogenous Mcl-1

In normal cells, Bak is sequestered by Mcl-1 and Bcl-xL.[10, 36] To test the hypothesis that HDACi-induced apoptosis could be due in part to the increase in Noxa binding to Mcl-1 so allowing Bak to exert its pro-apoptotic function, we initially examined whether Noxa was associated with Mcl-1. Endogenous Mcl-1 was immunoprecipitated and examined for Mcl-1, Noxa and Bak. An increased expression of Noxa (5-6 fold) but not Mcl-1 or Bak (Figure 4a and b lanes 1-2) was observed following exposure of Jurkat or CLL cells to LBH589. Although no significant increase in cellular levels of Mcl-1 was observed, the amount of Mcl-1 immunoprecipitated increased (1.6- 2.4-fold) in LBH589 exposed cells (Figure 4a and b lanes 5-6). The more efficient binding to the Mcl-1 antibody may have been due to a conformational change in Mcl-1 after disruption of its binding to its normal partners by the increased Noxa. Exposure of both Jurkat and CLL cells to LBH589 followed by immunoprecipitation with the Mcl-1 mAb resulted in a marked increase in Noxa (Figure 4a and b lanes 5-6). In contrast no major change or possibly only a small

decrease in the amount of Bak immunoprecipitated by the Mcl-1 Ab was observed (Figure 4a and b lanes 5-6). These data demonstrate that the LBH589-induced an increase in Noxa binding to Mcl-1 in both Jurkat and CLL cells.

LBH589 induces BH3-only proteins in CLL cells

Depsipeptide and MS-275 induce apoptosis in CLL cells.[21, 37] We wished to examine the ability of LBH589 to induce apoptosis in CLL cells and to assess the involvement of BH3-only proteins. We have examined the effects of cycloheximide on LBH589-induced apoptosis in cells from 30 different patients, cells from 9 patients were excluded due to high spontaneous apoptosis (≥ 35 %) at 20 h and cells from 9 further patients were excluded due to sensitivity to cycloheximide. LBH589 induced apoptosis in CLL cells from the remaining 12 patients, which was partly but not entirely blocked by co-treatment with cycloheximide (Figure 5a). The cycloheximide concentration could not be increased because of its ability to induce apoptosis. Thus whilst there is a major component of LBH589-induced apoptosis in CLL cells that is protein synthesis dependent, there may also be a component that is protein synthesis independent. Valproate and MS-275 but not LBH589 and SAHA induced the early generation of ROS in CLL cells (Supplemental Figure 1b). No changes were observed in Bax, Bak or Bcl-2 following exposure of CLL cells to LBH589 despite a marked induction of apoptosis (Figure 5b). Bcl-x_L expression in CLL cells is either very low or undetectable (Inoue and Snowden – unpublished data).[38] However, significant processing of caspase-3 to its p19/17 catalytically active large subunit accompanied by cleavage of Mcl-1 was observed in cells from four individuals examined (Figure 5b lanes 5, 12, 19 and 26). z-VAD.fmk (20 μM) almost completely inhibited HDACi-induced apoptosis in cells from all four patients, when assessed by PS externalization but not by loss in $\Delta\Psi m$ (Figure 5b). z-VAD.fmk also markedly inhibited the processing of caspase-3, by blocking the formation of the p19/17 subunits of caspase-3, which was accompanied by the appearance of an ~20 kDa fragment as well as an accumulation of the unprocessed zymogen (Figure 3c lanes 6, 13, 20 and 27). This p20 fragment is most probably catalytically inactive and likely arises from the covalent binding of z-VAD.fmk to the large subunit of caspase-3 following its initial cleavage by caspase-9 at Asp 175 between the large and small subunits.[39] The effects of cycloheximide on HDACi-induced apoptosis in CLL cells were clearly different

from those of z-VAD.fmk. Cycloheximide protected against HDACi-induced loss of $\Delta\Psi m$ as well as causing a partial protection against the increase in PS externalization and partially blocked caspase-3 processing (Figure 5b).

Exposure to LBH589 resulted in a time-dependent induction of some BH3-only proteins with significant inter-individual variation observed (Figure 5c). In cells from all 7 patients, a rapid time-dependent induction of Noxa was observed that was inhibited by cycloheximide but not z-VAD.fmk (Figure 5c and Supplemental Figure 3). LBH589 caused a marked time- and protein synthesis-dependent induction of Bim in cells from 3 patients (Figure 5c #1 and 2 and Supplemental Figure 3 #7) with a much more modest increase in cells from 3 other patients (Figure 5c #3 and 4 and Supplemental Figure 3 #5) and no increase in cells from 1 patiens (Supplemental Figure 3 #6). Induction of Bmf was clearly observed in cells from some patients but not others (Figure 5c and Supplemental Figure 3). Little or no induction of Puma or Bid was observed following exposure to LBH589 (Figure 5c). However, a time-dependent loss of both Bid and Puma was observed, which was prevented by z-VAD.fmk (Figure 5c lanes 5, 6, 12, 13, 19, 20, 26 and 27). Taken together with the cell lines (Figure 2c), these data suggest that Puma, as well as Bid, may be a caspase substrate. In summary, although significant inter-individual variation was observed in the HDACi-mediated increases in BH3-only proteins in CLL cells, the most consistent response was a rapid increase in Noxa.

Discussion

Our data strongly support the suggestion that HDACi induce apoptosis primarily by the intrinsic pathway with caspase-9 as the apical caspase in agreement with previous studies.[3, 11, 12] In agreement with previous studies,[20, 21] some HDACi, such as MS-275 and valproate, caused ROS generation. However this ROS generation did not seem to play a general role in the early stages of HDACi-induced apoptosis in various leukemic cell lines and CLL cells (Supplemental Figure 1). Rather, our data highlight a requirement for *de novo* protein synthesis of BH3-only proteins in HDACi-mediated apoptosis. Recent studies have also reported that HDACi-induced apoptosis may be mediated by increases in BH3-only proteins particularly Bim and Bmf, although this may be cell type dependent.[14-17, 32, 40] In addition to HDACi-mediated increases in Bim and Bmf, we demonstrate a p53-independent HDACi-mediated increase in Noxa that precedes the induction of other BH3-only proteins.

HDACi-induction of Noxa has not been previously characterized. The mechanism whereby HDACi increase Noxa expression is beyond the scope of the current manuscript, although some of the increased expression is clearly due to an up-regulation of mRNA for Noxa (Figure 3d). Although some studies in melanoma cells have noted that HDACi do not induce Noxa expression [41], the increase in Noxa observed in haemopoietic cells in the present study may not be entirely surprising as Noxa shows an elevated expression in the haemopoietic system particularly in B cells.[42] In addition, it has been recently shown that CLL cells express high levels of Noxa mRNA.[43] Although Noxa was initially reported as a p53 responsive gene,[44, 45] more recent work has demonstrated a p53-independent induction of Noxa involving either E2F1 or p73.[46, 47] However, our preliminary studies suggest that HDACi-induced increase in Noxa in some cell lines is independent of E2F1 or p73 (Supplemental Figure 4). Proteasome inhibitors can also induce Noxa in a p53-independent manner in melanoma, multiple myeloma and mantle cell lymphoma cells and blocking this induction by antisense oligonucleotides or RNA interference protects against apoptosis.[48-50] Interestingly in these studies, Noxa was selectively increased in melanoma compared to normal melanocytes suggesting that upregulation of Noxa may be valuable therapeutic strategy for some malignancies.[48, 50] We demonstrate a rapid and extensive HDACi-mediated induction of Noxa in CLL and

other leukemic cells (Figures 2 and 5), that is functionally important (Figure 3). Although HDACi-mediated induction of Bim and Bmf was also observed, the most consistent change was the increase in Noxa observed in all 7 patient samples analyzed as well as in the different cell lines.

Unlike other BH3-only proteins, Noxa interacts and inactivates almost exclusively with Mcl-1 and to a lesser extent with Bfl-1/A1 but not with other anti-apoptotic Bcl-2 family members including Bcl-2, Bcl-X_L and Bcl-w.[7-10] In this regard, expression of Bcl-x_L and Bcl-w was below detectable levels in CLL cells (Inoue and Snowden–unpublished data).[38] HDACi-mediated up-regulation of Noxa was associated with endogenous Mcl-1 in both CLL and Jurkat cells (Figure 4). In some cells, the interaction of Noxa and Mcl-1 results in displacement of Bak from Mcl-1 followed by rapid proteasomal degradation of Mcl-1.[10] Mcl-1 may be an important regulator of apoptosis in response to changes in the cellular environment and is rapidly down-regulated in response to apoptotic stimuli, such as DNA damage.[10, 34, 36] In the present study, extensive HDACi-induced apoptosis can clearly occur in the absence of marked loss of Mcl-1 (Figures 2 and 5). Thus rapid loss of Mcl-1 accompanying apoptosis is both cell type and stimulus dependent and is not an absolute requirement for HDACi-induced apoptosis mediated by increased Noxa and Bim. Another possible consequence of the Noxa/Mcl-1 interaction could be the release of Bak with a consequent loss of $\Delta\Psi m$ and induction of apoptosis.[10] Somewhat surprisingly the increased Noxa binding resulted in only a small decrease if any in the amount of Bak immunoprecipitated by the Mcl-1 Ab (Figure 4). Our results raise the possibility that Noxa, in addition to its primary mode of inducing apoptosis through release of Bak from Mcl-1, may also exert additional effects, such as a direct activation of the mitochondrial permeability transition pore.[51] However such an effect of Noxa would still need to act in concert with Bak, as Bak knockdown resulted in marked protection (Figure 3c) confirming a key role for Bak in HDACi-induced apoptosis, at least in Jurkat cells that express little or no Bax (Figure 2b).[35] Furthermore Bak knockdown did not affect HDACi-mediated induction of either Bim or Noxa (Figure 3c) compatible with the hypothesis that BH3-only proteins act upstream of Bak and Bax.[4, 7]

Based on our findings, it is interesting to speculate in which paradigms the HDACi-mediated increases in Noxa may be significant. Increased Noxa neutralizes Mcl-1 so inactivating the major member of one group of anti-apoptotic Bcl-2 family members. Firstly, increased Noxa may be important in cells where the HDACi also causes a concomitant decrease in members of the other anti-apoptotic Bcl-2 pro-survival group, such as Bcl-x_L, Bcl-2, or Bcl-w. Such HDACi-mediated decreases in Bcl-x_L and Bcl-2 were observed in this (Figure 2) and other studies, respectively.[33] Secondly, the Noxa increase may be important in cells, such as Jurkat, that are Bax negative and Bak positive, as well as in tumors with deletions or mutations in Bax.[52] Thirdly, Noxa increases may be important in tumors such as CLL, where Mcl-1 is proposed to be an important anti-apoptotic protein.[53] In this regard, a very recent study showed that a decreased Noxa/Mcl-1 ratio in lymph nodes compared to peripheral CLL cells correlated with increased survival.[38] Additionally HDACi may decrease the elevated levels of Bcl-xL found in lymph nodes,[38] so facilitating the induction of apoptosis in lymph nodes. Furthermore, Noxa levels in CLL and mantle cell lymphoma cells can also be increased by proteasome inhibitors leading to the proposal that the Noxa/Mcl-1 ratio may be a key determinant of cell death in these diseases.[38, 49] Taken together with our present findings, these data suggest that Noxa may be an attractive clinical target in the treatment of CLL and possibly other haematological malignancies.

In the present study, we observed a late caspase-dependent loss of both Puma and Bid. Caspase-8-mediated cleavage of Bid is important in amplification of the extrinsic pathway.[54] However, Bid is also cleaved by effector caspases at later times following an apoptotic stimulus. To our knowledge, this is the first description that Puma may be a caspase substrate. The physiological or pathological importance of this cleavage is not known. Although this cleavage occurred late in the apoptotic process, Puma may also be cleaved early in apoptosis by an initiator caspase.

In summary, HDACi induce apoptosis by inducing *de novo* protein synthesis of BH3-only proteins, particularly Noxa and Bim, and consequent activation of the intrinsic pathway. Up-regulation of Noxa by different therapeutic strategies, including HDACi, may provide a novel and valuable approach to target certain malignancies, including CLL.

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Figure Legends

Figure 1. HDACi-mediated induction of apoptosis requires *de novo* protein synthesis.

(a) K562 and Z138 cells were incubated either alone or with LBH589 (LBH 25 μM) or SAHA (25 μM) for 24 and 16 h, respectively either alone or in the presence of the caspase inhibitor, z-VAD.fmk (Vad 25 μM) or cycloheximide (Chx 2.5 μM). Apoptosis was the assessed either by measuring the cells with lower mitochondrial membrane potential ($\Delta\Psi m$) or increase in phosphatidylserine (PS) externalization. Results shown are the Mean ± SD (n=3). **(b)** K562, Z138 and Jurkat cells were incubated with LBH589 (LBH 25 μM) and apoptosis measured as described in (a). The processing of caspase-9 and -3 and the cleavage of PARP was also assessed by western blotting.

Figure 2. HDACi-mediated induction of BH-3-only proteins in various cell lines.

(a-c) K562 and Z138 cells were incubated either alone or with LBH589 (LBH 25 μM) in the presence or absence of z-VAD.fmk (z-VAD 25 μM) or cycloheximide (CHX 2.5 μM) as indicated. Cells were harvested at the indicated times and cell lysates analyzed as follows. **(a)** Inhibition of HDAC activity was assessed by accumulation of acetylated tubulin (Ac-tubulin) and acetylated histone H3 or H4 (Ac-H3 or Ac-H4). *De novo* protein synthesis and its inhibition were assessed by the accumulation of p21 in the presence or absence of cycloheximide. **(b)** Effects on anti-apoptotic and Bax Bak like pro-apoptotic Bcl-2 family members assessed by western blotting. **(c)** Effects on BH3-only proteins analyzed by western blotting. **(d)** K562 and Z138 cells were incubated for 24 or 16 h, respectively, either alone, or with LBH589 (LBH 25 μM), MS-275 (MS 250 μM), or SAHA (25 μM) in the presence or absence of cycloheximide (CHX 2.5 μM) as indicated. Cell lysates were analyzed by western blotting for the indicated proteins. Apoptosis was also assessed by measuring PS externalization.

Figure 3. Knockdown of Noxa and Bim protects cells from HDACi-induced apoptosis.

Jurkat cells were transfected for 24 h with **(a** and **b)** two siRNAs for untargeted sequences (-), Bim (a, a'), Bmf (b, b') or Noxa (c, c') and **(c)** siRNA for untargeted sequences (-), Noxa or Bak. Cells were exposed to LBH589 (LBH) (25 μM) for 8h followed by western blotting with **(a** and **c)** Bcl-2 family proteins as indicated and **(b)** caspase-3 and –9. Apoptosis was also assessed by PS externalization and loss of ΔΨm **(b)**. **(d)** Jurkat cells were exposed to LBH589 (25 μM) for 1 or 2 h either alone or in the presence of Actinomycin D (Act D 5 μg ml^{-1}). Expression of Noxa and β-actin were analyzed by real-time RT-PCR. The results are expressed as the fold change in Noxa expression normalized to the expression of β-actin. The asterisk (*) indicates significantly different from the corresponding control ($p<0.05$).

Figure 4. Immunoprecipitation of Noxa and Mcl-1.

(a) Jurkat and **(b)** CLL cells were exposed to LBH589 (25 μM) for 6 h and 14 h, respectively. Cell lysates were analyzed by western blotting for the indicated proteins. Cell lysates were immunoprecipitated with mouse IgG or mouse anti-Mcl-1 mAb followed by western blotting with the indicated antibody. For detection of Bak in the lysate, a short exposure is shown (lanes 1-2) whereas the longer exposure was necessary to detect Bak in the immunoprecipitated samples. In the lysate samples each lane (lanes 1-2) was loaded with 10 μg or 20 μg protein for Jurkat or CLL cells, respectively. Cells were lysed with 1% CHAPS and solubilized protein (0.15 mg for Jurkat cells and 0.3 mg for CLL cells) was pre-cleared followed by immunoprecipitation (IP) and western blotting (lanes 3-6). Western blots were also analyzed by densitometry as described in Material and Methods and the results expressed as the ratio of Mcl-1, Bak or Noxa in either the lysate or the Mcl-1-immunoprecipitiate from LBH589 compared to control cells (lanes 1-2 and 5-6).

Figure 5. LBH589 induces Noxa and Bim in CLL cells.

(a) CLL cells from twelve patients were cultured for 20 h either alone (Con) or with cycloheximide (CHX 2.5 μM), LBH589 (LBH 25 μM) alone or in combination (LBH/CHX). Apoptosis was assessed either by measuring cells with lower mitochondrial membrane potential (ΔΨm) or increase in phosphatidylserine (PS) externalization. Each line represents the data from one individual patient. The mean values represent the means of the 12 patients. **(b** and **c)** CLL cells from four

individuals were incubated for either 5 or 20 h either alone or in the presence of LBH589 (LBH 25 μM), z-VAD.fmk (z-VAD 200 μM) or cycloheximide (CHX 2.5 μM) and cell lysates were analyzed by western blotting for the indicated proteins. Apoptosis was assessed by ΔΨm or PS externalization. Two Puma Abs were used in order to confirm the specificity and help distinguish Puma α and β.

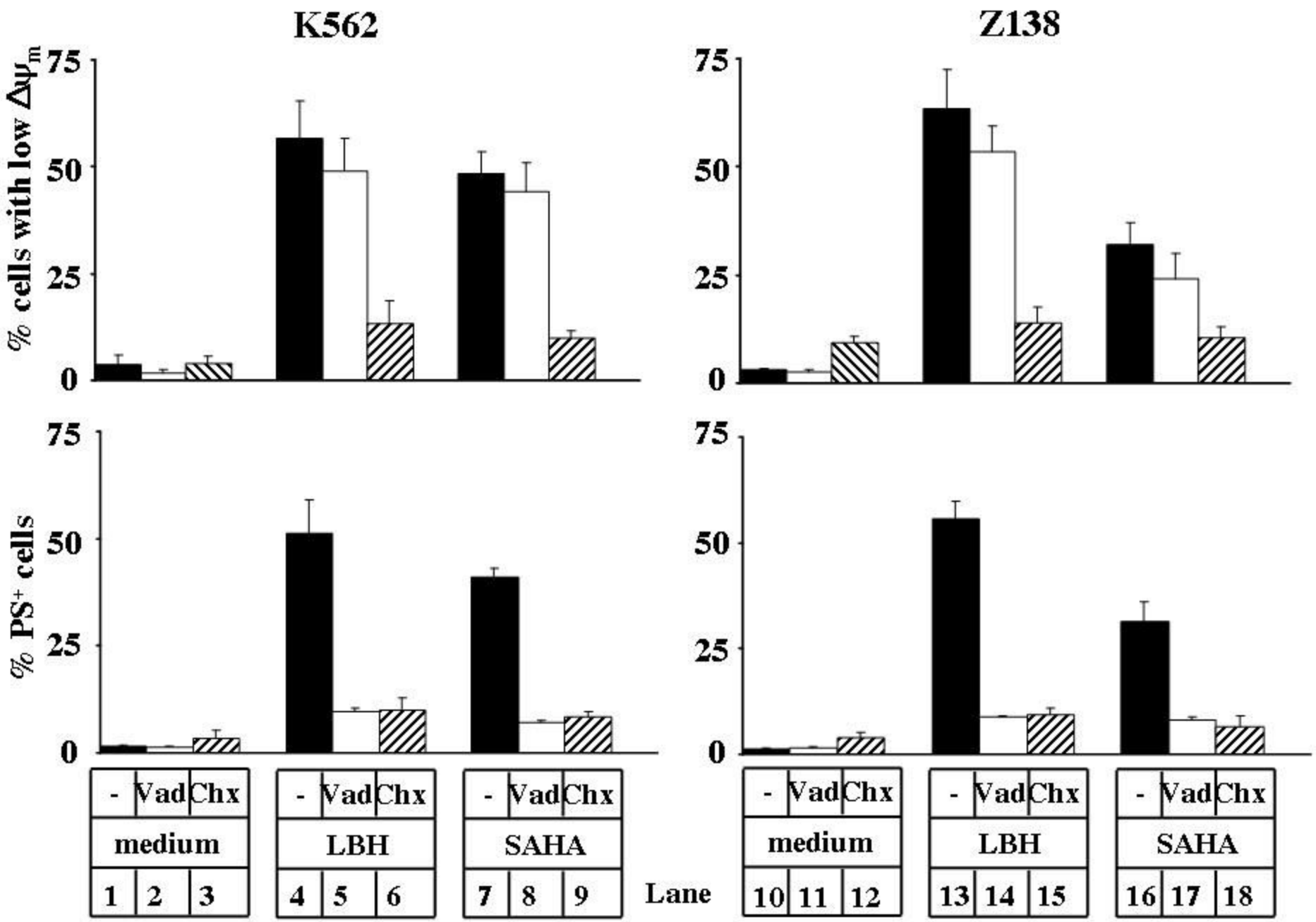

K562
Z138
% cells with low $\Delta\psi_m$
% PS+ cells
75
50
25
0
- Vad Chx
medium
LBH
SAHA
1 2 3
4 5 6
7 8 9
Lane
10 11 12
13 14 15
16 17 18

Figure 1a

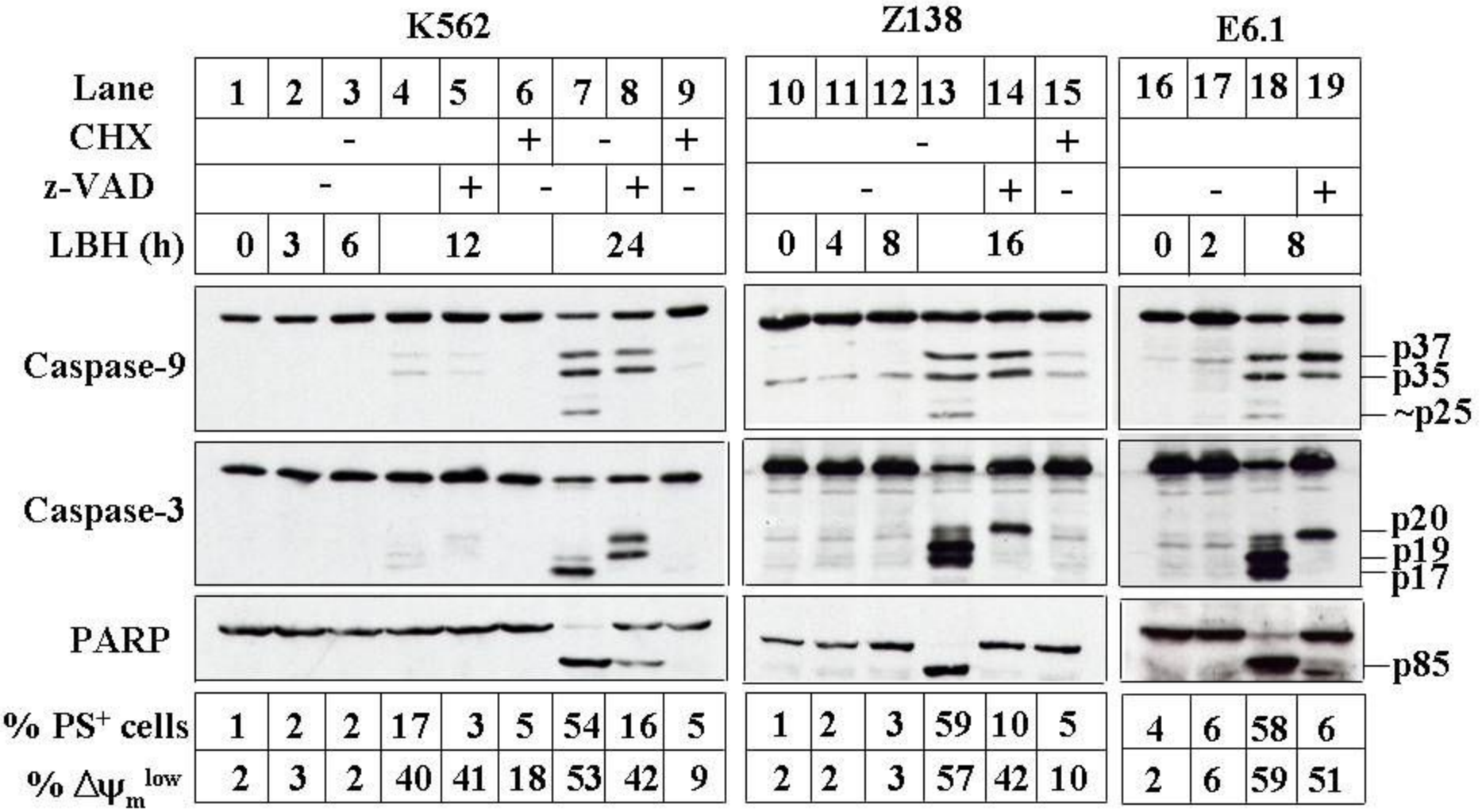
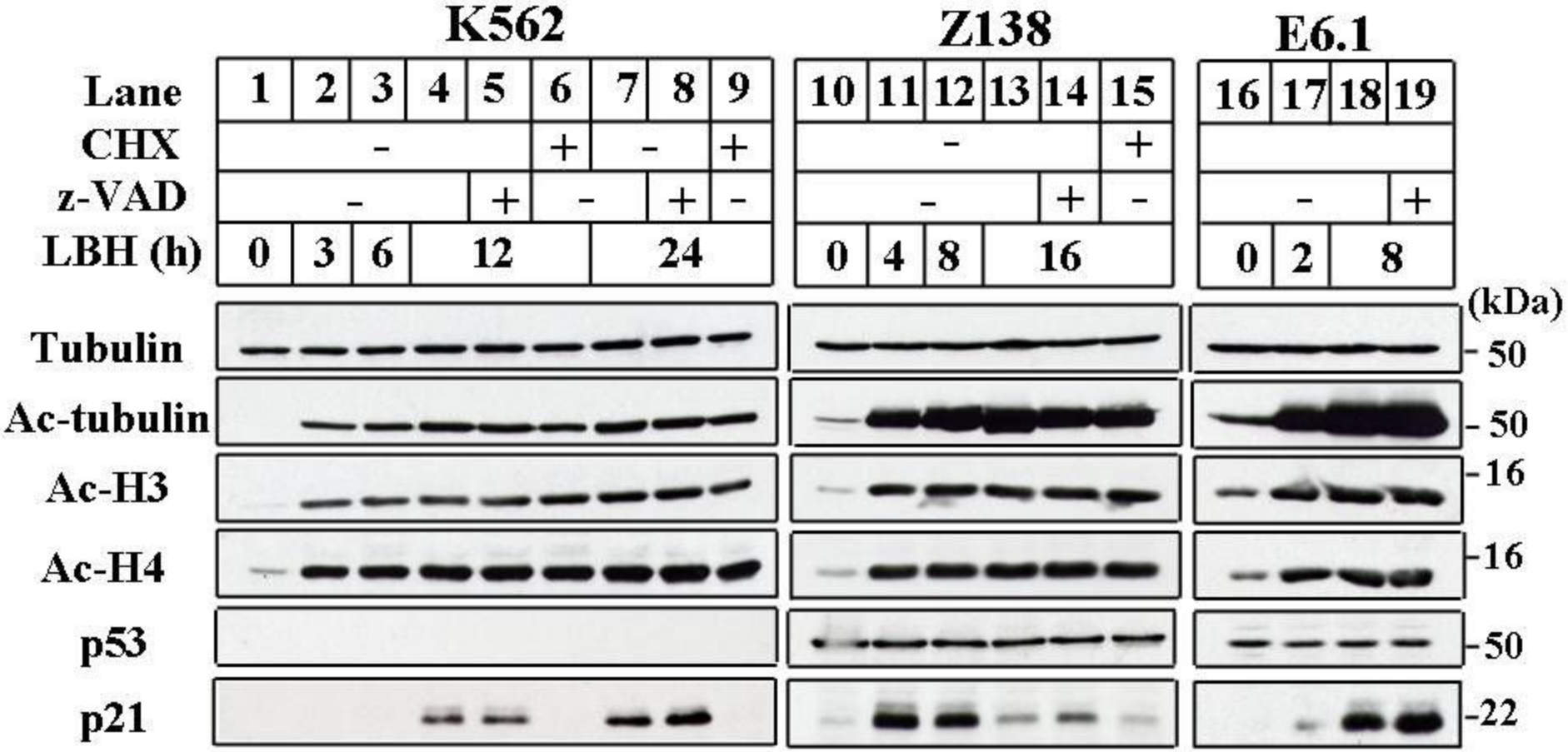

K562
Z138
E6.1
Lane
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15
16 17 18 19
CHX
- + - +
- +
z-VAD
- + - + -
- + -
- +
LBH (h)
0 3 6 12 24
0 4 8 16
0 2 8
Caspase-9
p37
p35
~p25
Caspase-3
p20
p19
p17
PARP
p85
% PS+ cells
1 2 2 17 3 5 54 16 5
1 2 3 59 10 5
4 6 58 6
% Δψm low
2 3 2 40 41 18 53 42 9
2 2 3 57 42 10
2 6 59 51

Figure 1b


K562
Z138
E6.1
Lane
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10 11 12 13 14 15
16 17 18 19
CHX
- - + - +
- +
z-VAD
- + - + -
- + -
- +
LBH (h)
0 3 6 12 24
0 4 8 16
0 2 8
(kDa)
Tubulin
Ac-tubulin
Ac-H3
Ac-H4
p53
p21
50
50
16
16
50
22

Figure 2a

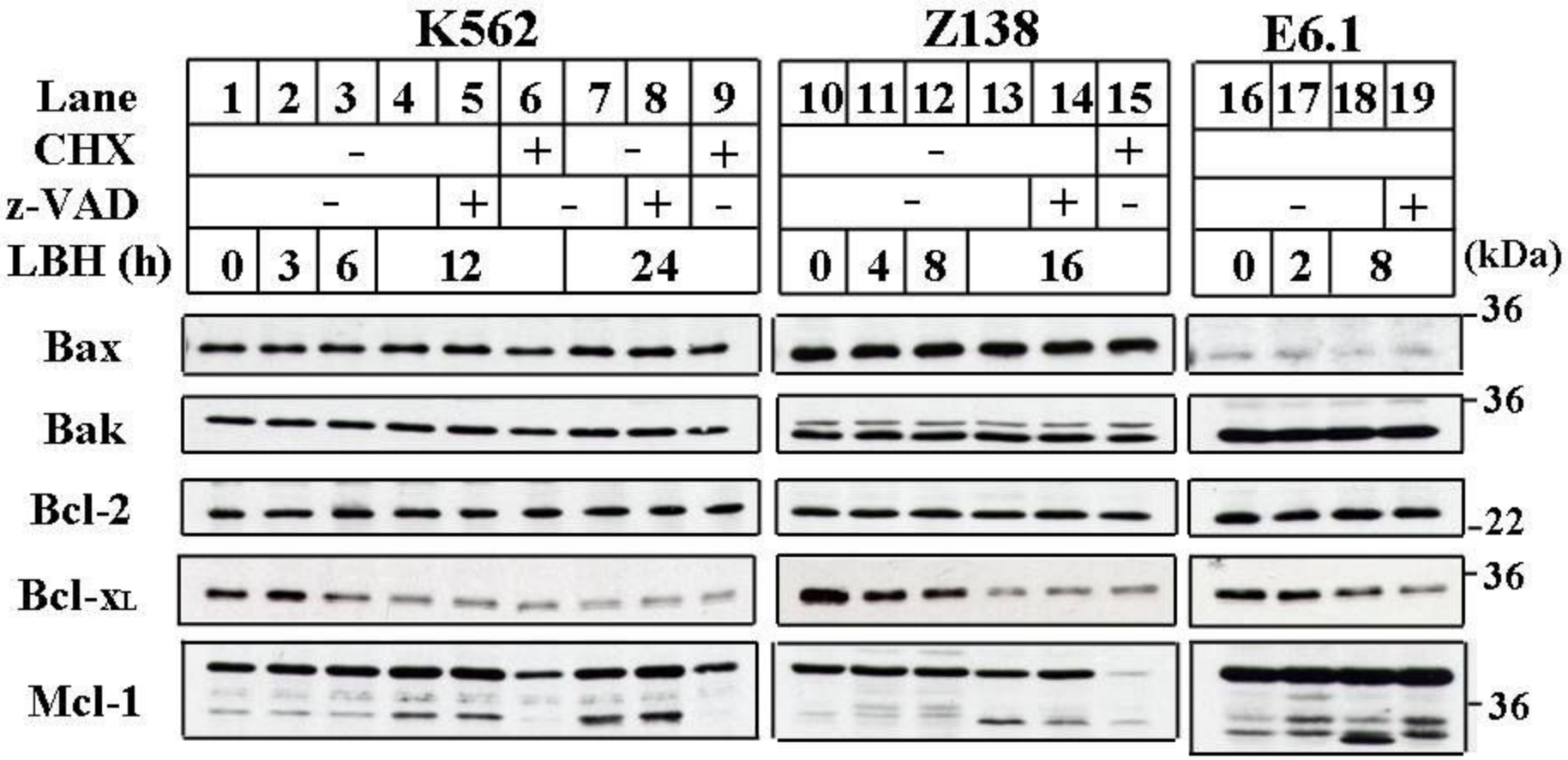
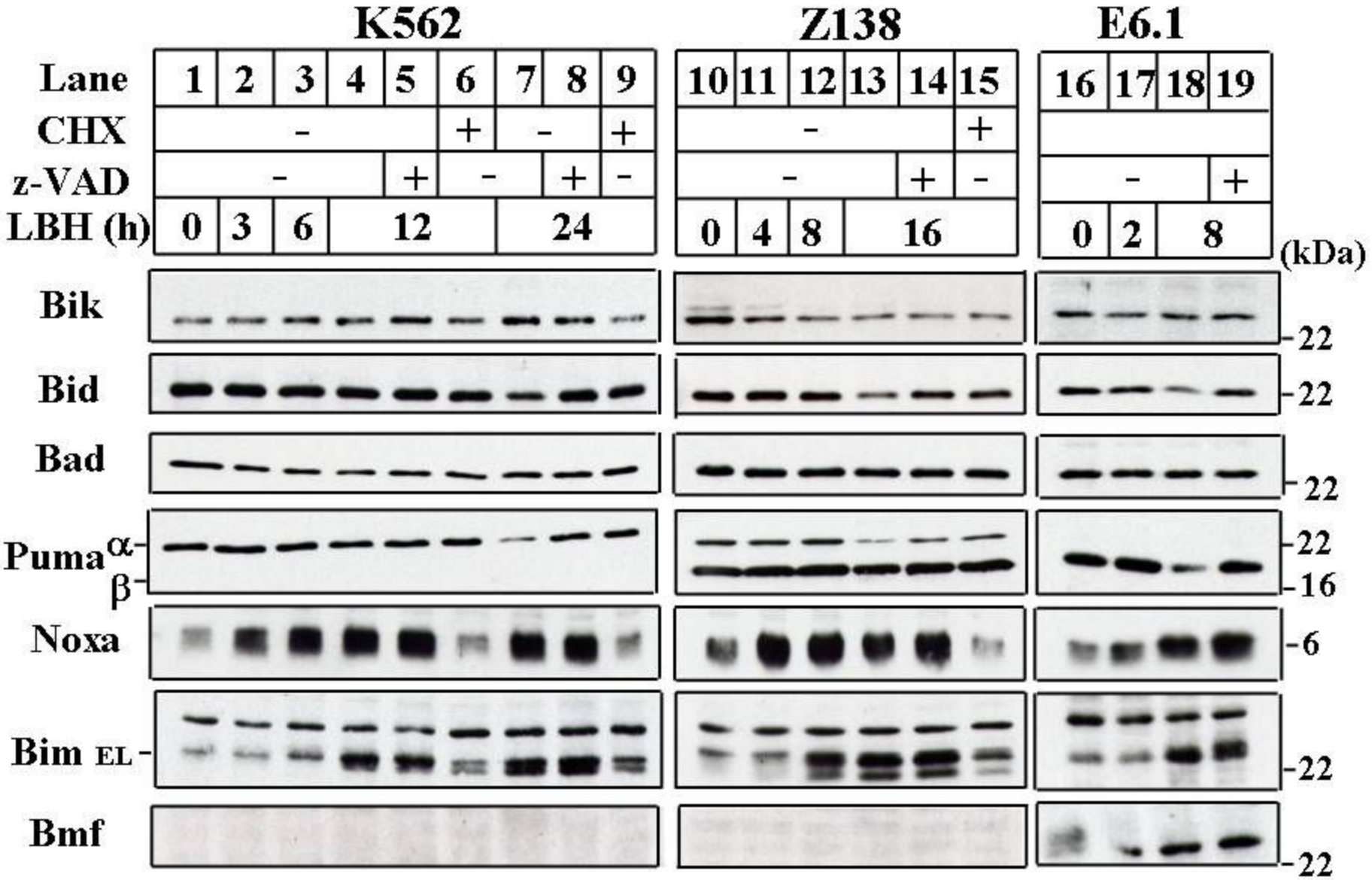

K562
Z138
E6.1
Lane
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15
16 17 18 19
CHX
- + - + - +
z-VAD
- + - + - +
LBH (h)
0 3 6 12 24
0 4 8 16
0 2 8
(kDa)
Bax
Bak
Bcl-2
Bcl-xL
Mcl-1
36
36
22
36
36

Figure 2b


K562
Z138
E6.1
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1 2 3 4 5 6 7 8 9
10 11 12 13 14 15
16 17 18 19
CHX
- - + - +
- +
z-VAD
- + - + -
- + -
LBH (h)
0 3 6 12 24
0 4 8 16
0 2 8
(kDa)
Bik
Bid
Bad
Puma α
β
Noxa
Bim EL
Bmf
22
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22
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6
22
22

Figure 2c

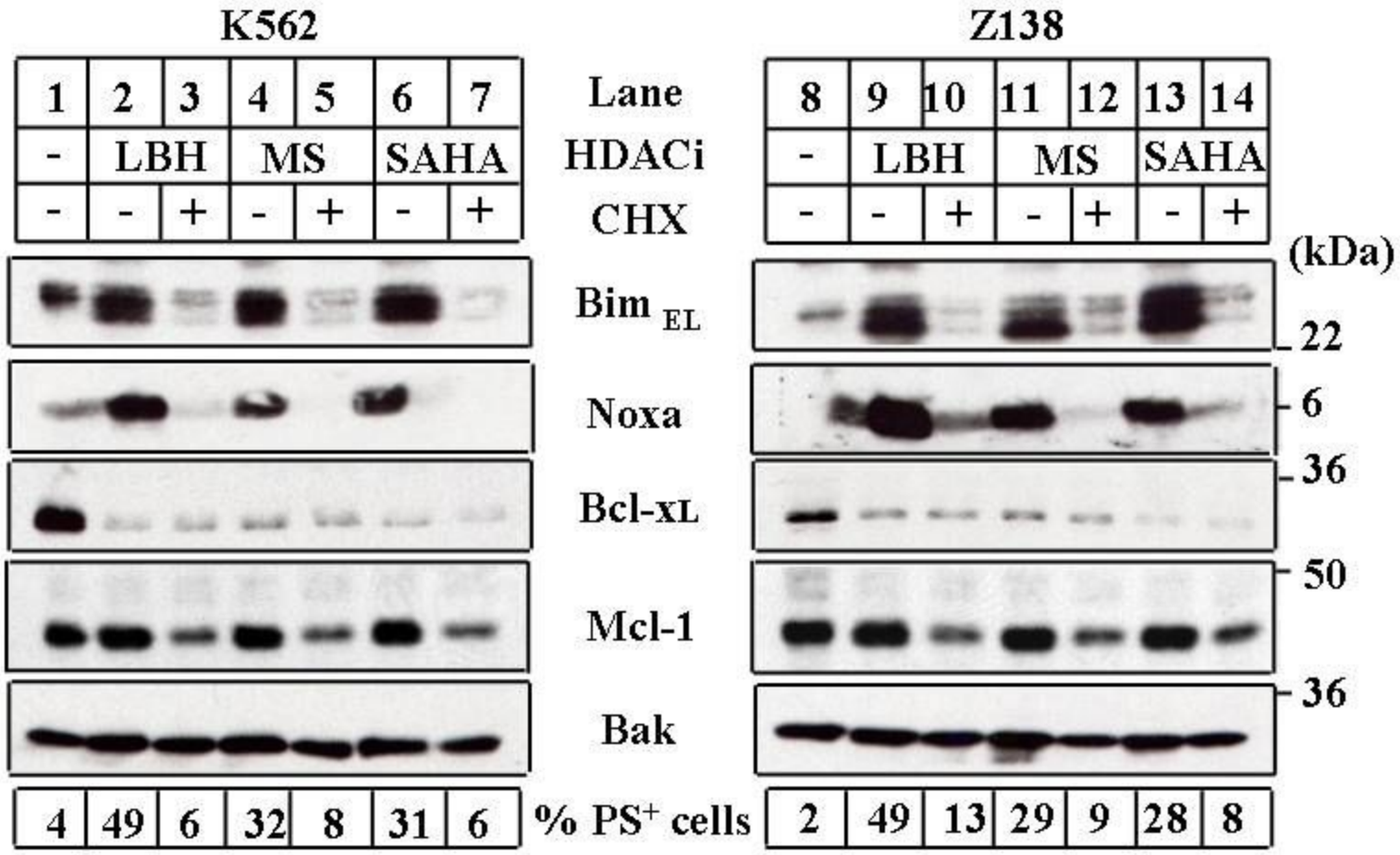

K562
Z138
1 2 3 4 5 6 7
Lane
8 9 10 11 12 13 14
- LBH MS SAHA
HDACi
- LBH MS SAHA
- - + - + - +
CHX
- - + - + - +
(kDa)
Bim EL
22
Noxa
6
Bcl-xL
36
Mcl-1
50
Bak
36
4 49 6 32 8 31 6
% PS+ cells
2 49 13 29 9 28 8

Figure 2d

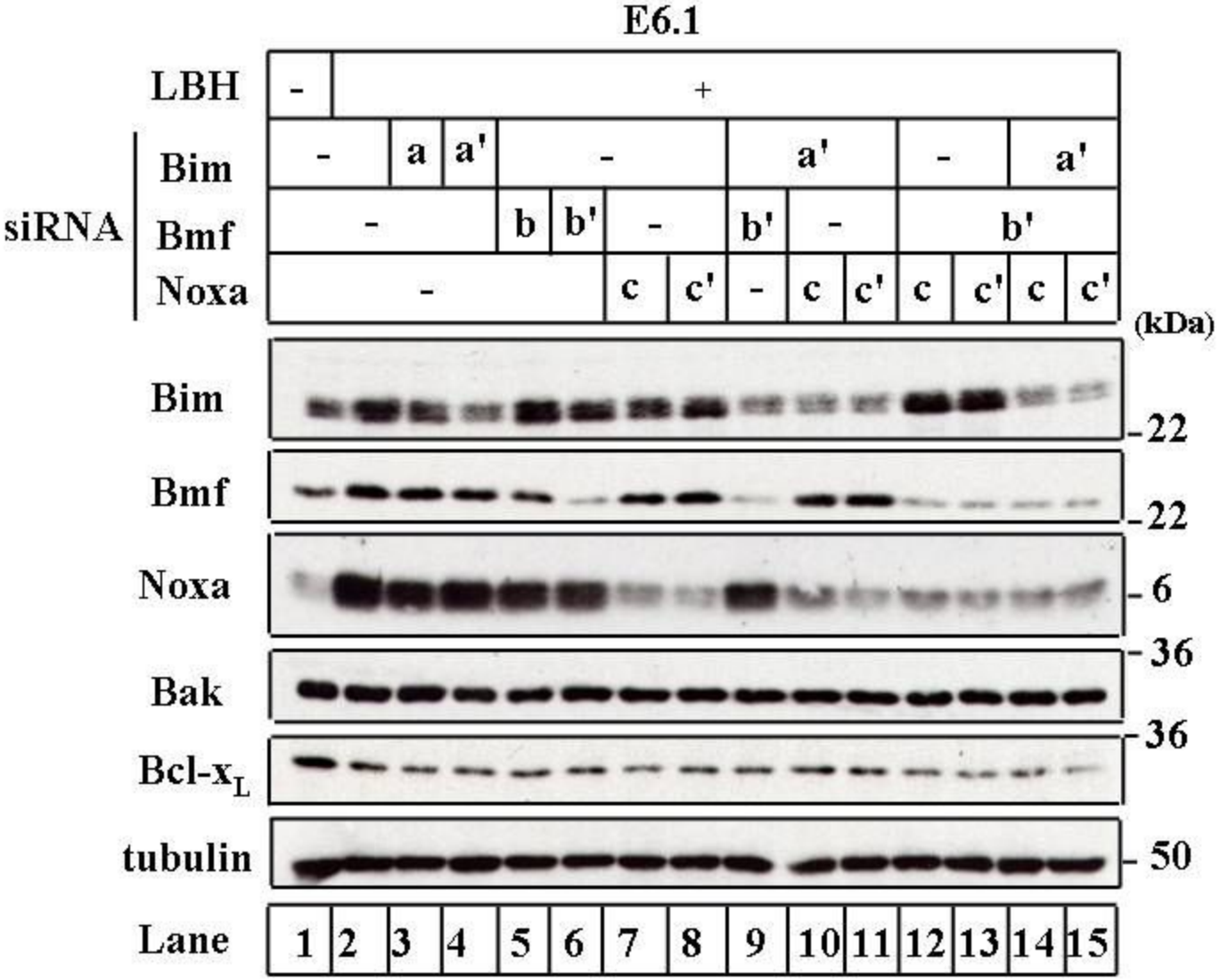
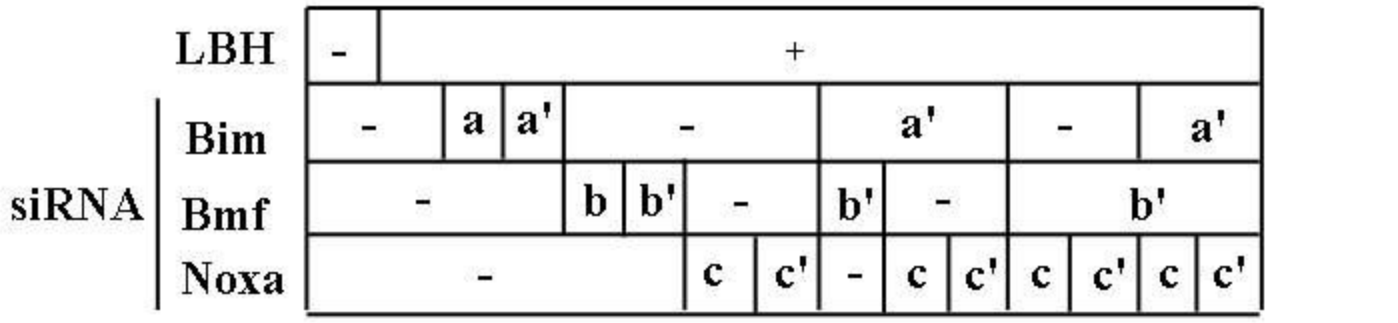
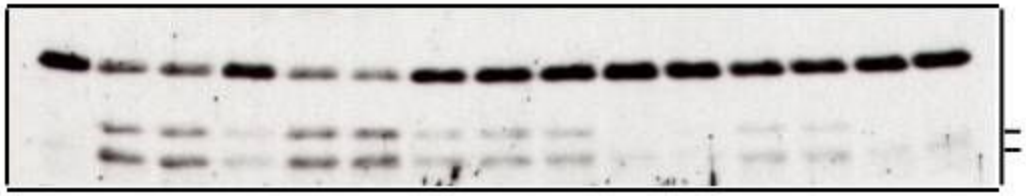
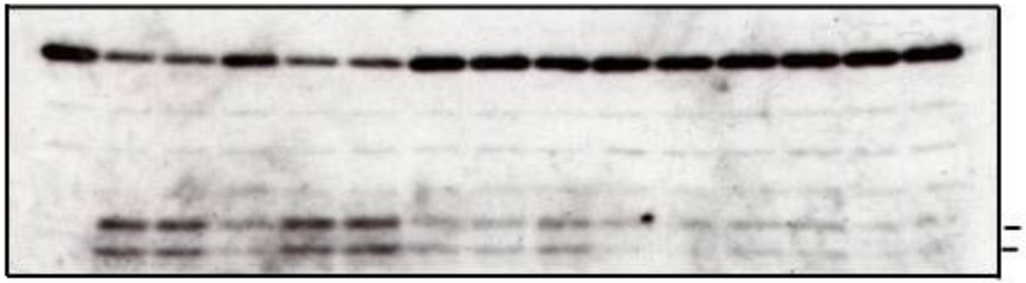

E6.1
LBH
siRNA
Bim
Bmf
Noxa
(kDa)
Bim
Bmf
Noxa
Bak
Bcl-x$_L$
tubulin
22
22
6
36
36
50
Lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 3a

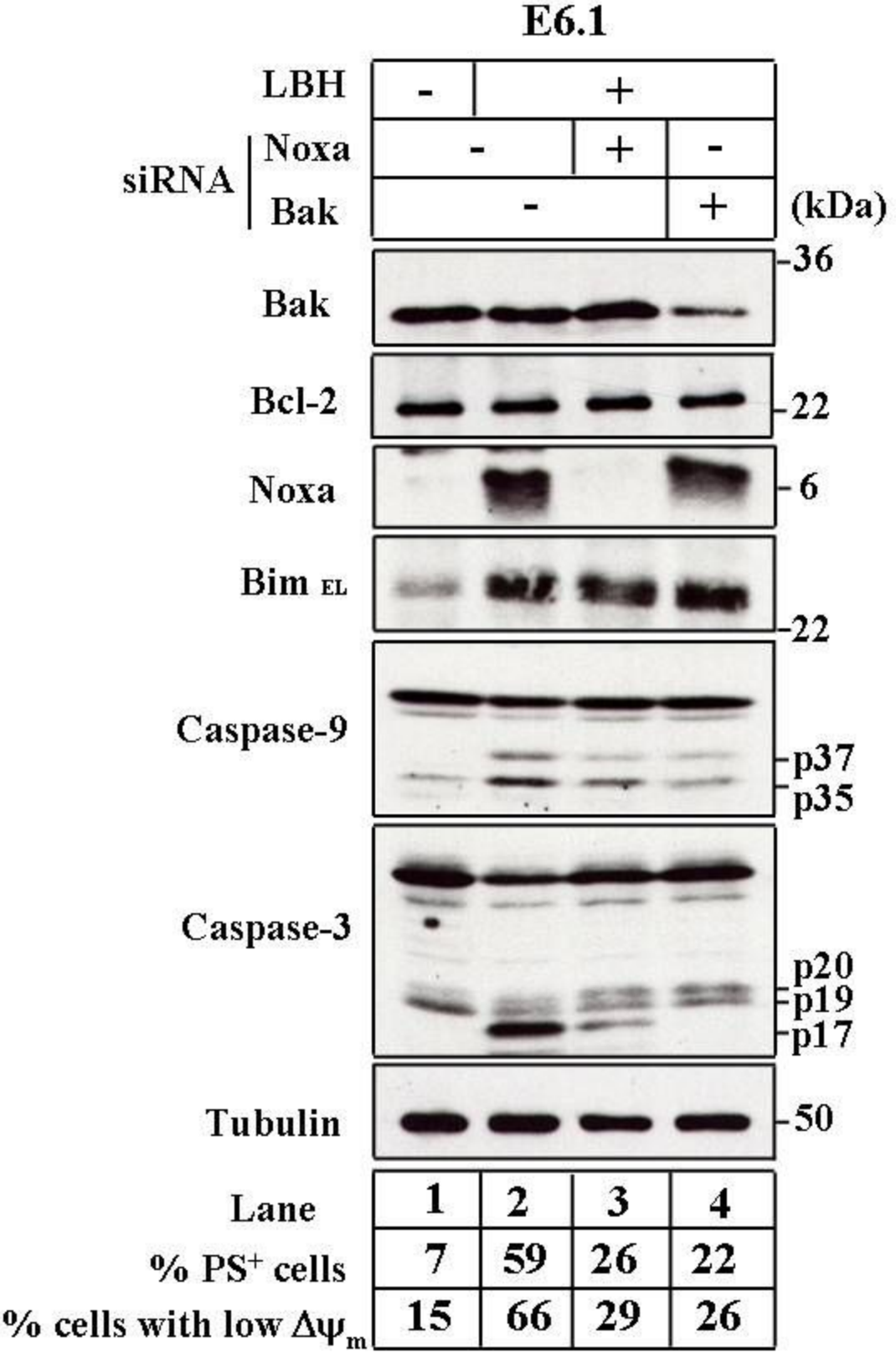






Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
%PS$^+$ cells	8	58	51	31	56	55	25	28	34	24	25	19	20	15	14
%cells with low $\Delta\psi_m$	14	61	63	49	60	60	34	29	44	35	25	34	40	23	24

Figure 3b



Lane	1	2	3	4
% PS^+ cells	7	59	26	22
% cells with low $\Delta\psi_m$	15	66	29	26

Figure 3c

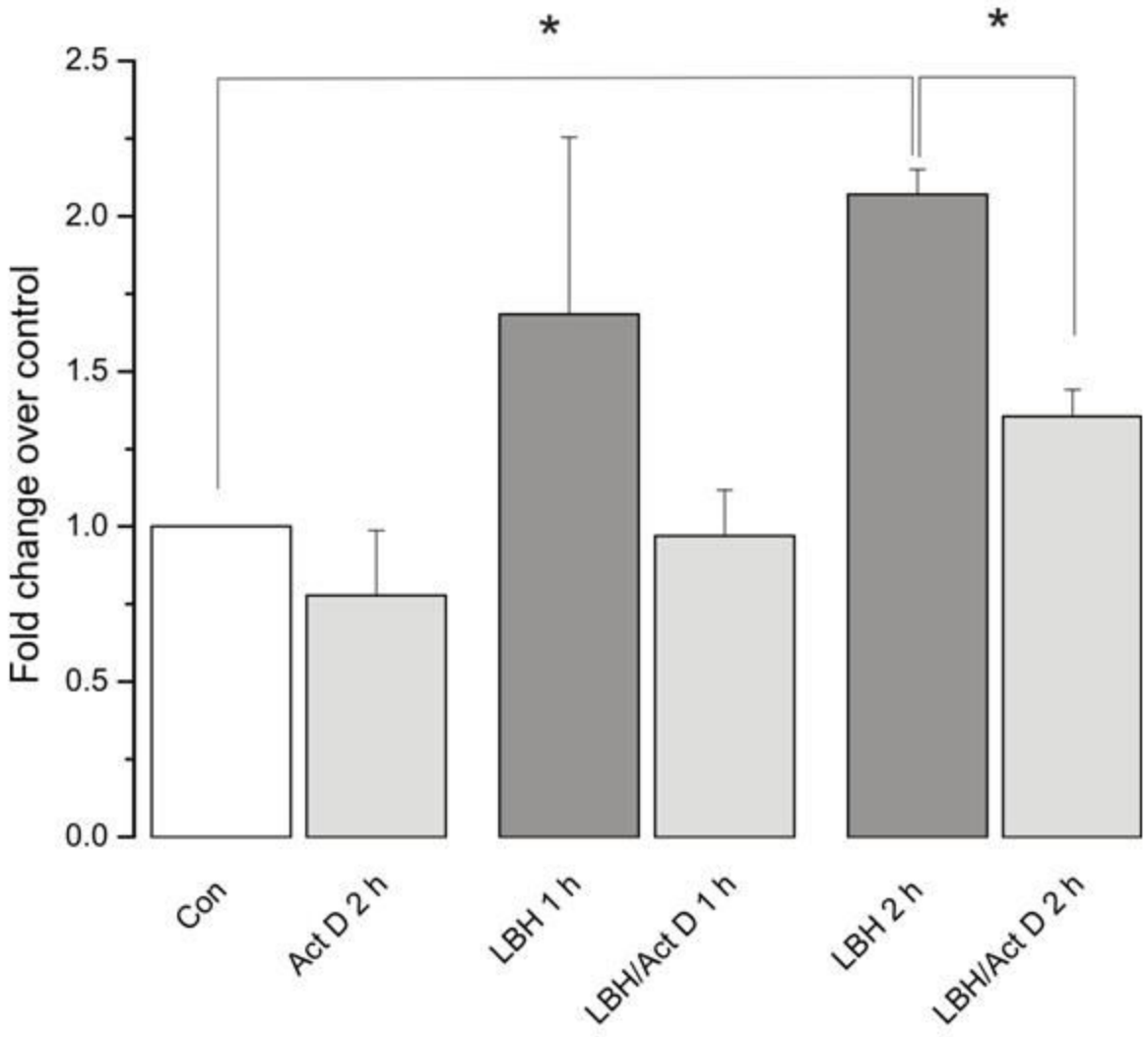

*
*
2.5
2.0
1.5
1.0
0.5
0.0
Fold change over control
Con
Act D 2 h
LBH 1 h
LBH/Act D 1 h
LBH 2 h
LBH/Act D 2 h

Figure 3d

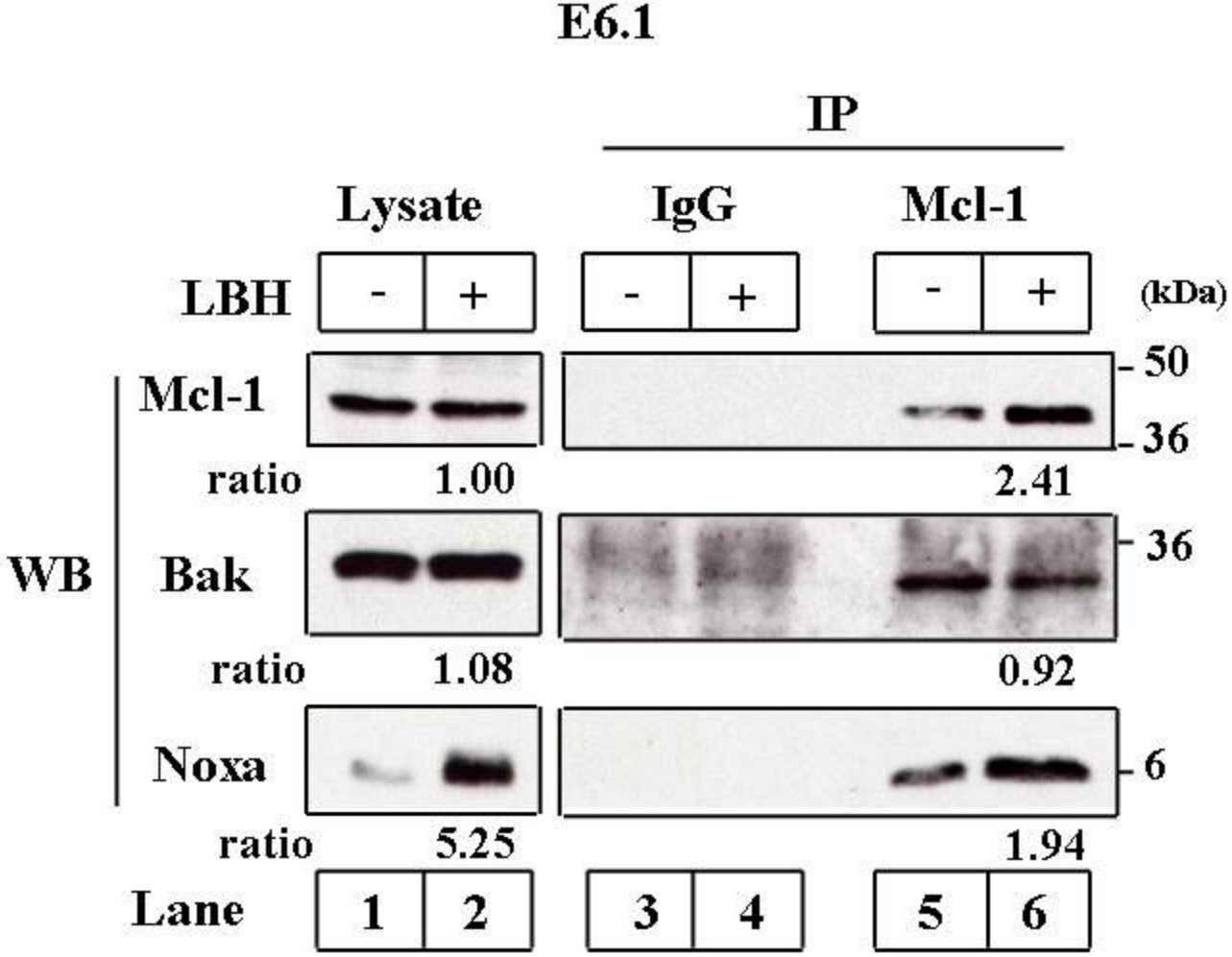

E6.1
IP
Lysate
IgG
Mcl-1
LBH
-
+
-
+
-
+
(kDa)
WB
Mcl-1
50
36
ratio
1.00
2.41
Bak
36
ratio
1.08
0.92
Noxa
6
ratio
5.25
1.94
Lane
1
2
3
4
5
6

Figure 4a

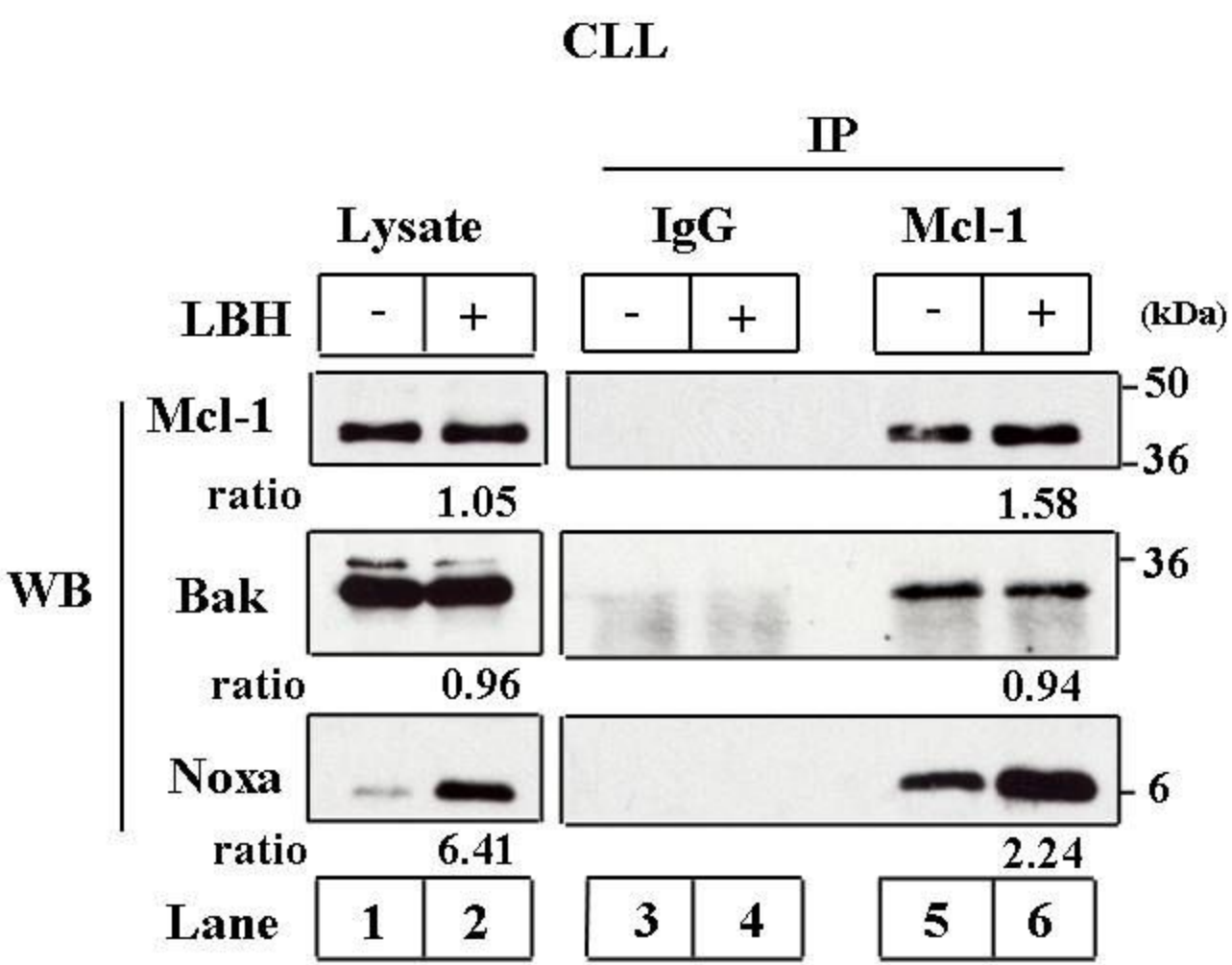
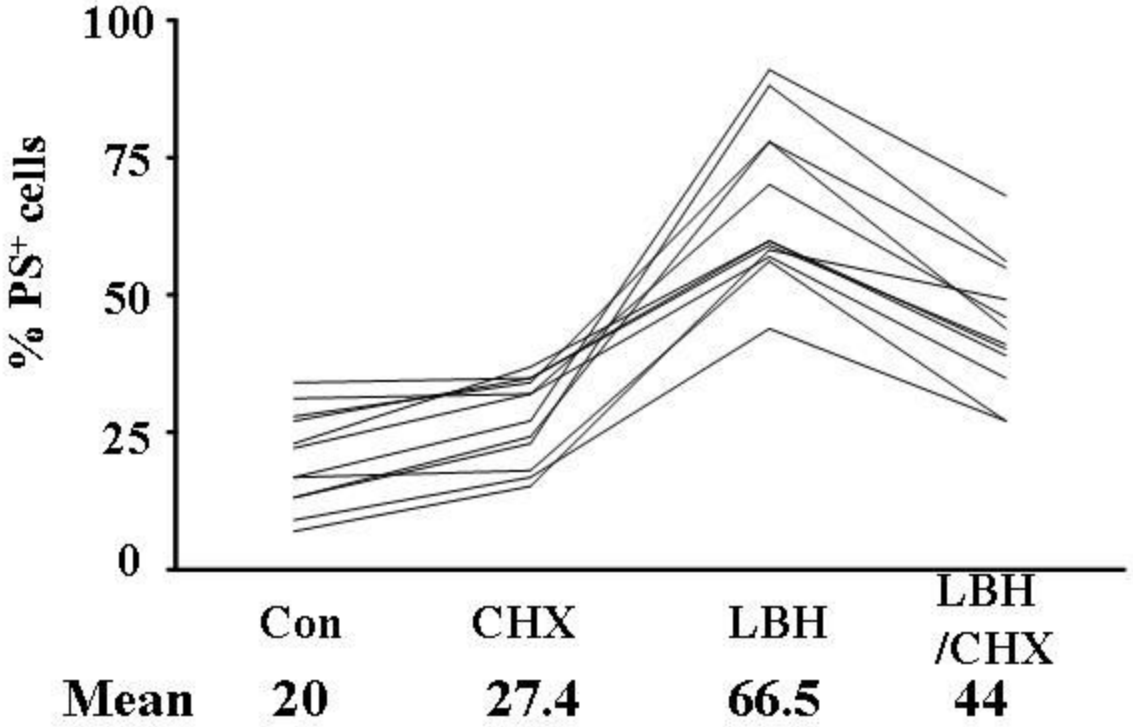

CLL
IP
Lysate
IgG
Mcl-1
LBH
-
+
-
+
-
+
(kDa)
WB
Mcl-1
50
36
ratio
1.05
1.58
Bak
36
ratio
0.96
0.94
Noxa
6
ratio
6.41
2.24
Lane
1
2
3
4
5
6

Figure 4b


100
75
50
25
0
% PS+ cells
Con
CHX
LBH
LBH /CHX
Mean
20
27.4
66.5
44

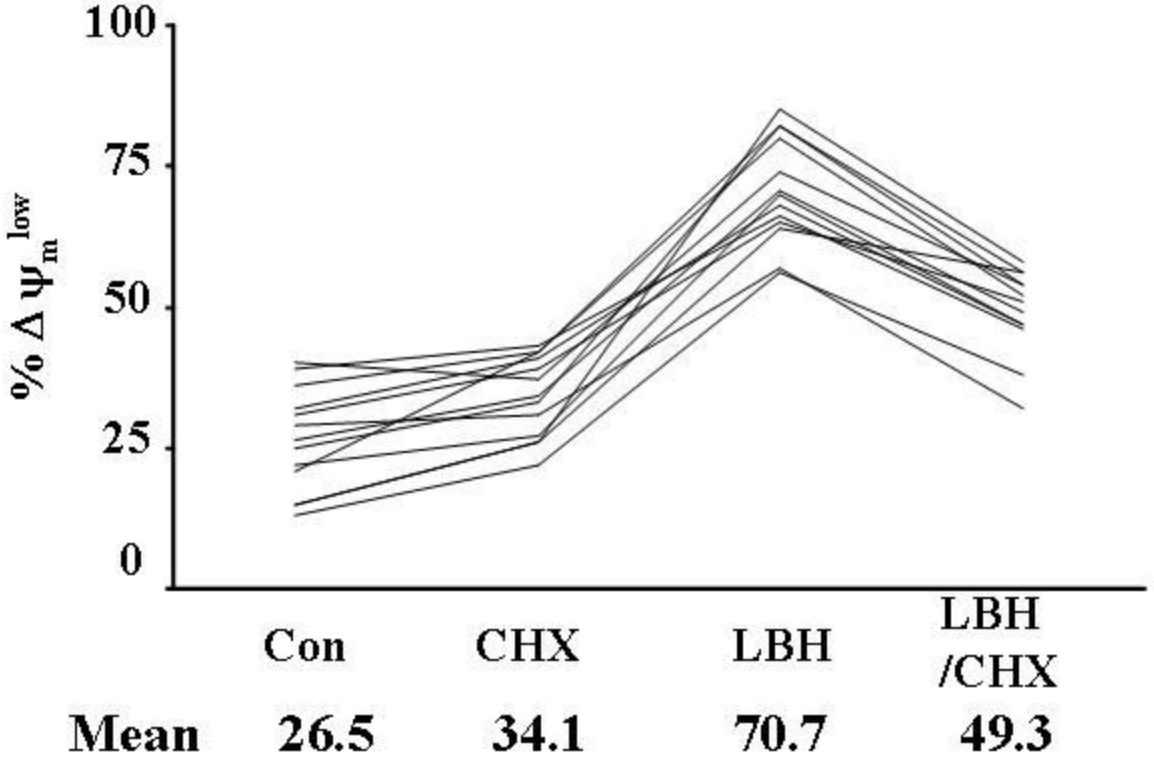
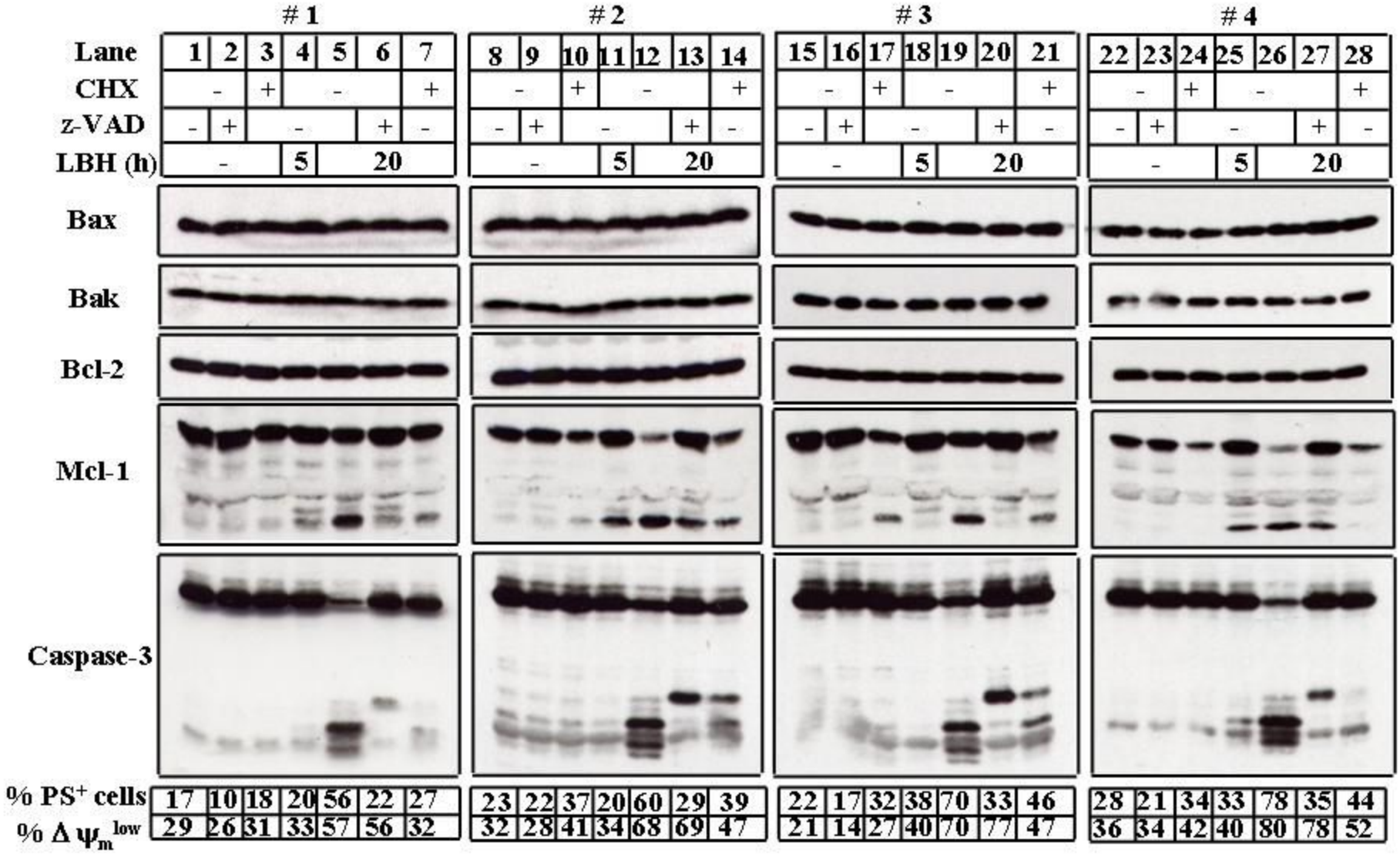

100
75
50
25
0
% Δ ψm low
Con
CHX
LBH
LBH /CHX
Mean
26.5
34.1
70.7
49.3

Figure 5a



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
CHX	-		+	-			+	-		+	-			+	-		+	-			+	-		+	-			+
z-VAD	-	+	-			+	-	-	+	-			+	-	-	+	-			+	-	-	+	-		+	-
LBH (h)	-			5		20		-			5		20		-			5		20		-			5		20	
% PS+ cells	17	10	18	20	56	22	27	23	22	37	20	60	29	39	22	17	32	38	70	33	46	28	21	34	33	78	35	44
% Δψm low	29	26	31	33	57	56	32	32	28	41	34	68	69	47	21	14	27	40	70	77	47	36	34	42	40	80	78	52

Figure 5b

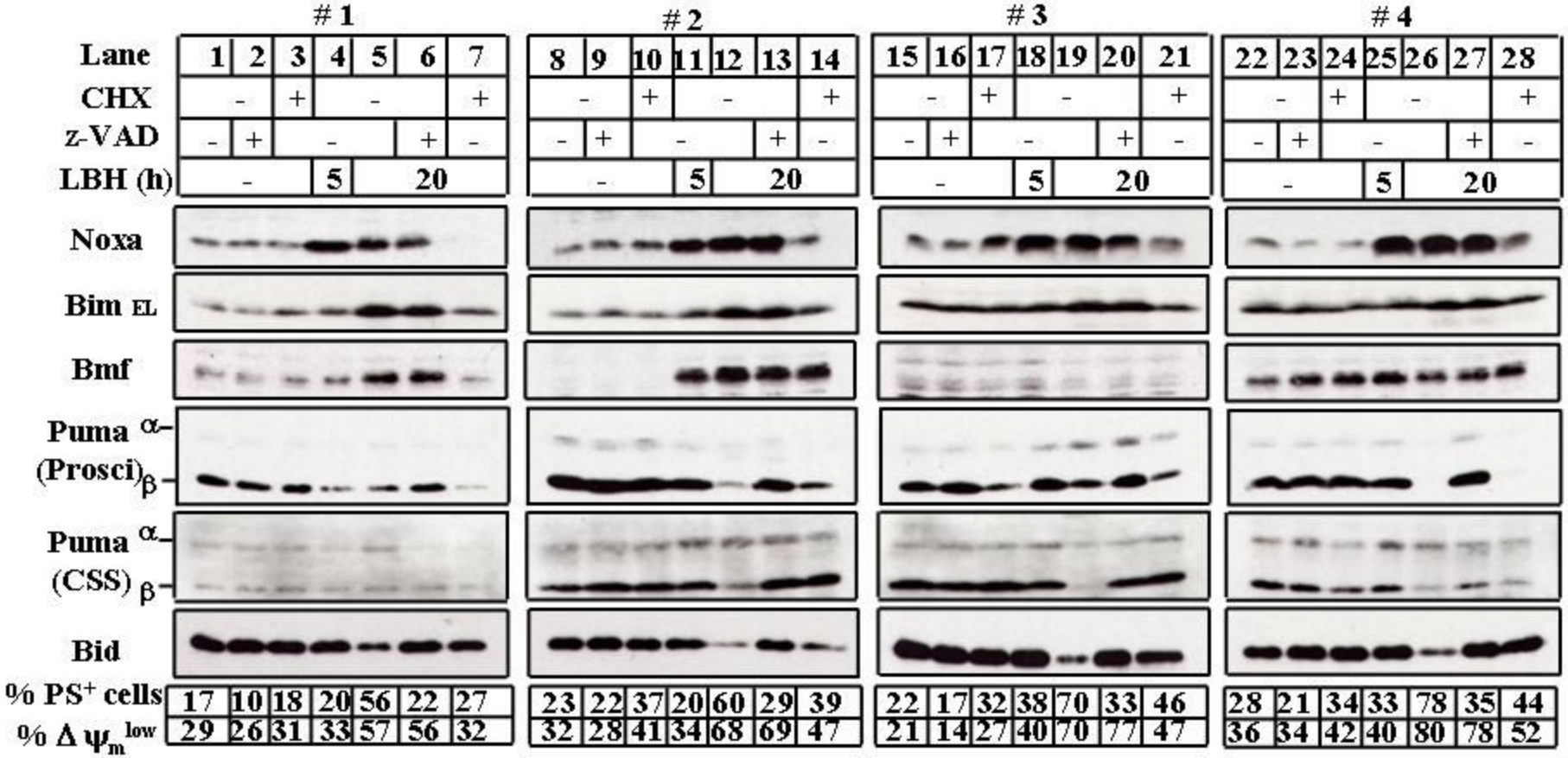

#1
#2
#3
#4
Lane
1 2 3 4 5 6 7
8 9 10 11 12 13 14
15 16 17 18 19 20 21
22 23 24 25 26 27 28
CHX
- + - +
z-VAD
- + - + -
LBH (h)
- 5 20
Noxa
Bim EL
Bmf
Puma α
(Prosci) β
Puma α
(CSS) β
Bid
% PS+ cells
17 10 18 20 56 22 27
23 22 37 20 60 29 39
22 17 32 38 70 33 46
28 21 34 33 78 35 44
% Δ ψm low
29 26 31 33 57 56 32
32 28 41 34 68 69 47
21 14 27 40 70 77 47
36 34 42 40 80 78 52

Figure 5c