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Tumor cell death in response to HAMLET is independent of Bcl-2 and P53.

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Running title: HAMLET-induced cell death is independent of *p53* and Bcl-2.

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ABSTRACT

HAMLET (Human α-lactalbumin Made Lethal to Tumor cells) is a protein-lipid complex that kills tumor cells while moving through the cytoplasm to the cell nuclei. Dying cells show features of classical apoptosis but it has remained unclear if cell death is executed only through this pathway. This study characterized the apoptotic response to HAMLET and the involvement of *p53* and Bcl-2 using chromatin condensation, caspases, phosphatidyl serine and viability as endpoints. Chromatin condensation showed characteristic of classical apoptosis and a low caspase response was detected, but in parallel, HAMLET induced caspase independent chromatin changes and cell death proceeded in the presence of caspase inhibitors. Over-expression of Bcl-2 modified the chromatin condensation pattern and the caspase response, but did not rescue the cells. Furthermore, *p53* deletions or gain of function mutations did not change the sensitivity to HAMLET. We conclude that HAMLET overrides classical apoptosis and kills tumor cells through a novel Bcl-2 and P53 - independent mechanism. (n=157).

INTRODUCTION

We have reported that a protein-lipid complex from human milk can trigger apoptosis in many different tumor cells lines (Håkansson et al., 1995). The complex, named Human α-lactalbumin Made Lethal to Tumor cells (HAMLET) consists of partially unfolded α-lactalbumin bound to oleic acid; C18:1 (Svensson et al., 2000). The unfolded state of the protein and the fatty acid cofactor are both required to trigger cell death (Svensson et al., 2003), but the mechanisms of cell death have not been defined. Dying cells show features of classical apoptosis, such as mitochondrial permeability transition, release of cytochrome c, caspase activation and DNA fragmentation (Håkansson et al., 1995; Kohler et al., 2001; Kohler et al., 1999) and HAMLET stimulates isolated mitochondria to release cytochrome c (Kohler et al., 2001). In addition, HAMLET migrates through the cytoplasm and accumulates in cell nuclei (Håkansson et al., 1995) where high affinity interactions between HAMLET and core histones within the nucleosomes have been detected *in vitro,* and HAMLET disturbs the association between histones and DNA (Duringer et al., 2003). The nuclear accumulation of the protein complex does not occur in healthy differentiated cells which are resistant to the effects of HAMLET suggesting that the nuclear accumulation may be a critical feature of cell death (Håkansson, 1999).

Different programmed cell death (PCD) pathways may be distinguished by the role of caspases and the patterns of chromatin condensation (Jaattela, 2002; Jaattela and Tschopp, 2003; Leist and Jaattela, 2001a). Classical apoptosis involves effector caspases, chromatin condensation, DNA fragmentation, apoptotic body formation and phosphatidyl serine exposure, and are controlled by the Bcl-2 family. The Bcl-2 protein family regulates cell survival (Vander Heiden et al., 1997) and especially Bcl-

2 and Bcl-xl at the mitochondrial outer membrane, may block the release of apoptogenic factors from the intermembrane space (Green and Reed, 1998; Gross et al., 1999; Kroemer and Reed, 2000). Bcl-2 and Bcl-xl inhibit the mitochondrial response to most death stimuli and overexpression protects cells from the pro-apoptotic Bcl-2 family members (Bossy-Wetzel et al., 1998; Kluck et al., 1997; Kroemer and Reed, 2000; Yang et al., 1997). Over-expression of Bcl-2 and Bcl-xl occurs in a variety of cancers, and contributes to their increased longevity (Reed, 1999).

The *p53* tumor suppressor initiates cell cycle arrest and DNA repair in response to DNA damage, but when cells harbor irreparable DNA damage, *p53* activates cell death programs and the cells undergo apoptosis (Bates and Vousden, 1999; Lane, 1993). Inactivation of P53 function facilitates carcinogenic transformation and approximately 50% of human tumors carry *p53* mutations (Cheng and Haas, 1990; Chiba et al., 1990; Hainaut et al., 1998; Hollstein et al., 1991; Lowe et al., 1994). Genomic *p53* mutations are usually manifested as deletions of one allele coupled to a mis-sense mutation of the other but in addition, *p53* mutations may create variant P53 proteins that contribute to tumorigenesis. These gain-of-function mutations are located in strictly conserved regions of the DNA binding domain, and generate a phenotype with altered P53 DNA binding specificity and new transcriptional targets (Roemer, 1999).

This study examined the contribution of classical apoptosis to tumor cell death in response to HAMLET. The cells showed features of classical apoptosis but died through a novel, Bcl-2 and P53 independent mechanism.

RESULTS

Evidence of classical apoptosis in HAMLET treated cells

Caspase activation, phosphatidyl serine (PS) exposure and DNA fragmentation were selected as parameters of classical apoptosis (Fig. 1) and etoposide was included as an agonist of classical apoptosis. Caspase 2, 3 and 9 activity increased in HAMLET treated cells but the response was much lower than the response to etoposide (Fig. 1A). PS surface exposure was detected by flow cytometry on 23 % of HAMLET treated compared to 54 % of etoposide treated cells after 6 hours (Fig. 1B). The PS response was caspase dependent as shown by zVAD-fmk inhibition. The formation of oligonucleosome DNA fragments was also caspase dependent in both HAMLET and etoposide treated cells (Kohler et al., 1999) (data not shown). zVAD-fmk inhibited the caspase response to HAMLET, but caused only a marginal change in cell viability from 50% to 64% at the LD_{50} concentration HAMLET (data not shown), suggesting that classical apoptosis is not the only cause of cell death.

Lack of Bcl-2 involvement in HAMLET-induced cell death

The effect of Bcl-2 on the response to HAMLET was examined using Bcl-2 over-expressing tumour cells. The transfected K562 cell line over-expressed Bcl-2, compared to the vector control, as shown by Western blot analysis (Fig. 2 A). The reduction in cell viability was similar between the Bcl-2 over-expressing cells S2 and S8, and the vector control (LD_{50} =18 μM) and there was no difference in the kinetics of cell death (Fig. 3 B). Jurkat cells over-expressing Bcl-2 showed an LD_{50} of 25 μM compared to 18 μM for the vector control. Etoposide treated Bcl-2 over-expressing cells showed the expected reduction in the caspase-3 response compared to the vector control, but no such difference was detected in response to HAMLET (Fig. 3D). Bcl-xl over-expressing cells died in response to HAMLET but were slightly more

resistant than vector control cells (LD_{50} 10 and 15 μM, respectively). *Bcl-2* family gene expression in HAMLET treated cells was examined using the RNA protection assay (Fig. 3 C). No significant change was detected after HAMLET treatment of the K562-vector, S2 and S8 cells.

The results suggest that cell death is independent of Bcl-2 and that the expression of Bcl-2 family members is not regulated by HAMLET.

Chromatin structure in HAMLET treated cells

Changes in chromatin structure in HAMLET treated cells were characterized by confocal microscopy. Classical apoptosis was defined by chromatin condensation into small and large spheres and crescents followed by the formation of apoptotic bodies. Apoptosis-like cell death was characterized by marginalization of the chromatin and by the absence of classical apoptosis (Jaattela and Tschopp, 2003; Leist and Jaattela, 2001a).

Chromatin condensation into small spheres, large spheres and crescents was observed in HAMLET treated Jurkat cells (Fig. 3A). This response was caspase dependent as shown by inhibition with zVAD-fmk and the pattern resembled that of cells treated with etoposide (Fig. 3B). More complex changes in chromatin structure occurred in parallel in the HAMLET-treated cells with marginalization of chromatin (Fig. 3C). This response was caspase independent and the marginalized chromatin pattern increased in frequency when classical apoptosis was blocked by zVAD-fmk (Fig. 3C).

The changes in chromatin structure were more rapid in HAMLET than in etoposide treated cells, with 33 % of cells showing condensed chromatin after 1 hour compared to 5 % for etoposide. We conclude that the chromatin condensation in HAMLET

treated cells proceeds in both a caspase dependent and a caspase independent manner and that a shift to the caspase independent morphologies occurs when caspases are blocked.

Chromatin condensation was compared between Bcl-2 over-expressing and vector control cells (Fig. 4). HAMLET caused the formation of small spheres, large spheres and crescent-shaped chromatin in both Bcl-2 over-expressing and vector control cells. In contrast, the chromatin response to etoposide was reduced by Bcl-2 over-expression and marginalized chromatin was not seen. We conclude that Bcl-2 controls chromatin condensation in response to etoposide but not the response to HAMLET.

HAMLET sensitivity of transfected cell lines with wt or mutant *p53*

As HAMLET accumulates in tumour cell nuclei and disrupts the chromatin, P53 dependent apoptotic responses might be activated (Fig. 5 A) (Duringer et al., 2003). The influence of P53 on the response to HAMLET was examined using two tumor cell lines with wild type or mutant *p53 (*Fig. 5 B). The H1299 lung carcinoma cells carry a homozygous *p53* deletion and are resistant to classical apoptosis. After transfection with the dominant-negative His-175 gain of function mutant, the cells become even more resistant to apoptotic stimuli (Blandino et al., 1999). The loss of viability was compared between the stably transfected line expressing mutant P53 (His-175) and a mutant lacking P53 expression His-175H (Tet-off) (Fig. 5 C). No difference in HAMLET susceptibility was detected in the presence or absence of Tetracycline (Fig. 5D). The colon carcinoma HCT116 +/+ carries a *wt p53* and HCT116 -/- carries a *p53* deletion. No difference in HAMLET sensitivity was detected between these clones. The difference in *p53* expression was confirmed by

Western blots. We conclude that the cellular response to HAMLET is not controlled by *p53*.

Cellular spectrum of HAMLET

Tumor cell lines and primary cells with known *p53* status were screened for HAMLET susceptibility (Table I). There was no correlation between *p53* status and HAMLET sensitivity in lymphoid cells, carcinoma and glioma cells carrying mutant, non-expressing, null or wild-type *p53*. The results confirmed the conclusion that p53 does not control cell death in response to HAMLET.

DISCUSSION

HAMLET is a protein-lipid complex that triggers programmed cell death in tumor cells and immature cells but spares healthy cells (Håkansson et al., 1995; Svensson et al., 2000). The proposed cellular targets include mitochondria and chromatin, suggesting that HAMLET might trigger the Bcl-2 and P53 dependent apoptosis machinery (Duringer et al., 2003; Kohler et al., 2001; Kohler et al., 1999). This hypothesis was refuted in the present study, however. Dying cells showed features of classical apoptosis, but the level of caspase activation and PS exposure was low and caspase inhibitors failed to rescue the cells. Furthermore, P53 and Bcl-2 did not regulate the survival of HAMLET treated cells. Instead, caspase independent mechanisms of cell death and chromatin condensation were detected. We conclude that HAMLET triggers tumor cell death through novel pathways that can be activated in the majority of tumor cells but not in healthy cells.

The definition of apoptosis is controversial, but a consensus has emerged that classical apoptosis involves the mitochondria and relies on effector caspases. Other, apoptosis-like cell death pathways also involve the mitochondria but rely on alternative and caspase-independent proteases such as Apoptosis inducing factor (AIF) and Endonuclease G, which translocate from the mitochondria to the nuclei where they trigger chromatin condensation and the formation of 50 kbp DNA fragments (Li et al., 2001). Cathepsins and calpains can stimulate caspase-dependent cell death (Leist and Jaattela, 2001b; Mathiasen et al., 2001; Mathiasen et al., 1999) or participate in caspase-independent PCD responses (Foghsgaard et al., 2001; Hegde et al., 2002; Roberg et al., 1999; Roberts et al., 1999; Suzuki et al., 2001) and Omi/Htra2 can activate caspases via inhibition of inhibitors of apoptosis (IAPs) or act as a caspase-independent protease (Suzuki et al., 1997). These responses are all controlled

by the mitochondria and by Bcl-2, illustrating how different apoptotic responses converge at the mitochondrial level and then diverge during the nuclear effector phase. The mitochondria are essential also in the case of primary DNA damage, through p53 or nuclear caspases that travel to and activate the mitochondria (Robertson et al., 2002). In this study, both classical apoptosis and apoptosis-like responses to HAMLET were detected but they were independent of Bcl-2 and Bcl-xl and did not influence the kinetics of cell death.

Mitochondrial outer membrane permeabilisation (MOMP) is a crucial step in cell death and causes the release of pro-apoptotic proteases and protease activating proteins from the inter-mitochondrial space (Green and Kroemer, 2004). Anti-apoptotic Bcl-2 family proteins block MOMP, while pro-apoptotic family members promote it. In addition, the permeability transition pore, that is controlled by adenine nucleotide transporter and voltage-dependent anion channel (VDAC), may allow water to pass through the membranes causing the outer membrane to burst (Mattson and Kroemer, 2003). The pro-apoptotic Bax and Bak can interact with VDAC which allows cross-talking between the two mechanisms (Cheng et al., 2003; Deshmukh et al., 2000). In preliminary experiments, we have observed that HAMLET triggers a cytochrome c response in Bcl-2 over-expressing cells, suggesting that HAMLET might cause MOMP through a direct effect of the lipid on the mitochondria (Kohler et al., 2001).

Other than HAMLET, only a couple of cell death inducers have been suggested to trigger Bcl-2 independent cell death. The nucleocytosolic adaptor protein Bin1 may be inactivated in prostate cancers and melanomas but triggers caspase and Bcl-2 independent cell death when reintroduced in a hepatoma cell line (Elliott et al., 2000). The RasV12 mutant also induced Bcl-2 independent (Chi et al., 1999) non-apoptotic

cell death after transfection of malignant glioma cells. These agonists may share intracellular signalling pathways, but the mechanisms of cell death need further study.

The chromatin condensation pattern has been proposed as a useful tool to distinguish classical apoptosis from other forms of PCD (Jaattela and Tschopp, 2003; Leist and Jaattela, 2001a). The chromatin response in HAMLET treated cells showed features of classical apoptosis as well as morphologies compatible with apoptosis-like cell death. The classical apoptosis patterns were blocked by zVAD-fmk, but the other morphologies remained unchanged and increased in frequency after zVAD-fmk treatment of the cells. The PCD associated chromatin patterns with marginalisation to the nuclear periphery have been detected in several models of apoptosis-like cell death. Defects in the ubiquitin pathway can cause caspase-independent cell death with chromatin condensation in murine fibroblasts (Monney et al., 1998). The active form of Vitamin D3 triggers calpain-mediated apoptosis-like cell death independent of caspases and with marginalization of the chromatin in a human breast cancer cell line (Mathiasen et al., 2002). We conclude that HAMLET activates classical apoptosis and caspase-independent PCD in parallel.

P53 controls the cellular response to nuclear DNA damage and is a classical regulator of tumor cell apoptosis (Lane, 1993). Agonists that do not directly damage the DNA may also be expected to activate P53 dependent cell death pathways through effects on chromatin. The disruption of chromatin was recently suggested to initiate PCD in yeast where the deletion of the histone chaperone Asf1 caused G2/M arrest and cell death with morphological features of both apoptosis and necrosis (Yamaki et al., 2001). HAMLET accumulates in tumor cell nuclei and the disturbance of the chromatin structure might impair replication, transcription and DNA repair

mechanisms, all of which depend on a functional chromatin assembly machinery. Impaired chromatin assembly might thus facilitate DNA damage and activate P53. This mechanism might, in turn, activate classical apoptosis through signaling intermediates like caspase-2 that move from the nucleus to the cell death regulators and effectors in the mitochondria and cytoplasm (Enoksson et al., 2004; Robertson et al., 2002). We had expected an effect of P53, but no difference in HAMLET sensitivity was detected between P53 wt and mutant cells. HAMLET was shown to activate a low caspase-2 response, but cell death was executed by other mechanisms. The results of the present study support the notion that molecules directly affecting chromatin structure and function may trigger cell death (Yamaki et al., 2001) through unconventional mechanisms and suggest a link between the accumulation of HAMLET in tumor cell nuclei and their sensitivity to HAMLET.

HAMLET is a complex of partially unfolded □-lactalbumin and the fatty acid cofactor and presents an unusual challenge to tumor cells. HAMLET appears to activate parallel mechanisms of death in tumor cells. Dying cells showed features of classical apoptosis as well as apoptosis like death, suggesting that multiple effector pathways are involved. This is different from the agonists that induce cell death through a single signalling pathway involving the caspase cascade leading to classical apoptosis and which are affected by alterations in control molecules, such as *p53* or Bcl-2. HAMLET offers an advantage in terms of cell death efficiency, as the multiplicity of mechanisms, may activate several alternative death pathways and circumvent the anti-apoptotic strategies of the tumor cell. The results show that HAMLET induces cell death through several routes and suggest that this explains the ability of HAMLET to kill many different tumor cell types, regardless of genetic background. This complex mode of action may relate to the presence of both the unfolded protein and the fatty acid in the tumor cells, as these constituents have

different intracellular targets. It may be speculated that HAMLET may have evolved to selectively purge unwanted tumor progenitors from the intestine of the breast fed child. By understanding this activity it might b possible to understand novel aspects of the differences between healthy cells and tumor cells.

MATERIALS AND METHODS

Reagents

Dulbecco´s modified Eagle´s medium, fetal calf serum (FCS), gentamicin, G418, L-glutamine, sodium pyruvate, non-essential amino acids, penicillin/streptomycin solution, RPMI 1640, and sodium pyruvate were from Gibco/BRL, Life Technology Ltd. (Paisley, Scotland, UK). Dimethylsulfoxide, HEPES, Tween-20 and Tris(hydroxymethyl) amonomethane was from Kebo Lab (Stockholm, Sweden). Heparin and PD-10 column were from Pharmacia Biotech (Stockholm, Sweden). Antipain, bovine serum albumin, ethylenediaminetetraacetate (EDTA), ethylene glycol-bis(b-aminoethyl Ether), triton X-100, zVAD-fmk and trypsin were from Sigma Chemicals Inc. (St. Louis, MO, US). Trypan blue was from Chroma Gesellschaft, Schmid & Co (Stuttgart, Germany). Etoposide was from Bristol-Myers Squibb Co. (New York, NY, US). DEVD-AMC, VDVAD-AMC and LEHD-AMC were from (Peptide Institute, Osaka, Japan) Blasticidin S, Trizol, Bis-tris gels and buffers were from Invitrogen Gibco (Carlsbad, CA, US). PVDF membranes were from Osmonics Inc. (Trevose, US). Polymorphprep density gradient was from (Nycomed, Roskilde, Denmark) Alexa Fluor 568 and TO-PRO-3 was from Molecular Probes Inc. (Eugene, OR, US). Riboquant Multi-Probe RNase Protection assay Kit was from Pharmingen Becton Dickinson, USA), Paraformaldehyde was from VWR International (Stockholm, Sweden). Mouse anti-p53 (AB-2) was from Oncogene (Boston, MA, US). Rabbit anti-human Bcl-x, mouse anti-human Bcl-2, Rabbit anti-mouse HRP was from Dako (Glostrup, Denmark)

Purification of α-lactalbumin and conversion to HAMLET

HAMLET is a folding variant of human α-lactalbumin stabilized by a C18:1 fatty acid cofactor. In this study, native α-lactalbumin was purified from human milk and

converted to HAMLET on an oleic acid conditioned ion exchange matrix as previously described (Svensson et al., 2000).

Cells and cell culture

The A549, A-498, HT29, SK-BR-3, DU145, NCI, J 82, CaCO-2, HT-29, MCF-7, T47d, PC3, U37, U251, CRL 2356, D54, Jurkat, L1210 and cell lines were from the American Type Culture Collection (ATCC). The cell lines were cultured as described (Håkansson et al., 1994). The promyelocytic leukaemia cell lines HL-60, K562 and U-937 were kindly provided by U. Gullberg, Division of Hematology, Department of Laboratory Medicine, Lund University, Lund, Sweden. Rat thymocytes were harvested as described (Zhivotovsky et al., 1993). K562 cells transfected with the *bcl-2* sequence in the Pc-DNA vector (S2 and S8) or vector control were cultured as described above. The Bcl-2 overexpressing Jurkat cells and the Bcl-xl overexpressing Fl5.12 cells Jurkat cells was cultured in conditions described above, but in addition the Fl5.12 culture medium was conditioned with WEHI-3B medium as a source of IL-3. HeLa cells expressing GFP-tagged histone H3 were grown in Dulbeccos MEM with glutamax supplemented with penicillin (100U/ml)-streptomycin (100 μg/ml), Sodium Pyruvate (1 mM), 10% FCS and 2 μg/ml blasticidin S. The *p53*-null adenocarcinoma cell line H1299 was stably transfected with the retroviral vector pPS-tTA-hygro expressing tetracycline-dependent transactivator protein tTA. Selected cell clones were then infected with self-inactivating retroviral vectors (pSIT-neo) carrying the gain of function mutant His-175 under control of a tetracycline-dependent promoter. To suppress the p53 expression, cells were maintained in the presence of the tetracycline derivate doxycycline as described (Bykov et al., 2002; Pugacheva et al., 2002). HCT116 colon carcinoma cells express endogenous wild type p53 +/+. HCT116p53 -/- cells were obtained by deletion of both alleles of the p53

gene by homologous recombination and was cultured as described (Bunz et al., 1998).

Caspase activity

Cells were treated with HAMLET 18 μM under serum free conditions or with etoposide (10 μM). Pre-treatment with z-VAD-fmk (10 μM) was for 1 h. VDVAD-AMC, DEVD-AMC or LEHD-AMC cleavage was quantified using a modified version of a flourometric assay (21). Briefly, 10^5 cells were pelleted, washed once with ice-cold PBS and re-suspended in 25 μl of PBS. For DEVD- cleavage, cells were combined with substrate dissolved in 100 mM Hepes, pH 7.25, 10% sucrose, 10 mM dithiothreitol (DTT), 0.1% CHAPS. For VDVAD-AMC or LEHD-AMC cleavage, cells were combined with substrate dissolved in 100 mM MES, pH 6.5, 10% polyethylene glycol, 10 mM DTT, 0.1% CHAPS. Cleavage of the fluorogenic peptide substrates was monitored by AMC release in a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden) using 355 nm excitation and 460 nm emission wavelengths. Fluorescence units were converted to pmol using a standard curve generated with free AMC. Data from duplicate samples were analyzed by linear regression.

Phosphatidylserine exposure measurements

Phosphatidylserine exposure was detected using the Annexin V: FITC Apoptosis Detection Kit II (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's instructions. In brief, 5 x 10^5 cells were pelleted following drug treatment washed in PBS and resuspended in 100 μl of binding buffer containing annexinV-FITC and propidium iodide and 400 μl of binding buffer for flow cytometry. Fluorescence-activated cell sorter (FACS) analysis was on a Beckman Epics XL (Beckman-coulter, Fullerton, US). Double positive, necrotic cells were gated out before analysis.

Western blot

Total cell extracts were was washed once in PBS and resuspended in lysis buffer (150 mM NaCl, 20 mM TRIS-HCl, 2mM EDTA, pH 7.5) supplemented with protease inhibitors (Antipain 1:500 and Leupeptin 1:1000). Extracts were separated by Bis-Tris SDS gel electrophoresis according to the manufacturer's instructions. The proteins were electro-blotted to a PVDF membrane, blocked for 15 min in SAT-1 (ethanolamine 6.1g/l, glycine 9 g/l, polyvinylpirolidone 10 g/l, methanol 25%) and 15 min in Sat 2 (ethanolamine 6.1g/l, glycine 9 g/l, Tween-20 1.25 g/l, gelatina hydrolysate 5 g/l, methanol 25%). The membrane was probed with primary antibodies in PBS-BSA 0.01% in 4°C. Secondary antibody binding was detected with a horseradish peroxidase conjugated secondary and visualized by the addition of Acetate buffer (50 mM Sodium acetate, ph 5.0), hydrogen peroxidase and 1% 3-amino-9 ethyl-Carbasole in acetone.

Cell viability

Confluent cell layers were detached from culture flasks by the addition of versene (140 mM NaCl, 2.4 mM KCl, 8mM Na_2PO_4, 1.6 KH_2PO_4, and 0.5 mM EDTA, pH 7.29) and washed once in PBS at 37˚C, and resuspended in cell culture medium at a concentration of $2x10^6$ cells/ml. Cells growing in suspension were harvest by centrifugation and washed once in PBS. The concentration was set to $2x10^6$ cells/ml in cell culture medium. 1 ml of cell suspension was incubated in 24-well plates with HAMLET or a crude milk fraction (MAL) incubated at 37°C in 5% CO_2 and the cells were harvested by aspiration.

Multiprobe RNA protection assay

K562 cells were exposed to 21 μM of HAMLET and harvested at various times. Total RNA was extracted from cells using Trizol reagent according to the manufacturer's instructions. Briefly, the cells were lysed and homogenized in Trizol reagent. Cell lysates were chloroform treated and centrifuged at 4°C. Total RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. Precipitated tRNA was washed once in 75% ethanol and dried and resolved in RNase-free water and frozen until use. Bcl-2 family mRNA, were examined using the Riboquant Multi-Probe RNase Protection assay Kit, according to the manufacturer's instructions. The RNA was quantified by phosphorimager (Storm 840, Amersham Biosciences, Little Chalfont, UK)

Chromatin morphology

Jurkat cells were exposed to 14 μM of HAMLET or 10 μM etoposide for 6 hours and was washed once in PBS and fixed in 4% paraformaldehyd in PBS. Fixed cells were preserved on microscopy slides. The cells on the slides were washed in PBS, permeabilised in 0.2% Triton X-100 and incubated for 5 min with the far-red DNA

stain TOPRO-3. The cells were washed 3x5min in PBS prior to evaluation in an LSM 510 META confocal microscope (Carl Zeiss, Germany) with 60x objective. The LSM 510 acquisition software was used. The frequency of the different chromatin patterns was shown in percent of total cells after counting a minimum of 100 cells in each experiment.

Subcellular localization studies

HAMLET was labeled with Alexa Fluor 568 according to manufacturer's instructions. HeLa cells expressing GFP-tagged histone H3 or H2B (Kimura and Cook, 2001) were exposed to Alexa-HAMLET for 3 h. Confocal microscopy was in a Bio-Rad 1024 laser scanning confocal equipment (Bio-Rad Laboratories, Hemel-Hempstead, UK) attached to a Nikon Eclipse 800 microscope (Nikon, Japan) with a 60x objective (NA 1.40).

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ABBREVIATIONS

HAMLET, human α-lactalbumin made lethal to tumor cells; DEVD-AMC, benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; LEHD-AMC, benzyloxycarbonyl-Leu-Glu-His-Asp-7-amino-4-methyl coumarin; MOMP, mitochondrial outer membrane permeabilisation; PCD, Programmed Cell Death; PS, Phosphatidyl Serine; VDVAD-AMC,benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-7-amino-4-methylcoumarin; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone;

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FIGURE LEGENDS

Figure 1. HAMLET induces classical apoptosis but caspase inhibition does not prevent death. (A) Caspase response to HAMLET or etoposide. Jurkat cells were treated with 18 μM HAMLET (LD_{50} concentration) or 10 μM etoposide for 6 hours and caspase-2, 3 and 9 activity was monitored as the AMC release from specific substrates. *Indicates that the experiment was carried out under serum free conditions. Treated cells are compared with 100 % control **(B) PS-exposure.** Jurkat cells were treated as described in A. Pre-treatment with 10 μM zVAD-fmk was for 1h at 37°C. PS exposure was visualized by flow cytometry.

Figure 2. Effects of Bcl-2 or Bcl-xl over-expression on the response to HAMLET. (A) Bcl-2 and Bcl-xl expression. Western blot of cellular extracts from transfected K562, Jurkat and FL5.12 cells confirming Bcl-2 and Bcl-xl over-expression. S2 and S8 stably transfected Bcl-2 over-expressing clones. K562 is a human chronic myeloid leukaemia cell line, Jurkat cells are T-cell lymphomas cell line and FL5.12 a murine pro-B-cell lymphoid cell line. **(B) Bcl-2 and Bcl-xl independent apoptotic response.** Viability of Bcl-2 and Bcl-xl over-expressing cells or vector controls cells exposed to different concentrations of HAMLET for 6 hours. **(C) HAMLET does not influence *bcl-2* family mRNA levels.** Cells were treated with 18 μM HAMLET and the mRNAs were quantified using the RNA protection assay. **(D) Caspase 3 responses in Bcl-2 overexpressing cells.** Cells were treated with 18 μM HAMLET or 10 μM etoposide for 6 hours and caspase-3 activity was monitored as the AMC release from the specific substrate (DEVD-AMC). Treated cells are compared with 100 % control.

Figure 3. Chromatin changes in HAMLET or etoposide-treated Jurkat cells. Jurkat cells were exposed to 14 μM HAMLET or 10 μM etoposide for 1 or 3 and the

chromatin morphology was examined by confocal microscopy. Pre-treatment with zVAD-fmk was for 1h at 37°C. **(A) Chromatin condensation in response to HAMLET**. Four different chromatin morphologies were observed using TO-PRO-3 staining (red, upper panels). i) Homogenous chromatin, ii) chromatin fragmented and condensed to small compact spheres, iii) chromatin fragmented to large spheres and iv) crescent-shaped chromatin. v) chromatin marginalized to the nuclear periphery. **(B) Chromatin condensation in response to etoposide.** Similar morphologies as in **A** were observed. **(C) Frequency of chromatin morphologies.** Values are the means of three separate experiments and error bars are the standard deviations. Remaining cells had unchanged chromatin structure.

Figure 4. Chromatin condensation in Bcl-2 over-expressing Jurkat cells. *Bcl-2* transfected or vector control cells were exposed to 14 μM HAMLET or 10μM etoposide for 6h and the chromatin morphology was examined by confocal microscopy. Chromatin was visualized with the DNA specific dye TO-PRO-3 (red, upper panels). **(A) Chromatin morphologies in Bcl-2 overexpressing cells.** Four different chromatin morphologies were identified in Bcl-2 overexpressing cells in response to HAMLET or etoposide: i) Homogenous chromatin, ii) Fragmented and condensed to small compact spheres, iii) fragmented to large spheres, iv) crescent-shaped chromatin. v) chromatin marginalized to the nuclear periphery.**(B) Chromatin morphologies in vector control cells.** The chromatin morphologies described in A were also observed after exposure to HAMLET or etoposide of the control cells carrying the transfection vector. **(C) Frequency of changes in chromatin structure.** Values are the means of three experiments and error bars are the standard deviations.

Figure 5. HAMLET induced cell death is independent of p53.

(A) HAMLET accumulates in tumor cell nuclei, co-localizes with histones and perturbs the chromatin structure. HeLa cells expressing GFP-tagged histone H3 were treated with Alexa-HAMLET or medium for 3 h and analyzed by real time confocal microscopy (Duringer et al., 2003)**. (B) Genotype of cells with modified p53 status.** HCT116 is a human colon carcinoma cell line with a tumor derived *arf* deletion and wild-type *p53*, (+/+). The (-/-) clone carries a p53 deletion (Bunz et al., 1998). The lung carcinoma H1299 is *p53* negative, and was stably transfected with the p53 mutant R175H in a tetracycline repressible expression system. The +Tet cells were repressed for the mutant *p53* gene expression. **(C) P53 expression**. P53 expression was confirmed by Western. **(D) Viability of HAMLET-treated HCT116 and H1299 cells.** The cell lines were treated with HAMLET for 24h and the viability determined. Values are the means of three experiments and error bars are the standard deviations.

Table I - Sensitivity to HAMLET and *p53* status of tumor cells.

		Milk fraction		HAMLET
Lymphoid cells [c]	***p53* status**	**LD_{50}** [a] **(n** [b] **=14)**		**LD_{50}** [a] **(n** [b] **=10)**
Human	Mutant	28	(1)	10 (1)
	Non-expressing	35	(3)	20 (1)
Mouse	mutant	21	(1)	10 (1)
Rat	Wild-type	35	(1)	
Carcinomas [c]				
Human	Wild-type	88	(1)	30 (2)
	Mutant	79	(4)	
	Non-expressing	88	(1)	
	Null	63	(2)	50 (2)
Gliomas [c]				
Human	Wild-type			50 (1)
	Mutant			30 (2)

[a] The concentration (μM) required to kill 50% of the cells.
[b] Number of cell lines
[c] Jurkat, HL-60, K562, U-937, L1210, purified Rat Thymocytes, A549, A-498, MCF-7, HCT116, NCI, Caco-2, HT-29, SK-BR-3, T47d, DU-145, J 82, PC-3,U251, CRL 2356 and D54 cells were used.

Figure 1 - Caspase activity in response to HAMLET and etoposide in Jurkat cells

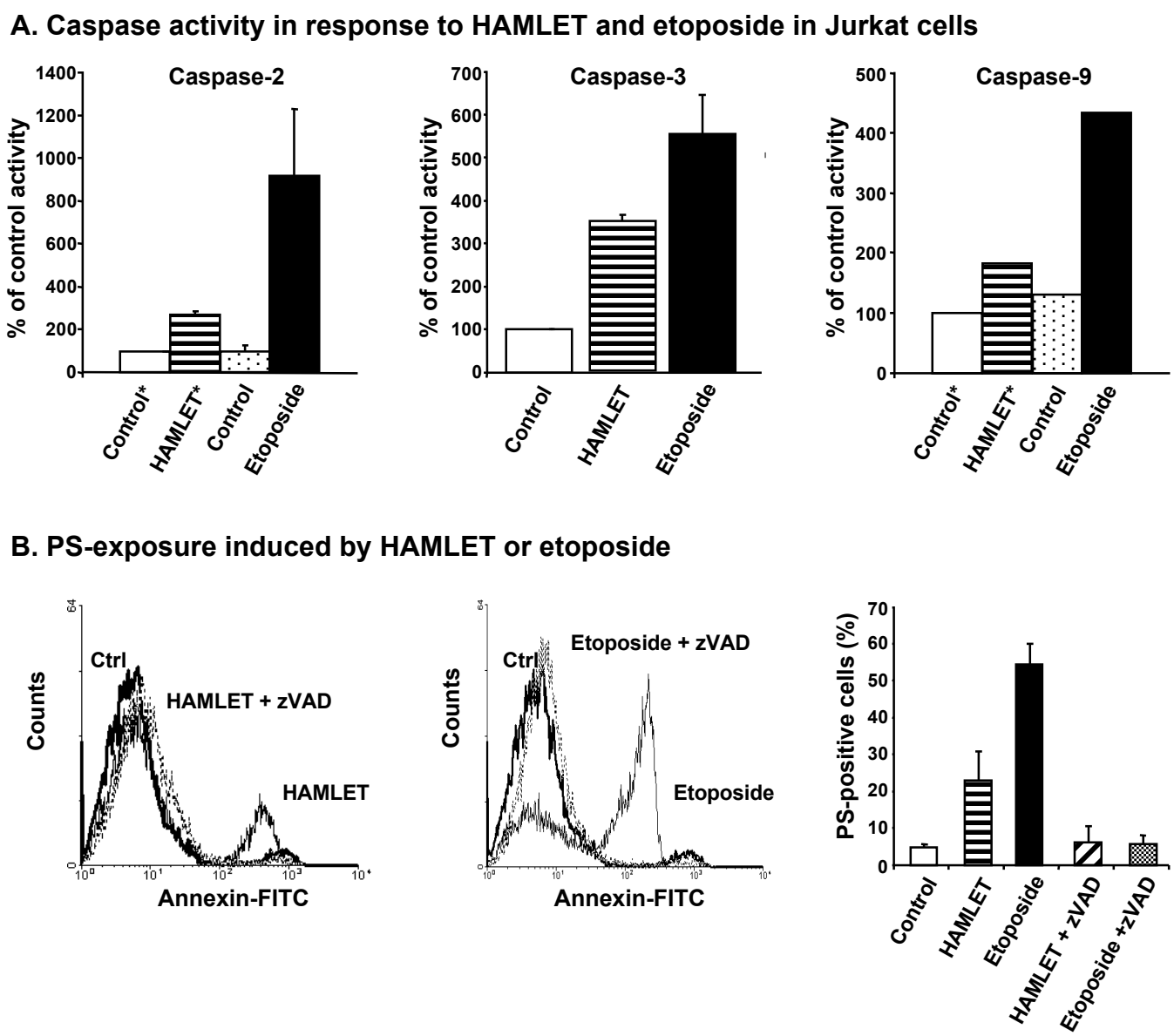


Figure 2 - HAMLET induce cell death irrespective of the Bcl-2 family.

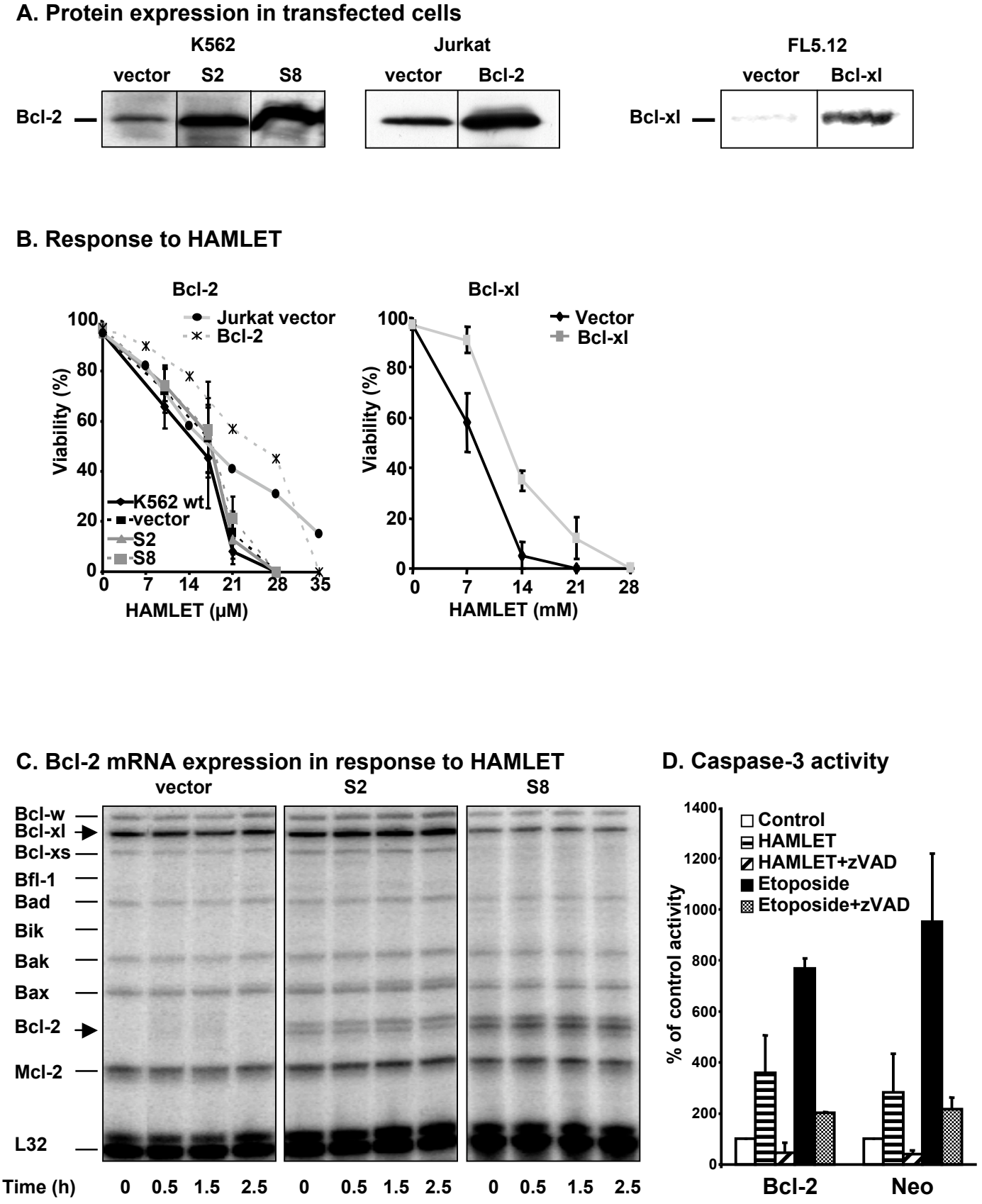
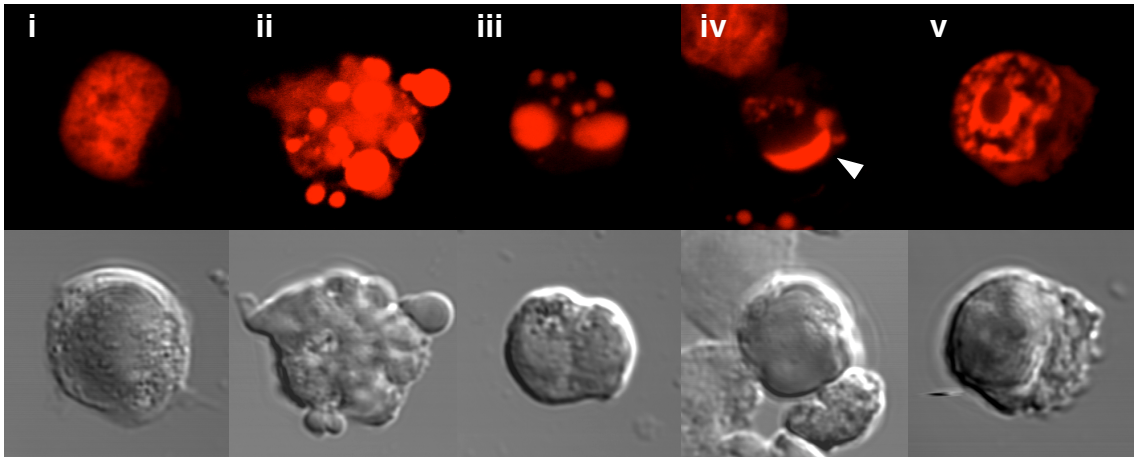
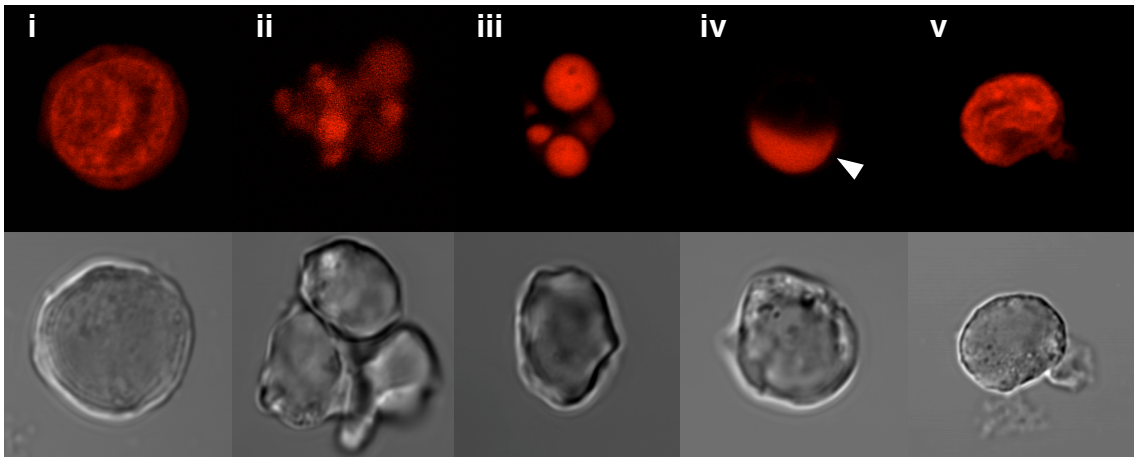


Figure 3 - Chromatin changes in Jurkat cells

A. Chromatin morphologies in HAMLET treated cells



B. Chromatin morphologies in Etoposide treated cells



C. Distribution of chromatin morphologies

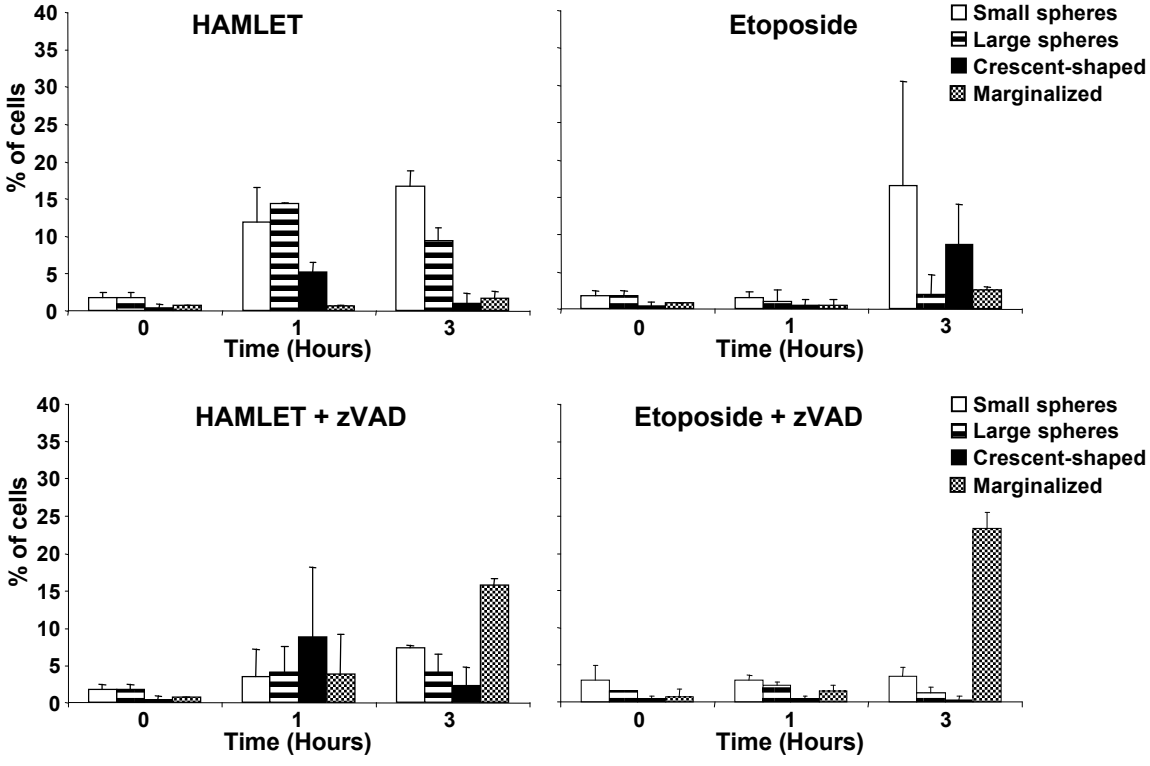
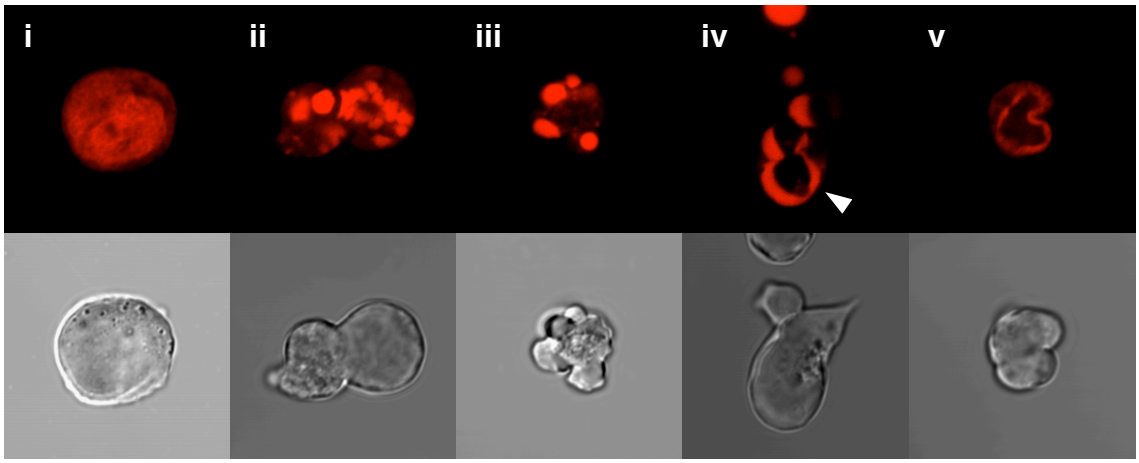
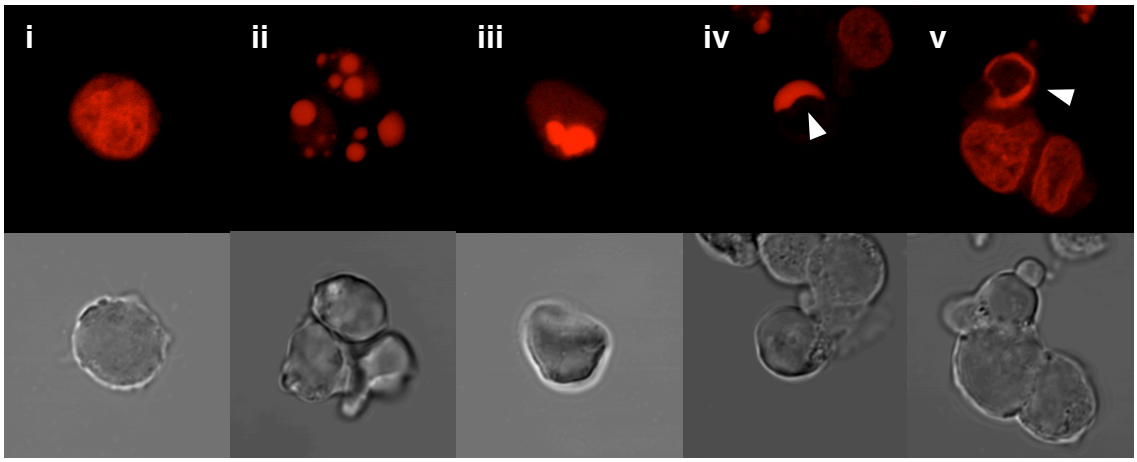


Figure 4 - Chromatin changes in bcl-2 overexpressing cells

A. Chromatin morphologies in bcl-2 overexpressing cells



B. Chromatin morphologies in vector control



C. Distribution of chromatin morphologies

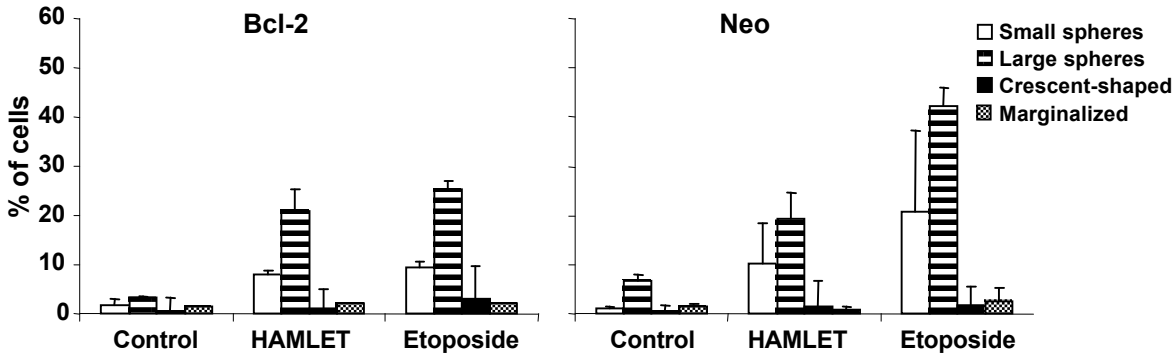
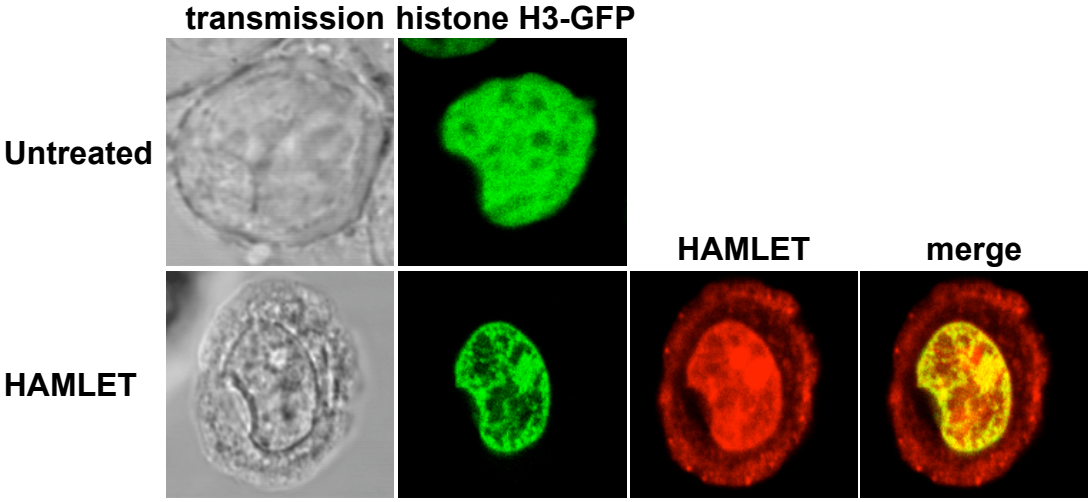


Figure 5 - HAMLET induce cell death irrespective of p53 status

A. HAMLET colocalizes with histones in tumor cell nuclei



B. P53 genotype

Cell	p53 expression
H1299 -Tet	His-175 mutant
+Tet	null
HCT116 +/+	wt
HCT116 -/-	null*

*deleted by homologous recombination

C. P53 protein expression

D. Sensitivity to HAMLET