Apoptosis 2006; 11: 221–233

DOI: 10.1007/s10495-006-3607-7

HAMLET triggers apoptosis but tumor cell death is independent of caspases, Bcl-2 and *p53*

O. Hallgren, L. Gustafsson, H. Irjala, G. Selivanova, S. Orrenius and C. Svanborg

Department of Microbiology, Immunology and Glycobiology (MIG), Institute of Laboratory Medicine, Lund University, Lund, Sweden (O. Hallgren, L. Gustafsson, H. Irjala, C. Svanborg); Department of Physics, Lund Institute of Technology and Lund University Medical Laser Centre, Lund, Sweden (L. Gustafsson); Medicity Research Laboratory, University of Turku, Turku, Finland (H. Irjala); Department of Oncology-Pathology, Cancer Center Karolinska (CCK), Karolinska Institutet, Stockholm, Sweden (G. Selivanova); Division of Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden (S. Orrenius)

Published online: xx

HAMLET (Human α-lactalbumin Made Lethal to Tumor cells) triggers selective tumor cell death *in vitro* and limits tumor progression *in vivo*. Dying cells show features of apoptosis but it is not clear if the apoptotic response explains tumor cell death. This study examined the contribution of apoptosis to cell death in response to HAMLET. Apoptotic changes like caspase activation, phosphatidyl serine externalization, chromatin condensation were detected in HAMLET-treated tumor cells, but caspase inhibition or Bcl-2 over-expression did not prolong cell survival and the caspase response was Bcl-2 independent. HAMLET translocates to the nuclei and binds directly to chromatin, but the death response was unrelated to the *p53* status of the tumor cells. *p53* deletions or gain of function mutations did not influence the HAMLET sensitivity of tumor cells. Chromatin condensation was partly caspase dependent, but apoptosis-like marginalization of chromatin was also observed. The results show that tumor cell death in response to HAMLET is independent of caspases, *p53* and Bcl-2 even though HAMLET activates an apoptotic response. The use of other cell death pathways allows HAMLET to successfully circumvent fundamental anti-apoptotic strategies that are present in many tumor cells.

Keywords: HAMLET; α-lactalbumin; programmed cell death; p53 and Bcl-2.

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; PS: Phosphatidylserine; VDVAD-AMC: benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-7-amino-4-methylcoumarin; zVAD-fmk: benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; COX-2: Cyclooxygenase-2.

Correspondence to: C. Svanborg, Section for MIG, Lund University, Solvegatan 23, 223 62, Lund, Sweden. Tel.: +46-46-173972; Fax: +46-46-137468; e-mail: Catharina.Svanborg@mig.lu.se

Introduction

The definition of apoptosis is debated, but classically, apoptosis involves the mitochondria and relies on initiator and effector caspases.[1] Apoptosis is accompanied by phosphatidyl serine exposure on the cell surface and by chromatin condensation, DNA fragmentation and apoptotic body formation.[2,3] Other, apoptosis-like cell death pathways also involve the mitochondria but rely on alternative and caspase-independent mediators such as Apoptosis Inducing Factor and Endonuclease G which translocate from the mitochondria to the nucleus, where they trigger chromatin condensation and the formation of DNA fragments.[4] The apoptosis and apoptosis-like cell death pathways are both controlled by the Bcl-2 family of proteins,[5] however. Bcl-2 and Bcl-xl block the release of apoptogenic proteins from the inter-membrane space of the mitochondria,[6–8] inhibit the mitochondrial response to many death stimuli, and protect cells from the pro-apoptotic Bcl-2 family members.[9–11] Over-expression of Bcl-2 and Bcl-xl occurs in a variety of tumors, and contributes to their resistance to chemotherapy.[12] Accordingly, many attempts have been made to identify agonists that might activate Bcl-2 independent pathways and trigger apoptosis also in tumor cells that over-express Bcl-2.[13,14]

Tumor cell apoptosis is also controlled by P53. The P53 tumor suppressor initiates cell cycle arrest and DNA repair, but when cells harbor irreparable DNA damage, P53 activates cell death programs and the cells undergo apoptosis.[15,16] Inactivation of *p53* facilitates carcinogenic transformation and approximately 50% of human tumors carry *p53* mutations.[17,18] Genomic *p53* mutations are usually manifested as deletions of one allele coupled to a missense mutation of the other and, 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.[19] The mitochondria are essential also in the case of primary DNA damage, through *p53* or nuclear caspases which can migrate into the cytoplasm.[20–22] Recently, pharmacological approaches to the normalization of *p53* function in tumor cells have been successful in experimental models.[23]

We have reported that a protein-lipid complex from human milk, named HAMLET, can trigger apoptosis in many different tumor cell lines, *in vitro*.[24] The effect is also maintained *in vivo* in man and animal models. HAMLET has been showed to induce apoptosis and to remove tumor cells in a human rat glioblastoma xenograft model and to reduce or remove the lesions in patients with skin papillomas.[25,26] The complex consists of partially unfolded α-lactalbumin bound to oleic acid and tumor cell death is rapid but healthy cells are resistant to the effect. This is coupled to a difference in uptake of HAMLET between tumor cells and healthy cells, and to proteasome activation (unpublished data). After the invasion of the tumor cells, HAMLET translocates to the nuclei where it binds histones and perturbs the chromatin.[27,28] Furthermore, HAMLET causes cellular changes compatible with apoptosis.[24,29] While these are essential steps in the tumor cell response to HAMLET, the mechanism of cell death and the basis of tumor selectivity are not fully understood.

This study, examined apoptosis in HAMLET-treated tumor cells, using caspase activity, chromatin condensation, phosphatidyl serine exposure and viability as end points. Furthermore, the influence of Bcl-2 and *p53* on the death response was assessed. The results show that HAMLET triggers apoptosis but that the cells die through other mechanisms.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium, fetal calf serum, gentamicin, G418, L-glutamine, sodium pyruvate, non-essential amino acids, penicillin/streptomycin solution, RPMI 1640 (GibcoBRL, Life Technology Ltd. Paisley, Scotland, U.K.). Dimethylsulfoxide, HEPES, Tween-20 and Tris (Kebo Lab, Stockholm, Sweden). Heparin and PD-10 column (Pharmacia Biotech, Stockholm, Sweden). Antipain, bovine serum albumin, EDTA, ethylene glycol-bis, triton X-100, zVAD-fmk and trypsin (Sigma Chemicals Inc. St. Louis, MO, USA). Trypan blue (Chroma Gesellschaft, Schmid & Co Stuttgart, Germany). Etoposide (Bristol-Myers Squibb Co. New York, NY, USA). Annexin V: FITC Apoptosis Detection Kit II (BD Pharmingen, San Jose, CA, USA). DEVD-AMC, VDVAD-AMC and LEHD-AMC (Peptide Institute, Osaka, Japan). Blasticidin S, Trizol, Bis-tris gels and buffers, Lipofectamine 2000 and pcDNA3.1/V5HisA plasmid (Invitrogen Gibco Carlsbad, CA, USA). pEGFP-C1 plasmid (Clontech, Becton Dickinson, USA). Alexa Fluor 568 and TO-PRO-1 (Molecular Probes Inc. Eugene, OR, USA). Riboquant Multi-Probe RNase Protection assay Kit (Pharmingen Becton Dickinson, USA). Mouse anti-p53 (AB-2) (Oncogene, Boston, MA, USA). Rabbit anti-human Bcl-x, mouse anti-human Bcl-2, Rabbit anti-mouse HRP, (Dako, Glostrup, Denmark). ECL PLUS kit (Amersham Biosciences, Little Chalfont, UK).

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.[28]

Cells and transfected cell lines

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 and L1210 cell lines were from the American Type Culture Collection (ATCC). The cell lines were cultured as described.[30] 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, Sweden. Rat thymocytes were harvested as described.[31] K562 cells transfected with the Bcl-2 sequence in the Pc-DNA vector (S2 and S8) or vector control were cultured as described above. Bcl-2 over-expressing Jurkat cells (pSFFV-Bcl-2) and vector control cells (pSFFV)[32] and Bcl-xl-over-expressing Fl5.12 cells (pSFFV-Bcl-xl) or vector control cells (pSFFV)[33] were cultured under 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/streptomycin (100 μg/ml), sodium pyruvate, FCS and 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 transfected 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 mutant expression, cells were maintained in the presence of the tetracycline derivate doxycycline as described.[23,34] HCT116 colon carcinoma cells express endogenous wild type P53 +/+. HCT116 *p53* −/− cells were obtained by deletion of both alleles of the *p53* gene by homologous recombination and were cultured as described.[35]

Plasmid pcDNA3.1-Bcl-xl was constructed by cloning the *Eco*RI fragment of pSFFV-neo-Bcl-xl[33] into

pcDNA3.1/V5HisA (Invitrogen) (not in frame with the V5HisA tag). pcDNA3.1-Bcl-xl or pcDNA3.1/V5HisA was transfected into A498 cells grown in six-well plates using Lipofectamine 2000 according to the manufacturer's instructions. The transfection efficiency was determined by co-transfection with the GFP expression plasmid pEGFP-C1 and evaluated on a Nikon Eclipse TE2000-U (Nikon, Japan) fluorescence microscope.

Caspase activity

Cells were treated with HAMLET (18 μM) or with etoposide (10 μM). Pre-treatment with z-VAD-fmk was for 1 h. The zVAD-fmk concentration of 10 μM was used in this paper as this concentration n had the same inhibitory effect as 50 μM. VDVAD-AMC, DEVD-AMC or LEHD-AMC cleavage was quantified using a modified version of a flourometric assay.[36] 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. The results of the daily control experiments were arbitrarily set as 100% and other results were compared to this value. Paired *t*-test (one-tailed) was used to determine significance.

Phosphatidyl serine exposure

Phosphatidylserine exposure was detected using the Annexin V: FITC Apoptosis Detection Kit II according to the manufacturer's instructions. Fluorescence-activated cell sorter (FACS) analysis was on a Beckman Epics XL (Beckman-coulter, Fullerton, USA). Double positive, necrotic cells were gated out before analysis.

Western blot

Harvested cells were washed once in PBS and re-suspended in lysis buffer (150 mM NaCl, 20 mM TRIS-HCl, 2 mM EDTA, pH 7.5) supplemented with protease inhibitors (Antipain 1:500 and Leupeptin 1:1000). Total protein concentrations were determined using the DC Protein Assay (Bio-RAD, CA, USA) according to the manufacturer's instructions. Equal amounts of proteins were separated by Bis-Tris SDS gel electrophoresis according to the manufacturer's instructions. Migrated proteins were electro-blotted to PVDF membranes ,blocked in SAT-1 (ethanolamine 6.1 g/l, glycine 9 g/l, polyvinylpirolidone 10 g/l, methanol 25%) and Sat 2 (ethanolamine 6.1 g/l, glycine 9 g/l, Tween-20 1.25 g/l, gelatina hydrolysate 5 g/l, methanol 25%). Membranes were probed with primary antibodies in PBS-BSA 0.01% in 4°C. Secondary antibody binding was either detected by the addition of acetate buffer (50 mM Sodium acetate, ph 5.0), hydrogen peroxidase and 1% 3-amino-9 ethyl-Carbasole in acetone or with the ECL Plus kit.

Cell viability

Confluent cell layers were detached from culture flasks by the addition of versene (140 mM NaCl, 2.4 mM KCl, 8 mM Na2PO4, 1.6 KH2PO4, and 0.5 mM EDTA, pH 7.29), washed once in PBS at 37°C, and re-suspended in cell culture medium at a concentration of 2×10^6 cells/ml. Cells growing in suspension were harvested by centrifugation and washed once in PBS. The concentration was set to 2×10^6 cells/ml in cell culture medium and 1 ml of the cell suspension was incubated in 24-well plates with HAMLET or a crude milk fraction (MAL)[24,37] 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 using Trizol according to the manufacturer's instructions. Bcl-2 family mRNA was 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, U.K.).

Subcellular localization studies

HAMLET was labeled with Alexa Fluor 568 according to the manufacturer's instructions. HeLa cells expressing GFP-tagged histone H3 or H2B[38], kindly provided by Hiroshi Kimura 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, U.K.) attached to a Nikon Eclipse 800 microscope (Nikon, Japan) with a 60×objective (NA 1.40).

Chromatin condensation

HAMLET or etoposide-treated Jurkat cells were harvested, washed once in PBS and fixed in 4% paraformaldehyde in PBS. Fixed cells on microscopy slides were washed in PBS, permeabilised in 0.2% Triton X-100 and incubated for 5 min with the DNA stain and TO-PRO-1. The cells were washed 3 × 5 min. in PBS prior to evaluation in an LSM 510 META confocal microscope (Carl Zeiss, Germany) with

a 63× objective. The frequency of each chromatin patterns is shown in percent of total cells after counting a minimum of 100 cells in each experiment.

Results

Evidence of apoptosis in HAMLET- treated cells

Jurkat leukemia cells and A549 lung carcinoma cells were exposed to HAMLET and the apoptotic response was studied using caspase activation, phosphatidyl serine exposure, DNA fragmentation and viability as end points. In parallel, the cells were treated with etoposide, as a positive control of apoptosis. The caspase response is shown in Figure 1A. There was an increase in caspase-2, caspase-3 and caspase-9 in HAMLET-treated cells, but the levels were significantly lower than the response to etoposide ($p < 0.05$ for caspase-2 and -3 and $p < 0.001$ for caspase-9) and did not increase further at higher HAMLET concentrations (data not shown). Phosphatidyl serine exposure was detected by flow cytometry, using FITC-labelled annexin (Figure 1B and C). HAMLET stimulated a 4.8-fold increase in PS exposure compared to an 11.2-fold increase in etoposide-treated cells and PS was detected on fewer HAMLET-treated cells (23 per cent) than etoposide-treated cells (54 per cent) ($p < 0.001$). The response was caspase-dependent, as shown by the reduction in PS exposure in the presence of the pan-caspase inhibitor, zVAD-fmk. Oligonucleosome length DNA fragments were detected in both HAMLET- and etoposide-treated cells. The formation of oligonucleosome length DNA fragments was caspase-dependent as it was inhibited by zVAD-fmk (data not shown). High molecular weight DNA fragments were formed in HAMLET-treated cells, but independently of caspases.

The viability of Jurkat and A549 cells in response to HAMLET in the presence or absence of zVAD-fmk is shown in Figure 1D. Cell death was caspase independent. The LD_{50} of HAMLET-treated Jurkat cells were 20 μM in the absence of zVAD-fmk and 17 μM in the presence of zVAD-fmk. The LD_{50} of HAMLET-treated A549 cells were 46 μM in the absence of and 41 μM in the presence of zVAD-fmk.

The results showed that HAMLET stimulates a low apoptotic response including effector caspases and suggested that tumor cell death mainly relies on caspase independent mechanisms.

HAMLET-induced cell death is independent of Bcl-2 and Bcl-xl

The mechanisms controlling the apoptotic response to HAMLET were further studied using Bcl-2 or Bcl-xl over-expressing tumor cells. The transfected K562 clones S2 and S8 were shown by Western blot analysis to over-express Bcl-2 as compared to the vector control cells (Figure 2A). A similar increase in Bcl-2 expression was found in transfected Jurkat cells. Bcl-2 family gene expression in HAMLET-treated cells was examined using the RNA protection assay. The increased Bcl-2 mRNA levels in the transfected S2 and S8 cell clones were confirmed. To examine if HAMLET influences the expression of the Bcl-2 gene family, mRNAs were quantified by the RNA protection assay. There was no detectable change after HAMLET treatment of the K562 cells, suggesting that Bcl-2 gene expression is not affected by HAMLET treatment (Figure 2B).

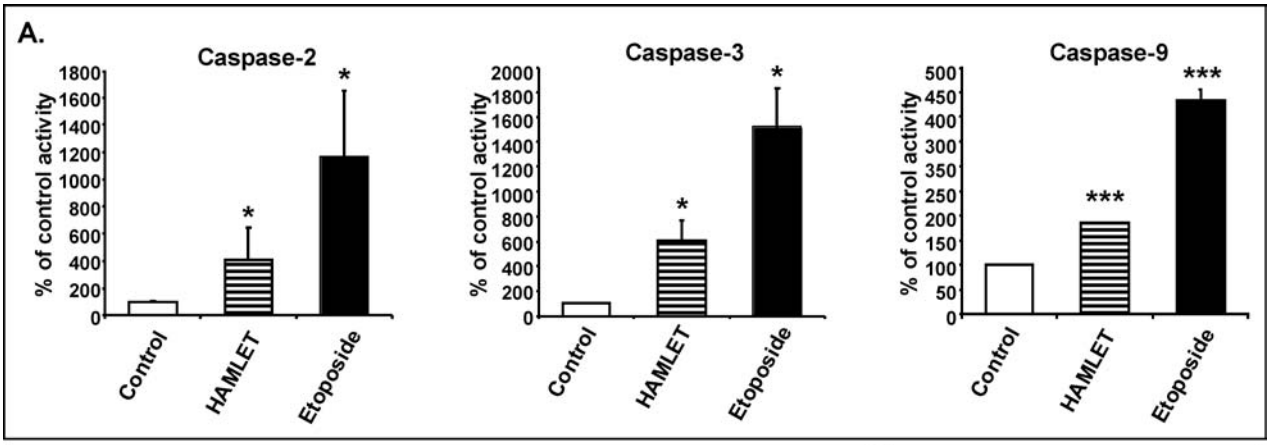
The influence of Bcl-2 on the caspase response to HAMLET was subsequently examined in Jurkat cells. Etoposide was used as a control of Bcl-2 dependent responses (Figure 2B). Bcl-2 over-expression was shown to abrogate the caspase-3 response to etoposide ($p < 0.05$) but not the caspase-3 response to HAMLET (Figure 2B). zVAD-fmk reduced the caspase-3 activity to background levels in both HAMLET and etoposide-treated cells.

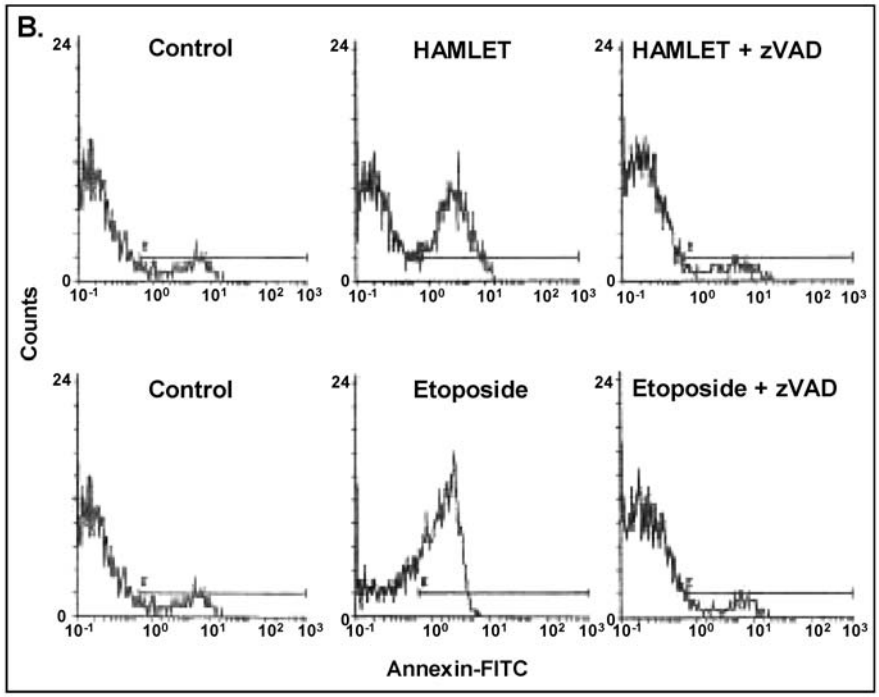
To determine if Bcl-2 might modify the death response to HAMLET, the loss of cell viability was compared between the Bcl-2 over-expressing K562 cells and the vector control cells (Figure 2C). There was no difference in the concentration of HAMLET required to kill 50 percent of the cells ($LD_{50} = 15$ μM for wild-type cells, 18 μM for vector control and S2 and 19 μM for the Bcl-2 over-expressing clone S8). Furthermore, there was no difference in the kinetics of cell death (Figure 2C). Similar results were obtained using Bcl-2 over-expressing Jurkat cells (LD_{50} of 16 μM compared to 14 μM for the vector control) as shown in Figure 2C.

The apoptotic response to HAMLET was further examined in Bcl-xl over-expressing FL5.12 cells. The transfected cells were shown by Western blot analysis to over-express Bcl-xl as compared to the vector control cells (Figure 3A). The concentration of HAMLET required to kill the cells and the kinetics of cell death are shown in Figure 3B and C. There was no significant difference in HAMLET sensitivity between Bcl-xl over-expressing or vector control cells (LD_{50} of 9 μM) and no significant difference in the kinetics of death between Bcl-xl over-expressing cells as compared to the vector control cells (Figure 3C). After 1 h. the viability was below 50% in both Bcl-xl over-expressing and vector control cells. Furthermore, Bcl-xl over-expression was achieved by transient transfection of the human kidney carcinoma A498 cell line. The transfection resulted in over-expression of Bcl-xl as compared to the vector control as shown by western blot (Figure 3A). There was no difference in sensitivity to HAMLET between Bcl-2 over-expressing or vector control cells (LD_{50} of 13 μM) as shown in Figure 3D.

The results show that cell death in response to HAMLET is independent of Bcl-2 and Bcl-xl.

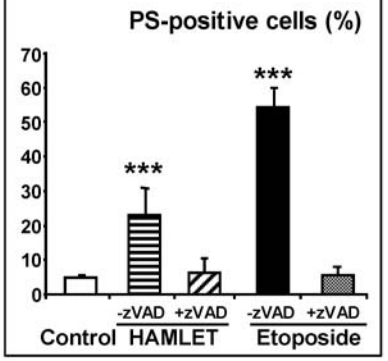
Figure 1. (A) Caspase response to HAMLET or etoposide. Jurkat cells were treated with 18 μM HAMLET or 10 μM etoposide for 6 h. and caspase-2, 3 and 9 activity was monitored as the AMC release from specific substrates. Results are given in percent of the control. (B) PS-exposure in HAMLET-treated Jurkat cells were detected by flow cytometry using Annexin-FITC. Pre-treatment with 10 μM zVAD-fmk was for 1 h at 37°C. (C) PS exposure was quantified as the median fluorescence intensity or in percent of the total cell number. (D) Cell death is caspase independent. Jurkat and A549 cells were treated with different concentrations of HAMLET for 6 or 24 hours in the presence or absence of zVAD-fmk and the viability was determined by trypan blue exclusion. Pre-treatment with zVAD-fmk was for 1 h at 37°C. Values are means of three experiments ± standard deviations.





C.

Fluorescence Intensity	
Control	0.103
HAMLET	0.241
HAMLET+zVAD	0.167
Etoposide	1.480
Etoposide+zVAD	0.163



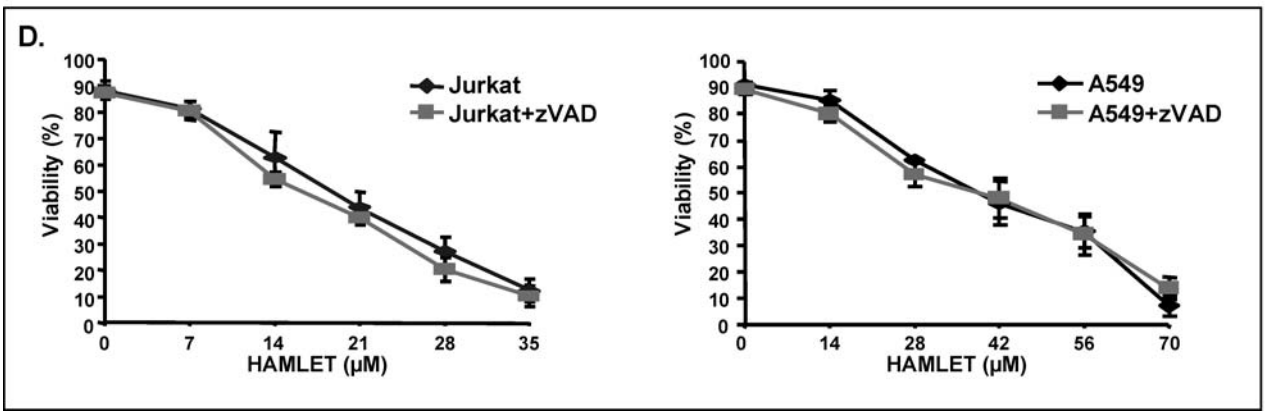
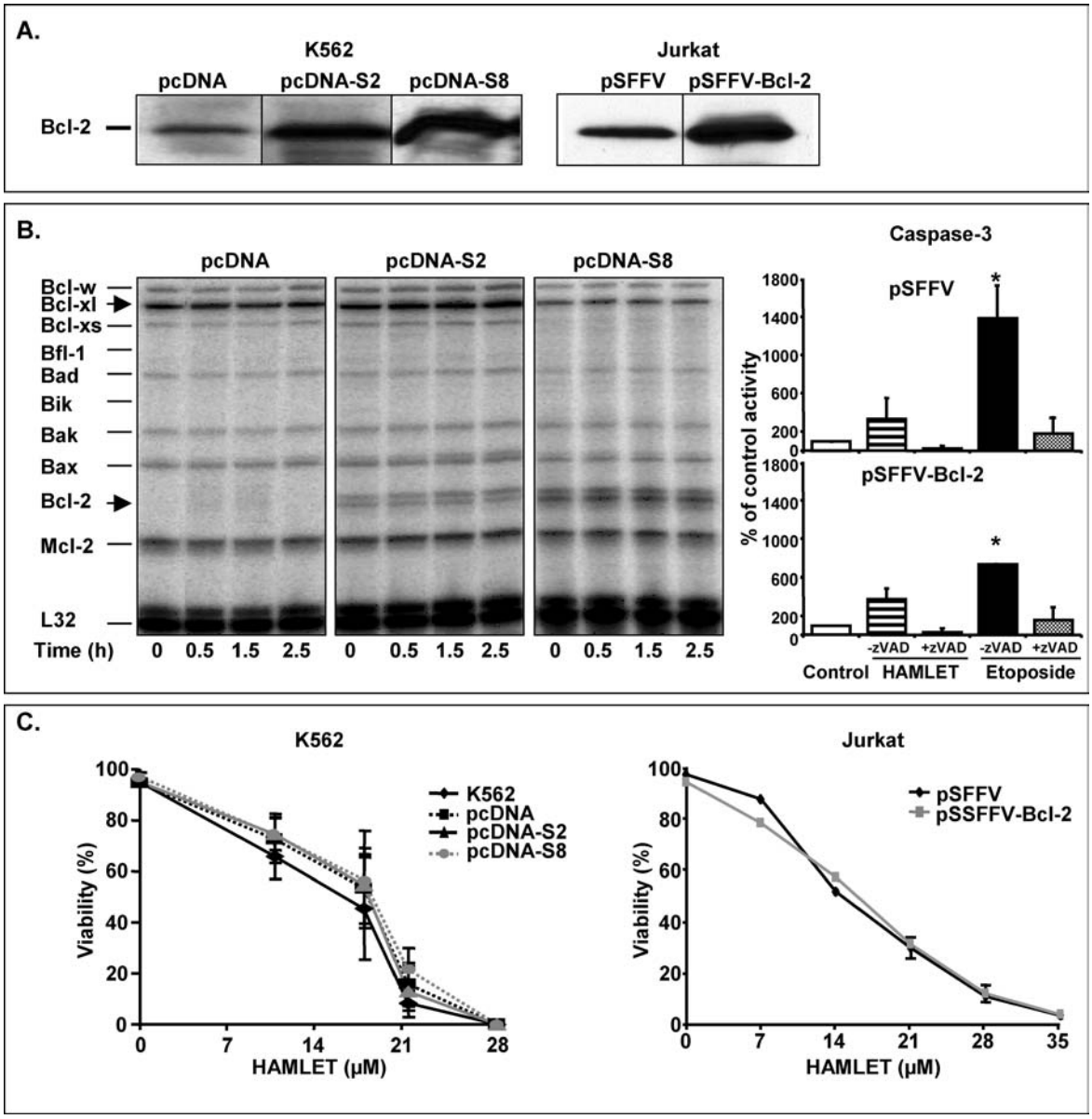


Figure 2. Bcl-2 over-expression does not protect tumor cells against HAMLET. (A) Western blots confirming Bcl-2 over-expression in the two stably transfected K562 cell clones (pcDNA-S2 and pc-DNA-S8) and transfected Jurkat cells (pSFFV-Bcl-2). The protein concentrations of the cell extracts were determined and equal amounts of protein were loaded in each lane. (B) HAMLET does not influence *bcl-2* family mRNA levels. Vector control (pcDNA) or Bcl-2 over-expressing K562 cells were treated with 18 μM of HAMLET and the mRNAs were quantified using the RNA protection assay. Bcl-2 over-expression did not reduce the caspase-3 response to HAMLET. Transfected Jurkat cells were treated with 18 μM HAMLET or 10 μM etoposide for 6 h and the caspase-3 activity was monitored as the AMC release from specific substrates. Pre-treatment with 10 μM zVAD-fmk was for 1 h at 37°C. Results are given in percent of control. There was a significant difference in caspase-3 activity between etoposide-treated bcl-2 overexpressing cells (pSFFV-Bcl-2) and vector control cells (pSFFV) ($p < 0.05$). (C) Cell death was Bcl-2 independent. Viability of Bcl-2 over-expressing cells or vector control cells exposed to different concentrations of HAMLET for 6 h. Values are means of three experiments ± standard deviations.

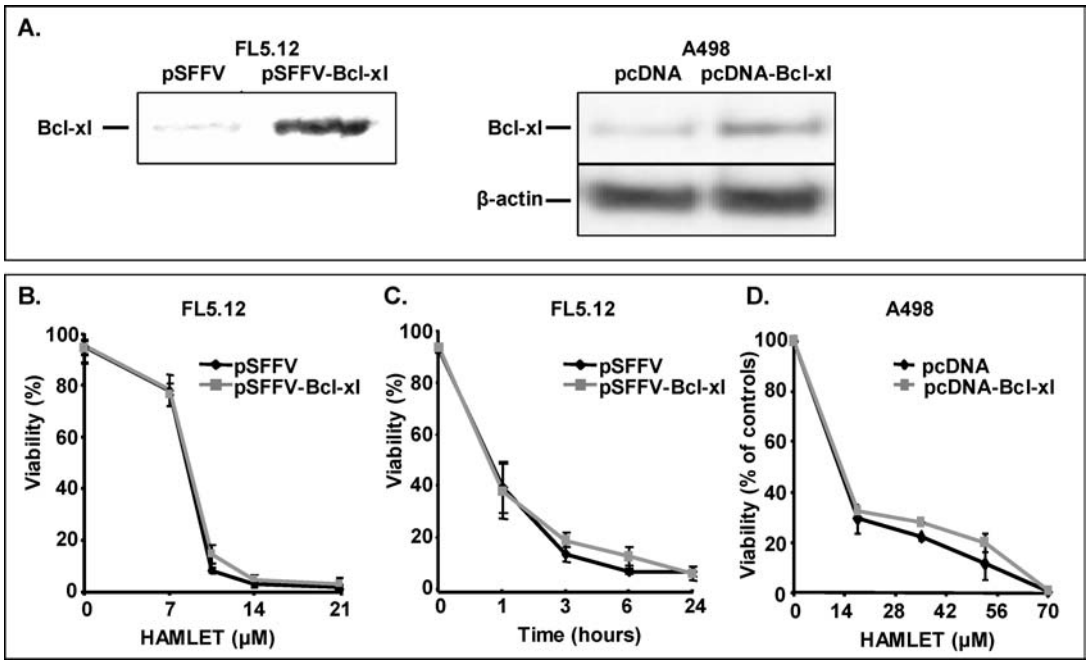


p53 involvement in HAMLET-treated tumor cells

HAMLET has been shown to accumulate in tumor cell nuclei where it binds to histones and disrupts the chromatin.[27] The changes in chromatin structure in HAMLET-treated cells were examined by real time confocal microscopy, using Alexa-Flour labeled HAMLET and HeLa cells expressing histone H3, with a gfp reporter (Figure 4A). HAMLET was shown to co-localize with histone H3 in the nuclei, and changes in chromatin structure were observed. As a consequence of this chromatin perturbation, a *p53*-dependent apoptotic response might be activated.

The influence of *p53* on the response to HAMLET was examined using two tumor cell lines with wild type or mu-

Figure 3. Bcl-xl over-expression does not alter the sensitivity to HAMLET. (A) Bcl-xl over-expression was confirmed by Western blots of extracts from stably transfected FL5.12 cells or transiently transfected A498 cells. The protein concentrations of the extracts were determined and equal amounts of protein were loaded in each well. (B) Dose dependent death of Bcl-xl over-expressing Fl5.12 cells (pSFFV-Bcl-xl) or vector control cells (pSSFV) exposed to HAMLET (10 μM) for 6 h. (C) Kinetics of cell death in Bcl-xl over-expressing Fl5.12 cells or vector control cells exposed to HAMLET (11 μM). (D) Dose dependent cell death of Bcl-xl over-expressing A498 cells (pcDNA-Bcl-xl) or vector control cells (pcDNA) exposed to HAMLET for 24 h. Values are means of three experiments ± standard deviations.



tant *p53*. The stably transfected H1299 lung carcinoma cells carry a homozygous *p53* deletion and are resistant to apoptosis. After transfection with the dominant-negative His-175 gain of function mutant, the cells become even more resistant to apoptotic stimuli.[39] The colon carcinoma cell line HCT116 +/+ carries a wt *p53* gene and HCT116 −/− carries a *p53* deletion. In previous studies, these cells have been shown to differ in sensitivity to apoptotic stimuli.[35]

The effect of *p53* on the response to HAMLET was first examined by comparing the H1299 cells expressing mutant P53 (His-175) or cells with repressed P53 expression (Tet-off). The difference in P53 expression was confirmed by Western blots (Figure 4B). The cells were exposed to HAMLET but there was no difference in cell death related to their *p53* genotype (Figure 4C). Subsequently, HCT116 cells carrying wild-type or deleted *p53* were used. The difference in P53 expression was confirmed by Western blots, and the cells were exposed to HAMLET (Figure 4B and C). No *p53* dependent difference in HAMLET sensitivity was detected suggesting that *p53* does not control the susceptibility of tumor cells to HAMLET (Figure 4C). This was supported by studies of tumor cell lines and tumor cells in primary culture (Table 1). There was no consistent relationship between *p53* status and HAMLET sensitivity in lymphoid cells, carcinoma cells or gliomal cells.

The results suggest that cell death in response to HAMLET is *p53* independent.

Chromatin structure in HAMLET-treated cells

The chromatin condensation pattern has been proposed to distinguish apoptosis from other forms of PCD.[40,41] Apoptotic cells show chromatin condensation into small spheres, large spheres and crescents followed by the formation of apoptotic bodies. Cells undergoing apoptosis-like cell death have other chromatin changes, including marginalization but do not show classical apoptosis.

The changes in chromatin structure in response to HAMLET were studied by confocal microscopy. Chromatin condensation into small spheres, large spheres and crescents occurred in Jurkat cells (Figure 5A and B) and this response was caspase dependent as shown by inhibition with zVAD-fmk. The chromatin condensation pattern resembled the caspase dependent response to etoposide (Figure 5A). In addition, more complex changes in chromatin structure occurred in the HAMLET-treated cells. There was marginalization of chromatin (Figure 5A and B) and this response was caspase-independent. The proportion of cells with marginalized chromatin increased when the apoptotic response was blocked by zVAD-fmk. The total number of cells with condensed chromatin was constant, however (Figure 5C). Chro-

Table 1. Sensitivity to HAMLET and *p53* status of tumor cells

	p53 status	Milk fraction LD_{50}* ($n^{\dagger} = 14$)	HAMLET LD_{50}* ($n^{\dagger} = 10$)
Lymphoid cells‡			
Human	Mutant	28 (1)	10 (1)
	Non-expressing	35 (3)	20 (1)
Mouse	Mutant	21 (1)	10 (1)
Rat	Wild-type	35 (1)	
Carcinomas§			
Human	Wild-type	88 (1)	30 (2)
	Mutant	79 (4)	
	Non-expressing	88 (1)	
	Null	63 (2)	50 (2)
Gliomas‖			
Human	Wild-type		50 (1)
	Mutant		30 (2)

*The concentration (μM) required to kill 50% of the cells. Viability was determined by trypan blue exclusion.
†Number of cell lines.
‡Jurkat, HL-60, K562, U-937, L1210 and purified Rat Thymocytes were treated for 6 hours and viability was determined.
§A498, A549, MCF-7, HCT116, NCI, Caco-2, HT-29, SK-BR-3, T47d, DU-145, J 82 and PC-3 were treated for 24 hours and viability was determined.
‖U251, CRL 2356 and D54 were treated for 24 hours and viability was determined.

matin condensation was more rapid in HAMLET-treated cells (33% after 1 hours) than in etoposide-treated cells (5% after 1 hours) (data not shown). The results show that 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 pathway occurs when caspases are blocked.

Chromatin condensation was subsequently examined in Bcl-2 over-expressing Jurkat cells (Figure 6A). HAMLET treatment caused the formation of small spheres, large spheres, crescents and chromatin marginalized to the nuclear periphery also in Bcl-2 over-expressing cells and there was no difference compared to vector control cells. In contrast, Bcl-2 over-expression decreased chromatin condensation in response to etoposide (Figure 6B). The results show that chromatin condensation in HAMLET-treated cells is independent of Bcl-2.

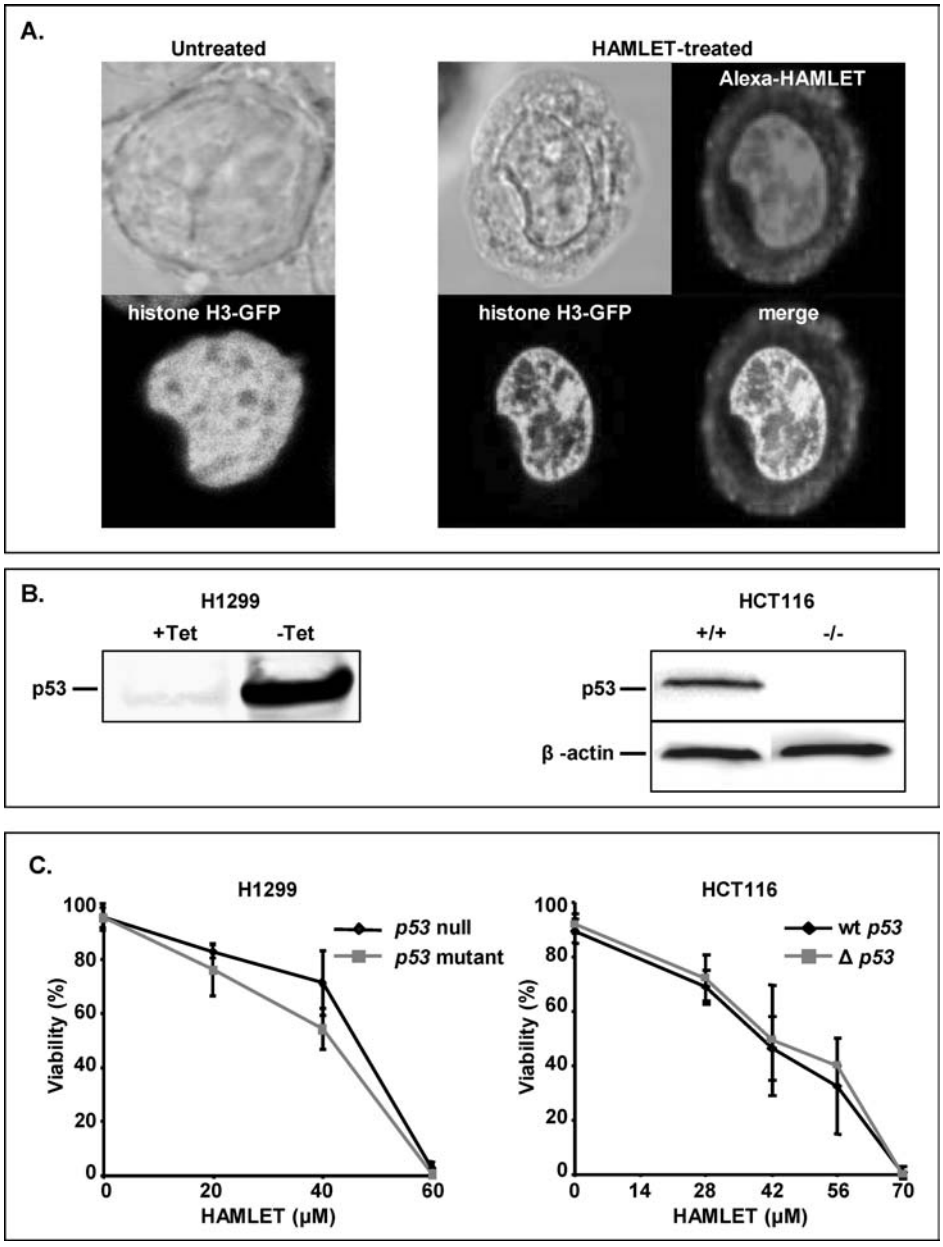
Discussion

HAMLET is a protein-lipid complex that kills tumor cells and immature cells but spares healthy, differentiated cells.[24,28,37] Earlier experiments have suggested that HAMLET triggers apoptosis, but have not defined if the apoptotic pathway is responsible for cell death.[27,29,42] This study re-examined the apoptotic response to HAMLET in tumor cells and compared the response to etoposide, which is a well-known apoptosis inducer. HAMLET was shown to activate pro-apoptotic caspases and to stimulate PS exposure but the response was lower than in etoposide-treated cells. The caspases caused chromatin condensation but were not the cause of cell death as caspase inhibitors failed to rescue the tumor cells. The death response to HAMLET was further examined as a function of Bcl-2-related mechanisms but caspase activation and cell death were independent of Bcl-2 and Bcl-xl. Furthermore, cell death was independent of *p53* even though HAMLET bound to and perturbed the chromatin. The results show that HAMLET triggers tumor cell death by circumventing the pathways controlled by *p53* or Bcl-2 and suggest that the apoptotic response is a bystander phenomenon rather than a cause of cell death. HAMLET thus appears to activate fundamental cell death pathways in many of tumor cells but not in healthy, differentiated cells.

The caspase response to HAMLET was not controlled by Bcl-2 and HAMLET was a less potent activator of the pro-apoptotic caspases than etoposide. The Bcl-2 dependent activation of caspases by etoposide has been extensively characterized. Etoposide activates the mitochondria and the effector caspases either directly or via a nuclear caspase-2 response, and both mechanisms are controlled by Bcl-2.[20–22] HAMLET has also been shown to perturb the mitochondria and to stimulate the release of cytochrome c.[42] In this study, a low caspase-2 response was detected. Despite these similarities, HAMLET and etoposide activate caspases through different mechanisms, as the response to HAMLET is not controlled by Bcl-2. A few other examples of Bcl-2 independent caspase responses have been reported. The COX-2 inhibitors induce cell death via caspase-independent as well as caspase dependent mechanisms, and neither of the pathways is controlled by Bcl-2 although the caspase dependent pathway involves mitochondrial activation with cytochrome c release and apoptosome complex formation.[43,44] The nucleocytosolic adaptor protein Bin1 may be inactivated in prostate cancers and melanomas, but triggers caspases and Bcl-2 independent cell death when reintroduced in a hepatoma cell line.[45] The RasV12 mutant also induces Bcl-2 independent non-apoptotic cell death after transfection of malignant glioma cells.[46] Further studies are needed to assess if HAMLET and these agonists activate similar cell death pathways.

The tumor cells respond by directing HAMLET to the nuclei and by sequestering the complex there.[24,37] HAMLET disturbs the association between histones and DNA through high affinity interactions with core histones.[27] 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 of HAMLET may be critical for cell death.[37] HAMLET does not interact directly with DNA, however, and there is no evidence of direct DNA damage as a basis for *p53* involvement but the disruption of chromatin may initiate programmed cell death. There are examples of agonists that do not directly damage the DNA but activate *p53*-dependent

Figure 4. *p53* does not control the sensitivity to HAMLET. (A) HeLa cells expressing GFP-tagged histone H3 were treated with Alexa-HAMLET or medium for 3 h and analyzed by confocal microscopy.[27] The nuclear localization of HAMLET, the co-localization with histones and the change in chromatin structure are shown. (B) P53 expression determined by Western blots. The *p53* negative lung carcinoma H1299 was stably transfected with the *p53* mutant His-175 in a tetracycline repressible expression system. In the absence of Doxycycline (–Tet) the cells expressed mutant P53 but Doxycycline (+Tet) repressed P53 expression. The HCT116 human colon carcinoma with wild-type *p53* (+/+) was compared to a clone carrying a *p53* deletion (–/–).[35] The protein concentrations of the extracts were determined and equal amounts of protein were loaded in each lane. (C) The HAMLET concentration required for cell death was compared between *p53* wt and mutant HCT116 and H1299 cells. The cell lines were treated with HAMLET for 24 h. Values are means of three experiments ± standard deviations.



cell death pathways through effects on chromatin. In yeast, the deletion of the histone chaperone Asf1 caused G2/M arrest and cell death with morphological features of both apoptosis and necrosis.[47] This mechanism might, in turn, activate classical apoptosis through signaling intermediates like caspase-2, which translocates to the cytoplasm and mitochondria and release cytochrome c.[20,22] The present study showed, however, that the cell death response to HAMLET proceeds regardless of *p53* genotype. The results support the notion that molecules directly affecting chromatin structure and function may trigger cell death through *p53* independent mechanisms.[47]

The chromatin condensation pattern has been used as a tool to distinguish apoptosis from other forms of pro-

Figure 5. Chromatin condensation in response to HAMLET or etoposide. (B) Jurkat cells were exposed to 18 μM of HAMLET or 10 μM of etoposide for 1 or 3 h and the chromatin morphology was examined by confocal microscopy, using TO-PRO-1 staining. Pre-treatment with zVAD-fmk was for 1 h at 37°C. (A) Overview of the chromatin response to HAMLET or etoposide in the presence or absence of zVAD-fmk. (B) Close-up of the five different chromatin morphologies i) homogenous chromatin, ii) fragmented chromatin condensed to small compact spheres, iii) crescent-shaped chromatin, iv) chromatin fragmented into large spheres and v) chromatin marginalized to the nuclear periphery. (B) The patterns shown in panels ii, iii and iv were partially inhibited by zVAD-fmk. (C) Frequency of the chromatin morphologies after 3 h of incubation. Remaining cells had unchanged chromatin structure. Values are means of three separate experiments ± standard deviations.

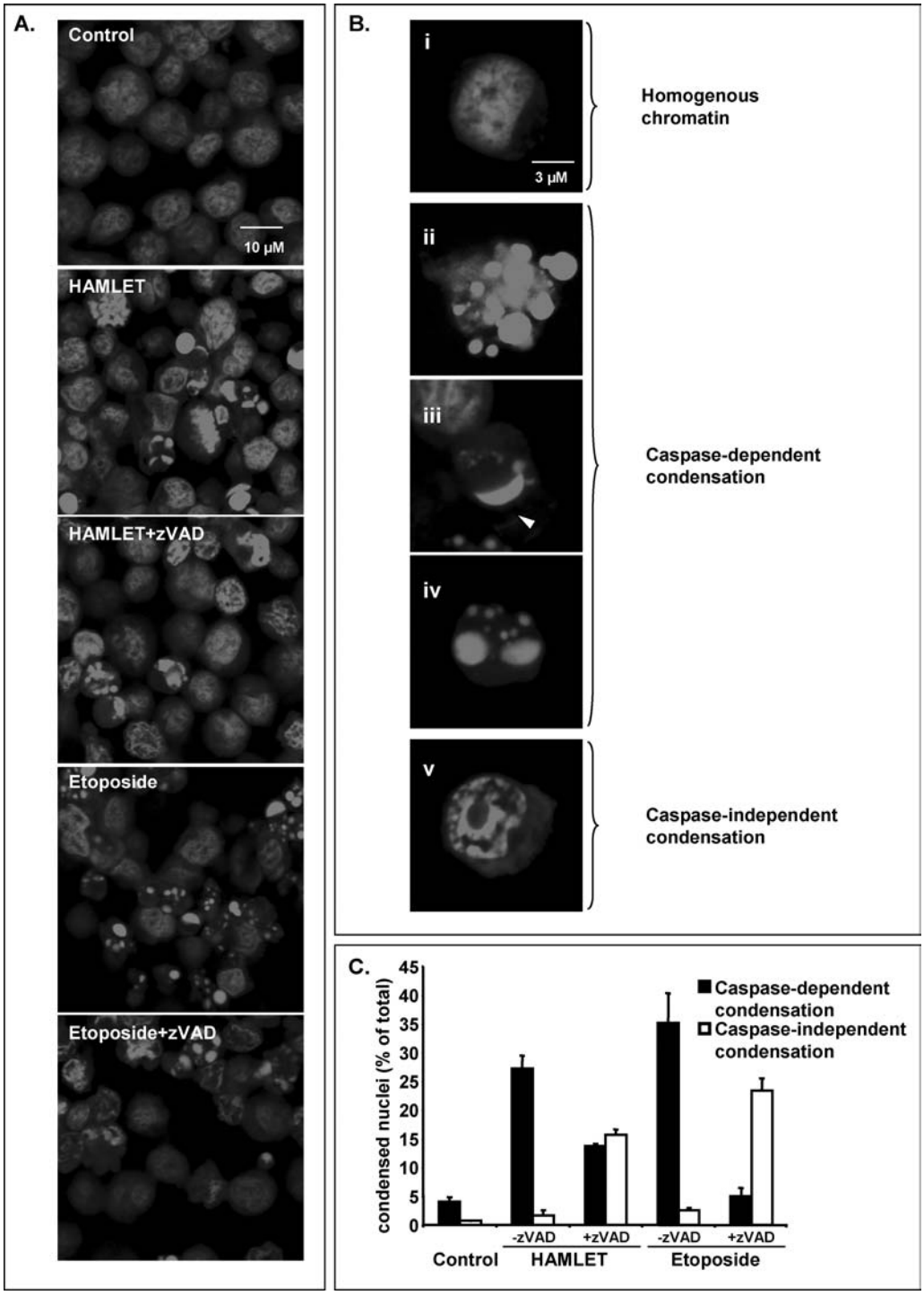
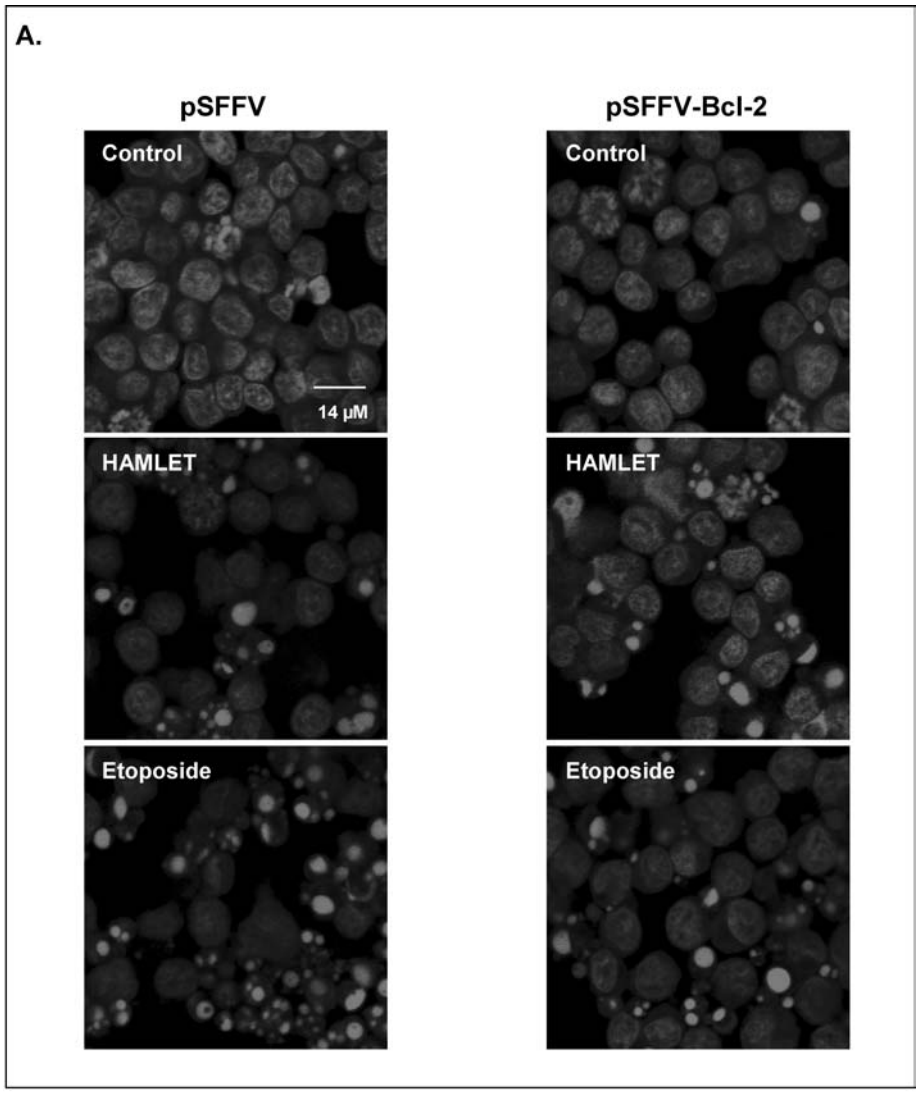


Figure 6. Chromatin condensation in Bcl-2 over-expressing Jurkat cells. Bcl-2 transfected (pSFFV-Bcl-2) or vector control cells (pSFFV) were exposed to 14 μM HAMLET or 10 μM etoposide for 6 h and the chromatin morphology was examined by confocal microscopy, using TO-PRO-1. (A) Overview of the chromatin morphology. (B) Frequency of the chromatin morphologies. Remaining cells had unchanged chromatin structure. Values are means of two experiments $\pm$ standard deviations.



grammed cell death.[40,41] The chromatin in HAMLET-treated cells showed features of apoptosis as well as morphologies compatible with apoptosis-like cell death. Marginalization to the nuclear periphery has been detected in several models of apoptosis-like cell death, and HAMLET caused such changes. The apoptotic chromatin changes were blocked by zVAD-fmk, but a compensatory increase in the other types of chromatin condensation was observed after zVAD-fmk treatment of the cells. This shift from an apoptotic to an apoptosis-like pattern was also observed in etoposide-treated cells. Some other examples of caspase-independent cell death with chromatin condensation have been reported. Defects in the ubiquitin pathway caused caspase independent chromatin condensation in murine fibroblasts[48] and the active form of Vitamin D3 has been shown to trigger calpain-mediated, caspase independent apoptosis-like cell death with marginalization of the chromatin in a human breast cancer cell line.[49] We conclude that the chromatin response to HAMLET involves both classical apoptosis and caspase-independent cell death pathways.

The sensitivity of tumor cells to HAMLET reflects the massive accumulation of HAMLET in their cytoplasm. Healthy cells also bind HAMLET to the surface but in contrast to the tumor cells they restrict the entry of HAMLET into the cytoplasm and the nuclear translocation does not occur in those cells. We have examined how the cell death response is triggered by the overload of unfolded protein and how the tumor cells die as a function of this response. We have shown that HAMLET activates the 20S proteasomes which attempt to digest the complex, but HAMLET is partially resistant to proteolytic degradation and causes proteasome damage (unpublished data). The tumor cells respond by transporting the complex into the cell nucleus causing what resembles an autophagic response. Thus, HAMLET has several intracellular targets, including the proteasomes, the mitochondria and the chromatin. HAMLET differs from many other apoptosis or cell death inducing agents in that it attacks multiple cellular targets and activates multiple signaling cascades in tumor cells. This multiplicity of targets may have evolved to ensure that the functional end-point of cell death may be reached in many different cell types and that unwanted cells can be removed from the gastro-intestinal tract of the breast-fed child.

Cancer cells prolong their survival by blocking death pathways that limit the longevity of normal cells. Novel approaches to tumor therapy attempt to circumvent these blocks and to activate cell death pathways that remain intact in tumor cells. One approach has been to rectify the effects of mutant *p53*, and recently wild type *p53* activity was restored in tumor cells, using a molecule that induces a conformational change in *p53*.[23] Bcl-2 independent activators of caspases are also being explored. Recently, the synthetic Bcl-2 inhibitor YC137 was shown to induce apoptosis in breast cancer cells and to sensitize other cancer cell types.[13,14] Tumor cells are notorious for developing resistance to most agonists, however, and this fact explains the common practice of combining several therapies. An alternative approach would be to use substances with multiple cellular targets, where synergy may be achieved and where resistance development may be less likely to occur. HAMLET is an interesting candidate in view of the tumor cell selectivity and the ability to kill cells with altered *p53* or Bcl-2 genotype. *In vivo* studies in animal models and human patients have shown that HAMLET retains the selectively for tumor cells *in vivo* and that HAMLET can be used as a topical treatment of human skin papillomas.[25,26] Naturally occurring molecules like HAMLET may thus offer new tools to selectively remove tumor cells *in vivo* and to develop alternative therapeutic approaches.

Acknowledgments

We thank Hiroshi Kimura for generously supplying the H3-GFP expressing cells, Stanley Korsmeyer for sharing the Bcl-2 and Bcl-xl over-expressing cells and L. Anderson for the K562 transfectants, A. Håkansson for early experiments and J. Robertson for valuable advice. This thesis was funded by the Medical Faculty at Lund University, the Royal Physiographic Society in Lund, the Swedish Cancer Foundation, the Swedish Pediatric Cancer Foundation, the American Cancer Society, the Segerfalk Foundation, the Anna-Lisa and Sven-Erik Lundgren Foundation for Medical Research, the Knut Alice Wallenberg Foundation, the Lund City Jubileumsfond, the John and Augusta Persson Foundation for Medical Research, the Maggie Stephen Foundation and the HJ Forssman Foundation for Medical Investigations.

References

1. Jaattela M. Programmed cell death: Many ways for cells to die decently. *Ann Med* 2002; **34**: 480–488.
2. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; **26**: 239–257.
3. Ellis HM, Horvitz HR. Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* 1986; **44**: 817–829.
4. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 2001; **412**: 95–99.
5. Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell* 1997; **91**: 627–637.
6. Kroemer G, Reed JC. Mitochondrial control of cell death. *Nat Med* 2000; **6**: 513–519. l.
7. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281**: 1309–1312.
8. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999; **13**: 1899–1911.
9. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for

Bcl-2 regulation of apoptosis [see comments]. *Science* 1997; **275**: 1132–1136.
10. Yang J, Liu X, Bhalla K, *et al*. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked [see comments]. *Science* 1997; **275**: 1129–1132.
11. Bossy-Wetzel E, Newmeyer DD, Green DR. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD- specific caspase activation and independently of mitochondrial transmembrane depolarization. *Embo J* 1998; **17**: 37–49.
12. Reed JC. Dysregulation of apoptosis in cancer. *J Clin Oncol* 1999; **17**: 2941–2953.
13. Real PJ, Cao Y, Wang R, *et al*. Breast cancer cells can evade apoptosis- mediated selective killing by a novel small molecule inhibitor of Bcl-2. *Cancer Res* 2004; **64**: 7947–7953.
14. Huang Z. Bcl-2 family proteins as targets for anticancer drug design. *Oncogene* 2000; **19**: 6627–6631.
15. Lane DP. Cancer. A death in the life of p53 [news; comment]. *Nature* 1993; **362**: 786–787.
16. Bates S, Vousden KH. Mechanisms of p53-mediated apoptosis. *Cell Mol Life Sci* 1999; **55**: 28–37.
17. Cheng J, Haas M. Frequent mutations in the p53 tumor suppressor gene in human leukemia T-cell lines. *Mol Cell Biol.* 1990; **10**: 5502–5509.
18. Lowe SW, Bodis S, McClatchey A, *et al*. p53 status and the efficacy of cancer therapy in vivo. *Science* 1994; **266**: 807–810.
19. Roemer K. Mutant p53: Gain-of-function oncoproteins and wild-type p53 inactivators. *Biol Chem* 1999; **380**: 879–887.
20. Robertson JD, Enoksson M, Suomela M, Zhivotovsky B, Orrenius S. Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. *J Biol Chem* 2002; **277**: 29803–29809.
21. Robertson JD, Gogvadze V, Kropotov A, *et al*. Processed caspase-2 can induce mitochondria-mediated apoptosis independently of its enzymatic activity. *EMBO Rep* 2004; **5**: 643–648.
22. Enoksson M, Robertson JD, Gogvadze V, *et al*. Caspase-2 permeabilizes the outer mitochondrial membrane and disrupts the binding of cytochrome c to anionic phospholipids. *J Biol Chem* 2004.
23. Bykov VJ, Issaeva N, Shilov A, *et al*. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med* 2002; **8**: 282–288.
24. Håkansson A, Zhivotovsky B, Orrenius S, Sabharwal H, Svanborg C. Apoptosis induced by a human milk protein. *Proc Natl Acad Sci USA* 1995; **92**: 8064–8068.
25. Fischer W, Gustafsson L, Mossberg AK, *et al*. Human alpha-lactalbumin made lethal to tumor cells (HAMLET) kills human glioblastoma cells in brain xenografts by an apoptosis-like mechanism and prolongs survival. *Cancer Res* 2004; **64**: 2105–2112.
26. Gustafsson L, Leijonhufvud I, Aronsson A, Mossberg AK, Svanborg C. Treatment of skin papillomas with topical alpha-lactalbumin-oleic acid. *N Engl J Med* 2004; **350**: 2663–2672.
27. Duringer C, Hamiche A, Gustafsson L, Kimura H, Svanborg C. HAMLET interacts with histones and chromatin in tumor cell nuclei. *J Biol Chem* 2003; **278**: 42131–42135.
28. Svensson M, Hakansson A, Mossberg AK, Linse S, Svanborg C. Conversion of alpha-lactalbumin to a protein inducing apoptosis. *Proc Natl Acad Sci USA* 2000; **97**: 4221–4226.
29. Kohler C, Håkansson A, Svanborg C, Orrenius S, Zhivotovsky B. Protease activation in apoptosis induced by MAL. *Exp Cell Res* 1999; **249**: 260–268.
30. Håkansson A, Kidd A, Wadell G, Sabharwal H, Svanborg C. Adenovirus infection enhances in vitro adherence of Streptococcus pneumoniae. *Infect Immun* 1994; **62**: 2707–2714.
31. Zhivotovsky B, Nicotera P, Bellomo G, Hanson K, Orrenius S. Ca^{2+} and endonuclease activation in radiation-induced lymphoid cell death. *Exp Cell Res* 1993; **207**: 163–170 issn: 0014–4827.
32. Armstrong RC, Aja T, Xiang J, *et al*. Fas-induced activation of the cell death-related protease CPP32 Is inhibited by Bcl-2 and by ICE family protease inhibitors. *J Biol Chem* 1996; **271**: 16850–16855.
33. Boise LH, Gonzalez-Garcia M, Postema CE, *et al.* bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 1993; **74**: 597–608.
34. Pugacheva EN, Ivanov AV, Kravchenko JE, *et al.* Novel gain of function activity of p53 mutants: activation of the dUTPase gene expression leading to resistance to 5–fluorouracil. *Oncogene* 2002; **21**: 4595–4600.
35. Bunz F, Dutriaux A, Lengauer C, *et al*. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 1998; **282**: 1497–1501.
36. Nicholson DW, Ali A, Thornberry NA, *et al*. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 1995; **376**: 37–43.
37. Håkansson A, Andreasson J, Zhivotovsky B, *et al.* Multimeric alpha-lactalbumin from human milk induces apoptosis through a direct effect on cell nuclei. *Exp Cell Res* 1999; **246**: 451–460.
38. Kimura H, Cook PR. Kinetics of core histones in living human cells: little exchange of H3 and H4 and some rapid exchange of H2B. *J Cell Biol* 2001; **153**: 1341–1353.
39. Blandino G, Levine AJ, Oren M. Mutant p53 gain of function: Differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. *Oncogene* 1999; **18**: 477–485.
40. Leist M, Jaattela M. Four deaths and a funeral: From caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* 2001; **2**: 589–598.
41. Jaattela M, Tschopp J. Caspase-independent cell death in T lymphocytes. *Nat Immunol* 2003; **4**: 416–423.
42. Kohler C, Gogvadze V, Hakansson A, *et al*. A folding variant of human alpha-lactalbumin induces mitochondrial permeability transition in isolated mitochondria. *Eur J Biochem* 2001; **268**: 186–191.
43. Johnson AJ, Smith LL, Zhu J, *et al*. A novel celecoxib derivative, OSU03012, induces cytotoxicity in primary CLL cells and transformed B-cell lymphoma cell line via a caspase- and Bcl-2-independent mechanism. *Blood* 2005; **105**: 2504–2509.
44. Jendrossek V, Handrick R, Belka C. Celecoxib activates a novel mitochondrial apoptosis signaling pathway. *Faseb J* 2003; **17**: 1547–1549.
45. Elliott K, Ge K, Du W, Prendergast GC. The c-Myc-interacting adaptor protein Bin1 activates a caspase-independent cell death program. *Oncogene* 2000; **19**: 4669–4684.
46. Chi S, Kitanaka C, Noguchi K, *et al*. Oncogenic Ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells. *Oncogene* 1999; **18**: 2281–2290.
47. Yamaki M, Umehara T, Chimura T, Horikoshi M. Cell death with predominant apoptotic features in Saccharomyces cerevisiae mediated by deletion of the histone chaperone ASF1/CIA1. *Genes Cells* 2001; **6**: 1043–1054.
48. Monney L, Otter I, Olivier R, *et al*. Defects in the ubiquitin pathway induce caspase-independent apoptosis blocked by Bcl-2. *J Biol Chem* 1998; **273**: 6121–6131.
49. Mathiasen IS, Sergeev IN, Bastholm L, *et al*. Calcium and calpain as key mediators of apoptosis-like death induced by vitamin D compounds in breast cancer cells. *J Biol Chem* 2002; **277**: 30738–30745.