

Histone Deacetylase Inhibitors Promote the Tumoricidal Effect of HAMLET

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Abstract

Histone deacetylase inhibitors (HDIs) and HAMLET (human α -lactalbumin made lethal to tumor cells) interact with histones, modify the structure of chromatin, and trigger tumor cell death. This study investigated how the combination of HDIs and HAMLET influences cell viability, histone acetylation, and DNA integrity. The pretreatment of tumor cells with HDIs was shown to enhance the lethal effect of HAMLET and the histone hyperacetylation response to HDIs increased even further after HAMLET treatment. HDIs and HAMLET were shown to target different histone domains as HAMLET bound tailless core histones, whereas HDIs modify the acetylation of the histone tail. DNA damage in response to HAMLET was increased by HDIs. The DNA repair response (p21WAF1 expression) was induced by both agonists but abolished when the two agonists were combined. The results suggest that the synergy of HDIs and HAMLET is based on different but converging death pathways, both involving chromatin alterations. We speculate that HAMLET and HDIs might be combined to promote tumor cell death *in vivo*. [Cancer Res 2007;67(23):11327–34]

Introduction

The chromatin undergoes constant structural modifications in living cells, and this dynamic process is essential in controlling gene expression. In eukaryotes, the transcription process is tightly controlled, mainly by the access of transcription factors to the target DNA. Nucleosomes are basic structural elements of chromatin consisting of 146 bp of DNA and a histone octamer formed by H2A, H2B, H3, and H4. The chromatin structure is modified by histone phosphorylation, methylation, acetylation, ubiquitination, and sumoylation and those posttranslational modifications form the basis of the “histone code” (1). Specifically, deacetylated chromatin is compact but histone acetylation has been shown to open the chromatin and to increase the access of transcription factors to DNA. Histone acetylases and histone deacetylases (HDACs) regulate the acetylation or deacetylation of lysine residues in the histone tails (1). Increased histone acetylation attenuates the electrostatic interaction with the negatively charged bases, and decreases the interaction of the histones with DNA,

thereby facilitating the access of transcription factors to target genes (2).

HDACs have emerged as molecular targets for the development of enzymatic inhibitors to treat human cancer. HDACs are generally overexpressed in tumors and promote tumor cell longevity by blocking the transcription of antitumoral genes (2). Many HDAC inhibitors (HDIs) are currently used *in vivo* because of their activity against many human malignancies. For example, Trichostatin A (TSA) and Vorinostat (also known as suberoylanilide hydroxamic acid or SAHA) are active against breast cancer and prostate cancer both *in vivo* and *in vitro* (3, 4), and early phase I/II trials showed that Romidepsin might be useful in the treatment of T cell lymphomas (5). In addition, HDIs have been shown to enhance the activity of other antitumoral drugs in cancer therapy. The mechanism is not fully understood, but the effect has been shown to be additive or synergistic, suggesting that different catalytic pathways may be involved.

HAMLET (human α -lactalbumin made lethal to tumor cells) is a molecular protein-lipid complex with tumoricidal activity (6). HAMLET is formed from human α -lactalbumin, which is the major protein in human milk (7–9). To produce HAMLET, α -lactalbumin is partially unfolded and bound to oleic acid during an ion exchange chromatography process (8, 9). The protein and lipid are both required for tumoricidal activity and structural studies have suggested that HAMLET represents a new type of cytotoxic entity. HAMLET triggers tumor cell death *in vitro* (10–12) but does not kill healthy differentiated cells, and *in vivo* studies have shown that HAMLET is active as a topical agent against skin papillomas (11) and bladder cancers (13). The mechanism(s) of cell death are not fully understood, but HAMLET is rapidly internalized by tumor cells and is translocated to the nuclei, where it accumulates (14). We have identified histones H3, H4, and H2B as HAMLET receptors in the nuclei, and showed that high-affinity interactions between HAMLET and histones perturb the chromatin structure in living tumor cells (14). The interaction of HAMLET with chromatin was proposed to mark the irreversible phase of tumor cell death in response to HAMLET. HAMLET and HDIs thus share the ability to alter the structure and function of chromatin.

This study examined if HDIs modify the cell death response to HAMLET. We also investigated if histone acetylation is modified after HAMLET treatment. The results show that HDIs and HAMLET in combination promote cell death and histone acetylation, and suggest that it might be useful to combine the substances *in vivo*.

Materials and Methods

Reagents. The HDIs TSA and Vorinostat were from Upstate and Alexis, respectively. ATP monitoring ViaLight HS kit was from Cambrex. zVAD-fmk was from Alexis and was added 30 min prior to the experiment at 50 μ mol/L.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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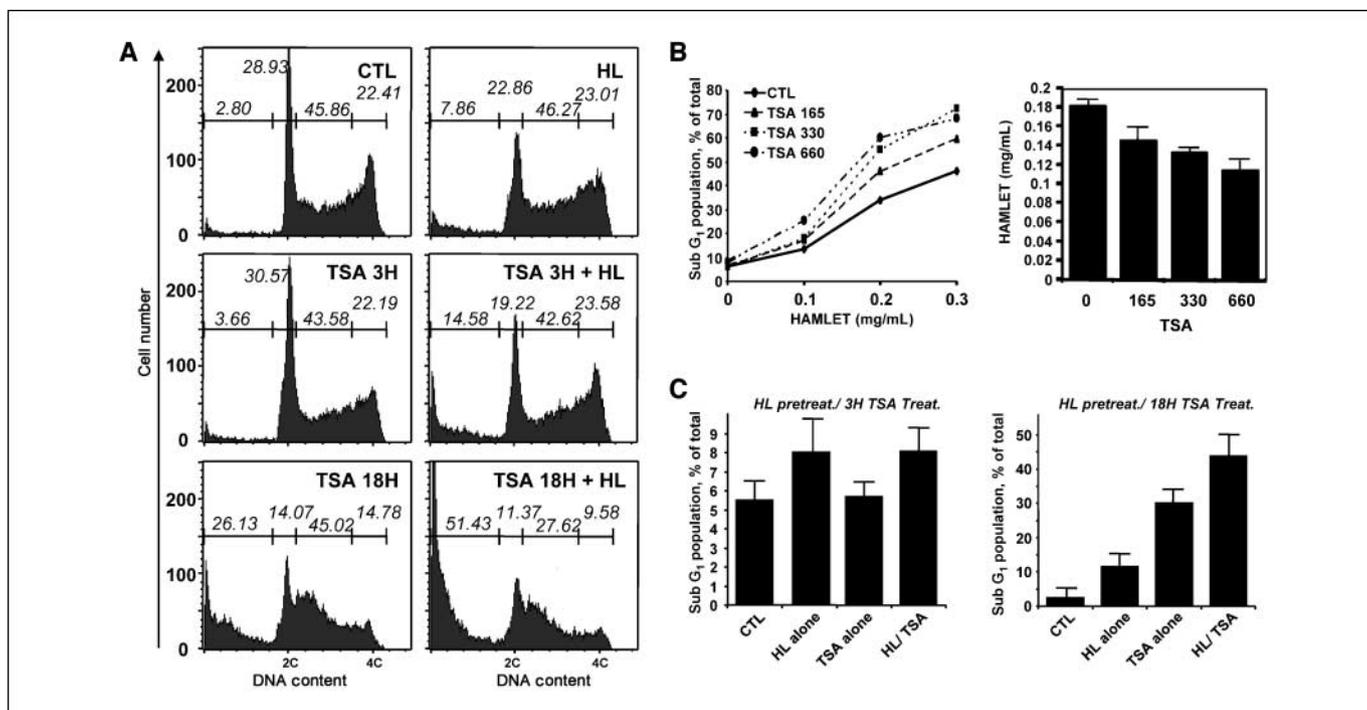


Figure 1. HAMLET and HDIs trigger tumor cell death. **A**, loss of cell viability, quantified by flow cytometry after propidium iodide staining. Jurkat cells were pretreated with TSA (330 nmol/L, 3 or 18 h) and then exposed to HAMLET (HL, 0.15 mg/mL, 3 h). Flow cytometry analysis of the enhanced cell death response to HAMLET and TSA. The proportion of dying cells (sub-G₁) and living cells (G₁, S, G₂) is indicated above the superscript line. Results from one of three experiments. **B**, concentration-dependent increase in Jurkat cell death. TSA pretreatment (165, 330, or 660 nmol/L, 3 h) followed by HAMLET (0.1, 0.2, and 0.3 mg/mL, 3 h). The sub-G₁ population was quantified by flow cytometry (left). Concentration effect calculations (right) of the amount of HAMLET required to kill 30% of the cells at different TSA concentrations. **C**, effect of HAMLET pretreatment on the response to TSA. Jurkat cells were pretreated with HAMLET for 3 h, followed by TSA for 3 or 18 h. The sub-G₁ population was quantified by flow cytometry ($n = 3$). After 3 h, cell death was only caused by HAMLET. After 18 h of TSA treatment, the effect on cell death was additive. Bars, SD.

Protease inhibitors were from Roche Applied Science. Polyvinylidene difluoride membrane (Immobilon-P) was from Millipore. Amplification grade DNase I was from Invitrogen. Primers were purchased from SuperArray Bioscience Corporation.⁴ HAMLET was produced from native purified human milk α -lactalbumin on an oleic acid-conditioned ion exchange matrix as previously described (8).

Cell culture. HeLa and Jurkat cells from the European Cell Culture Collection were cultured as previously described (9). HeLa cells were grown in DMEM with glutamax supplemented with penicillin (100 units/mL)/streptomycin (100 μ g/mL), sodium pyruvate (1 mmol/L; Invitrogen), and 10% FCS, and for cells expressing green fluorescent protein (GFP)-tagged histones, 2 μ g/mL of blasticidin S (Invitrogen).

Confocal microscopy. HeLa cells were grown in Lab-Tek Chamber slides and exposed to HAMLET or TSA as previously described (14). Cells were analyzed in an LSM 510 META confocal microscope (Carl Zeiss, Germany; $\times 63$). The frequency of each chromatin pattern shown is given after counting a minimum of 30 cells.

Flow cytometry. Harvested cells were fixed in 75% ice-cold ethanol (in PBS) for 2 h, centrifuged, washed with PBS, and treated with 0.25% Triton X-100 for 10 min at room temperature, incubated 30 min in swine serum (1% in PBS), and for 3 h with anti-acetyl histone H4 or anti-phosphorylated Ser¹³⁹ histone H2AX (clone JBW301) antibody (1:200, at room temperature). Cells were incubated with FITC anti-rabbit secondary antibodies (1:20 in PBS and 1% bovine serum albumin for 2 h), washed, resuspended in 2.5 μ g/mL of propidium iodide and 250 μ g/mL of RNase A in PBS, and incubated at 4°C overnight. Fluorescence intensity values FL2-A and FL2-W were quantified in a FACScalibur (Becton Dickinson). Red and green emissions from each cell were separated and quantified using standard optics.

Histones. Tailless *Drosophila melanogaster* histones were expressed in *Escherichia coli*, purified, and then assembled into octamers (15). The fold and functional integrity of the histones were confirmed by nucleosome assembly on DNA (data not shown). DNA-A 256-bp fragment containing a sea urchin 5S RNA gene (16) was gel-purified from an *Eco*RI or *Nci*I digest of plasmid pLV405-10 (17). The DNA was end-labeled with [γ -³²P]ATP (Amersham Pharmacia Biotech). Mixtures were analyzed by electrophoresis.

DNA fragmentation. High molecular weight DNA fragments were detected by field-inversion gel electrophoresis (FIGE). Briefly, cells (2×10^6) were embedded in low-melting agarose gel treated by proteinase K. Samples were run by electrophoresis at 180 V in 1% agarose gels in 0.5 TBE (45 mmol/L Tris, 1.25 mmol/L EDTA, 45 mmol/L boric acid; pH 8.0) at 12°C, with the ramping rate changing from 0.8 to 30 s for 24 h, using a forward to reverse ratio of 3:1. Quantification of high molecular fragmented DNA bands was performed using ImageJ software.

Immunoblot. Jurkat cells were extracted in ice-cold PBS and lysed [20 mmol/L Tris-Cl (pH 7.5), 100 mmol/L NaCl, 5 mmol/L MgCl₂, and 0.5% Nonidet P40] supplemented with protease inhibitors. Fifty micrograms of protein extracts were separated, electrotransferred onto a polyvinylidene difluoride membrane, and incubated with anti-p21waf1 polyclonal antibodies (1:2,000) or anti-PPP32 (1:1,000) overnight at 4°C. Goat anti-rabbit antibodies (horseradish peroxidase-conjugated 1:10,000; Dako) were then applied for 1 h at room temperature. Immunoreactive bands were revealed by enhanced chemiluminescence (Amersham).

Reverse transcription-PCR. RNA was DNase-treated with DNase I, cDNA were synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions, except that both random hexamers and Oligo(dT)₂₀ were mixed in the annealing step. Semiquantitative real-time PCR (RT-PCR) used RT² real-time SYBR Green technology from SuperArray Bioscience Corporation⁴ and SmartCycler II apparatus (Cepheid). Expression of target genes was

⁴ <http://www.medprobe.com/>

measured after normalization against glyceraldehyde-3-phosphate dehydrogenase, and values were expressed as the fold increase using the C_T method.

Results

Effects on tumor cell death. The effects of TSA and HAMLET on cell viability were examined by flow cytometry after propidium iodide staining of nuclear DNA. Cells in the sub- G_1 population were identified and defined as dead or dying by apoptosis (18). Cell

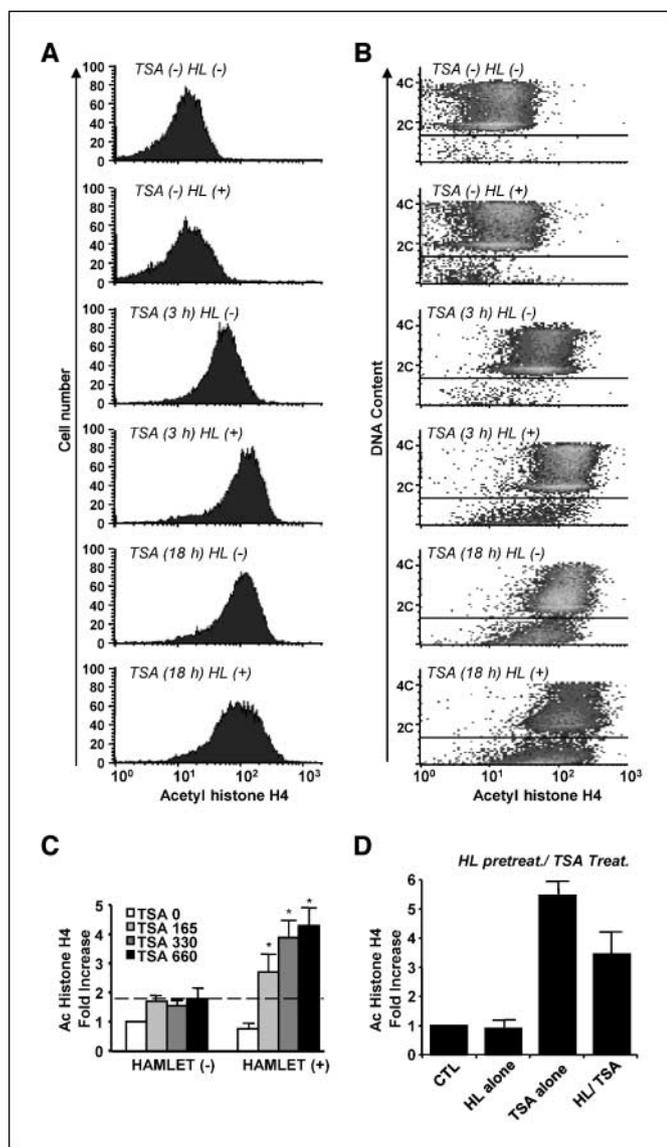


Figure 2. HAMLET and HDIs stimulate histone H4 acetylation. Jurkat cells were pretreated with TSA (330 nmol/L, 3 or 18 h) and then exposed to HAMLET (0.15 mg/mL, 3 h). The increase in histone H4 acetylation was quantified by flow cytometry after staining with specific antibodies. Results from one of four experiments. **A**, histone H4 acetylation levels increase synergistically after TSA pretreatment followed by HAMLET. **B**, acetylation in dying (sub- G_1) or intact cells (G_1 , S , G_2). Density plot with the DNA content on the Y-axis and histone H4 acetylation on the X-axis. **C**, histone H4 acetylation in Jurkat cells pretreated with TSA (165, 330, or 660 nmol/L, 3 h) followed by HAMLET (0.15 mg/mL, 3 h). Columns, mean of at least 5,000 cells in three independent experiments, bars, SD. **D**, HAMLET pretreatment prevents the hyperacetylation response to TSA. Jurkat cells were pretreated with HAMLET (0.15 mg/mL, 3 h) and then exposed to TSA (330 nmol/L, 3 h; columns, mean of at least 5,000 cells from three independent experiments; bars, SD.

viability was also assessed by trypan blue exclusion. Jurkat cells were first exposed to 330 nmol/L of TSA for 3 or 18 h. The longer exposure time increased the sub- G_1 population from 2.8% in the control to 26.1% in TSA-treated cells ($P < 0.01$; Fig. 1A) but the shorter exposure time had no effect (3.7% in TSA-treated cells, not significant). By trypan blue exclusion, the longer TSA exposure was shown to kill 30.2% of the cells compared with 5.3% in the control ($P < 0.01$), but after 3 h, TSA had no effect on cell viability (6.0% in TSA-treated cells, not significant; Supplementary Fig. S1A and B).

Jurkat cells were subsequently exposed to HAMLET, and the effect on viability was examined (Fig. 1B). A HAMLET concentration below the LD_{50} was selected (0.15 mg/mL) and the cells were incubated for 3 h. The short HAMLET exposure time was sufficient to increase the sub- G_1 population from 2.8% in the control to 7.7% ($P < 0.05$), and by trypan blue exclusion, 20% of the cells had died ($P < 0.05$; Supplementary Fig. S1B). The increase in the sub- G_1 population was directly proportional to the decrease in the G_1 peak (28.9% in the control to 22.9% in HAMLET-treated cells). The results show that the death response to HAMLET was direct and more rapid than the response to TSA.

To examine the combined effect of HAMLET and TSA, Jurkat cells were pretreated with TSA (330 nmol/L, 3 h) and exposed to HAMLET (0.15 mg/mL, 3 h). The combined treatment killed a significantly higher number of cells than either agonist alone (Fig. 1A). The sub- G_1 population increased from 2.8% in the control to 14.6% in the TSA/HAMLET-treated cells ($P < 0.01$, compared with HAMLET or TSA). The response to HAMLET was further enhanced by longer TSA pretreatment. After 18 h of pretreatment, HAMLET killed 51.4% of Jurkat cells compared with 26.1% for TSA alone and 7.7% for HAMLET alone (Fig. 1A). Similar results were obtained when trypan blue exclusion was used to quantify cell death. The combined treatment killed 41% of the cells, compared to 6% and 30% for TSA alone at 3 h and 18 h, respectively, and 25% for HAMLET treatment ($P < 0.01$, Supplementary Fig. S1A).

The dose-dependent cell death response is shown in Fig. 1B. In addition to flow cytometry, ATP levels were used to quantify cell death (19). TSA pretreatment increased the lethal effect of HAMLET at concentrations ranging from 0.1 to 0.3 mg/mL. By concentration effect calculations, the effect was shown to be synergistic as a lower concentration of HAMLET was required to kill TSA-pretreated cells [decrease from 0.18 to 0.12 mg/mL (660 μ mol/L TSA) to kill 30% of cells using sub- G_1 quantification (Fig. 1B) and from 0.26 to 0.18 mg/mL to kill 40% of cells using ATP levels; Supplementary Fig. S1C]. The results show that HAMLET and TSA promote cell death in a synergistic manner when TSA is given first.

To investigate if TSA and HAMLET act independently, the order of the agonists was changed. Jurkat cells were pretreated with 0.15 mg/mL of HAMLET for 3 h, followed by TSA for 3 or 18 h (Fig. 1C). TSA had no effect on cell viability at 3 h. HAMLET pretreatment followed by TSA treatment for 18 h further reduced cell viability compared to HAMLET alone, however, and a lethal effect of TSA alone was observed at 18 h. After concentration-median calculation, we conclude that this effect was additive (data not shown). The results suggest that a synergistic effect on cell death is achieved only when HDIs are given before HAMLET.

Effects on histone acetylation. HDIs increase histone acetylation by preventing HDAC activity (20). The effect of HDIs and HAMLET on histone acetylation was analyzed by flow cytometry in

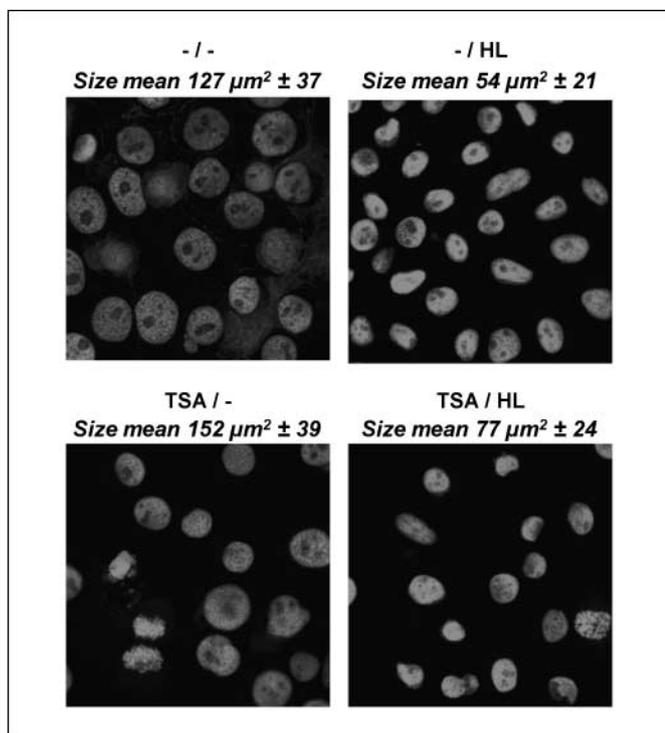


Figure 3. Nuclear morphology in cells treated with HAMLET and TSA. Confocal images of HeLa cells expressing GFP-tagged histone H4 were pretreated with TSA (330 nmol/L, 2 h) and exposed to HAMLET (0.3 mg/mL, 2 h). The influence of these treatments on the morphology of the nuclei as revealed by GFP-tagged histone H4. Quantification of the modification in nuclear size (μm^2). Results are the mean of three experiments (at least 30 cells in each experiment) \pm SD.

Jurkat cells, using antibodies specific for acetylated histone H4. Cells were pretreated with TSA (330 nmol/L, 3 or 18 h) and exposed to HAMLET (0.15 mg/mL, 3 h; Fig. 2). The responses to the combined treatment and to each agonist were compared. TSA increased histone H4 acetylation in a time-dependent manner. A significant increase above background levels had occurred after 3 h ($P < 0.001$), with a further increase after 18 h ($P < 0.001$ compared to 3 h, Fig. 2A and B; Supplementary Fig. S2A). These results were confirmed by using Vorinostat (Supplementary Fig. S2B). Three different concentrations of TSA were tested (165, 330, and 660 nmol/L). The maximum hyperacetylation response to HDIs was reached at the lowest TSA concentration (165 nmol/L, 3 h; Fig. 2C). The results confirmed the known hyperacetylation response to HDIs.

HAMLET treatment per se did not stimulate acetylation in Jurkat cells (Fig. 2) but in combination with TSA, an increase in hyperacetylation was observed ($P < 0.001$ compared with TSA alone). Hyperacetylation increased further when HAMLET was combined with increasing TSA concentrations ($P < 0.001$, Fig. 2). The results show that HAMLET increases the histone hyperacetylation response to HDIs and that this effect is TSA concentration-dependent.

Acetylation in intact and dying cells. To examine if cell death was influenced by hyperacetylation, intact and dying Jurkat cells were compared. Apoptotic cells in the sub- G_1 population were identified by propidium iodide staining of nuclear DNA and H4 acetylation by specific antibody staining and examined by two-dimensional flow cytometry (Fig. 2B). The sub- G_1 control cells showed a lower degree of acetylation than living cells ($P < 0.001$)

but TSA pretreatment increased the acetylation of both the sub- G_1 population and the intact cells (Fig. 2A and B). This effect was especially pronounced after 18 h.

HAMLET treatment increased hyperacetylation in TSA-treated, intact cells ($P < 0.001$, compared with TSA alone; Supplementary Fig. S2A). When dying cells were included, HAMLET/TSA treatment had an effect on acetylation after 3 h but not after 18 h (Fig. 2A and B). Time course experiments to dissociate acetylation from apoptosis showed that they occurred in parallel but that acetylation decreased during the fragmentation of the cells, prior to the sub- G_1 accumulation (data not shown). The results show that living and dying cells share the hyperacetylation response to TSA and HAMLET, suggesting that the effects on acetylation and death occur sequentially.

To investigate if TSA and HAMLET act independently on acetylation, the order of the agonists was changed (Fig. 2D). Jurkat cells were pretreated with 0.15 mg/mL of HAMLET followed by 330 nmol/L of TSA for 3 h. There was no increase in acetylation after 3 h but HAMLET was shown to antagonize TSA hyperacetylation (Fig. 2D). The results show that a combined effect of TSA and HAMLET on acetylation is observed only when cells were exposed to TSA before HAMLET. This implies that TSA exposes targets for HAMLET, whereas targets for TSA are unavailable after HAMLET binding.

Chromatin topology, visualized by confocal microscopy. The effect of HAMLET and TSA on chromatin topology and nuclear size was analyzed by confocal microscopy using stably transfected HeLa cells expressing histone H4-GFP. TSA treatment (330 nmol/L, 4 h) caused a slight increase in nuclear size ($P < 0.05$), whereas HAMLET decreased the nuclear size ($P < 0.001$; Fig. 3). A similar decrease occurred when the cells were exposed to TSA followed by HAMLET ($P < 0.001$ compared with TSA alone or control). The results suggested that TSA treatment opens the chromatin, whereas HAMLET causes chromatin condensation. These observations are compatible with the known ability of HDIs to open up chromatin (20), and with the nuclear condensation in HAMLET-treated cells (12).

HAMLET and TSA target different histone domains. HDIs and HAMLET have each been shown to modify the structure of

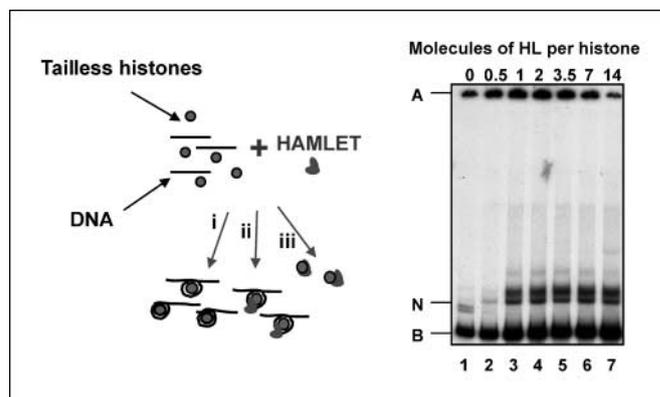
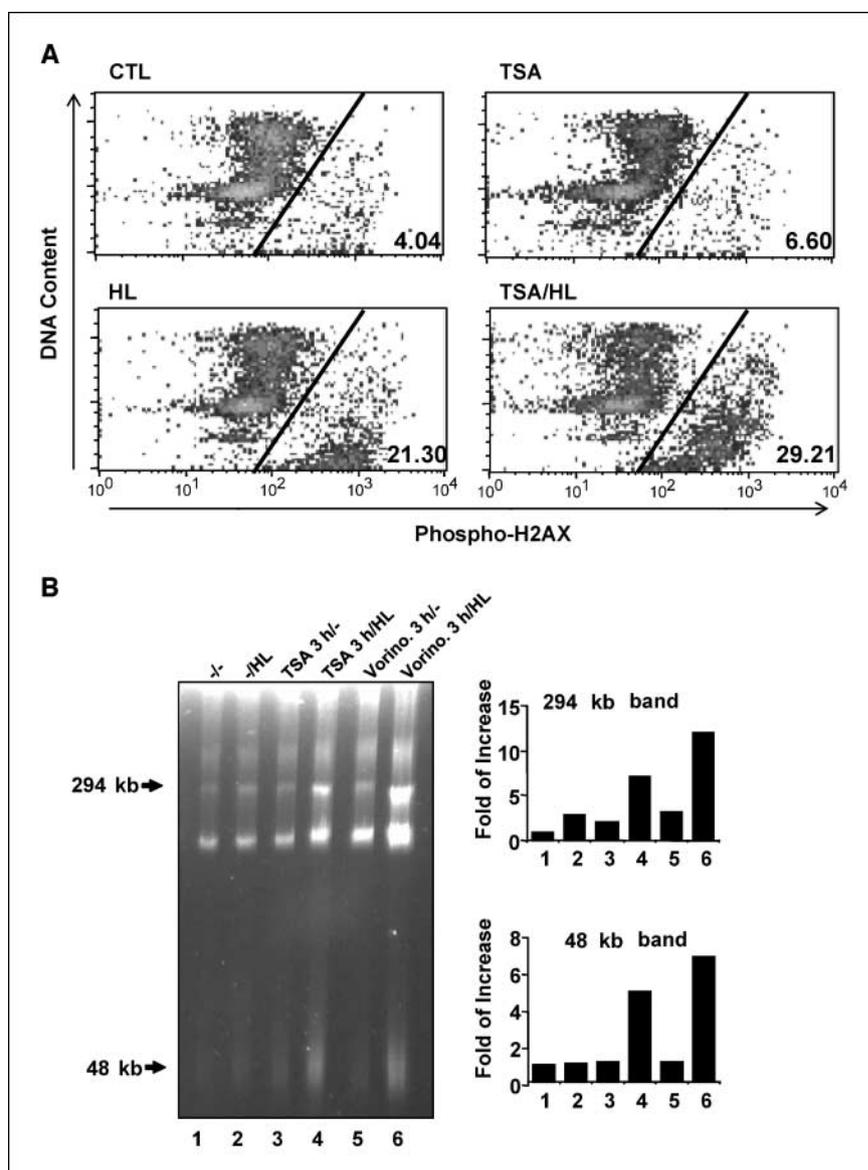


Figure 4. HAMLET binds to tailless histones. HAMLET was added to a mixture of radiolabeled 146 bp DNA fragments and recombinant tailless histones. In the absence of HAMLET, no nucleosomes were formed (lane 1). HAMLET triggered nucleosome assembly resulting in one single nucleosome species (N, lane 2). The band intensity increased with higher HAMLET concentrations, and HAMLET associated with the nucleosomes forming a second band (lanes 3–7). HAMLET also dissolved the unspecific histone-DNA aggregates (A); free probe (B).

Figure 5. Effect of HDIs and HAMLET on DNA damage. **A**, increase in the number of cells with DNA damage after HAMLET treatment of TSA-pretreated cells. Jurkat cells were pretreated with TSA (330 nmol/L, 3 or 18 h) and then exposed to HAMLET (0.15 mg/mL, 3 h). The increase in phosphorylation of Ser¹³⁹ of histone H2AX was quantified by flow cytometry after staining with specific antibodies. Cells were defined as dying (sub-G₁) or intact (G₁, S, G₂), as determined by a density plot with the DNA content on the Y-axis and the phosphorylated histone H2AX on the X-axis. Results from one of three experiments. **B**, accumulation of large DNA fragments in Jurkat cells pretreated with HDIs (330 nmol/L TSA or 2.5 μ mol/L Vorinostat, 3 h) followed by HAMLET (0.15 mg/mL, 3 h). Large DNA fragments were resolved by FIGE (*left*): results from one of three experiments. Bands were quantified using ImageJ software (histograms, *right*).



chromatin in tumor cells. HDIs bind to HDACs, which target the histone tail (1). HAMLET also binds to histones, but the more exact binding specificity is not known. We therefore investigated if the interaction of HAMLET with histones requires the histone tail. For this purpose, mutant *D. melanogaster* core histones lacking the NH₂-terminal histone tails were constructed by deletion of the sequences encoding residues preferentially cleaved from nucleosomal histones by trypsin but leaving behind the intact globular domains. The tailless histones were expressed in *E. coli*, purified, and then assembled into octamers (ref. 15; Fig. 4). Histone assembly on the 146 bp fragments resulted in the formation of nucleosomal core particles (Fig. 4, *band N*), similar to those observed with control histones (14). The results show that HAMLET binds histones independently of the histone tail, confirming that HAMLET and TSA target different histone domains.

Phosphorylated H2AX and DNA fragmentation. DNA damage was quantified as the phosphorylation of Ser¹³⁹ of histone H2AX by two-dimensional flow cytometry, as previously described

(21). Phosphorylated H2AX staining was rare in control cells (4.04%) and in TSA-treated cells (330 nmol/L, 3 h; 6.60%). In contrast, HAMLET induced an increase in phosphorylated H2AX staining (21.30%, $P < 0.01$). The increase was mainly seen in cells with low DNA content, corresponding to the sub-G₁ population (Fig. 5A). When cells were pretreated with TSA for 3 h, a further increase of phosphorylated H2AX (29.21%, $P < 0.05$) was observed in sub-G₁ as well as in G₁ cells.

DNA damage was further examined by FIGE as previously described (22). The accumulation of high molecular weight DNA fragments was recorded (294 and 48 kb; Fig. 5B). HAMLET treatment (0.15 mg/mL, 3 h) increased the formation of 294 kb fragments by 2.8-fold compared to the control. A 7-fold increase was detected after TSA pretreatment (330 nmol/L, 3 h), and compared with controls, a 12-fold increase occurred after Vorinostat pretreatment (2.5 μ mol/L, 3 h). The 48 kb DNA fragments were only observed after combined HDI/HAMLET treatment. These results show that HAMLET alone induces DNA damage and that this effect is enhanced by HDIs.

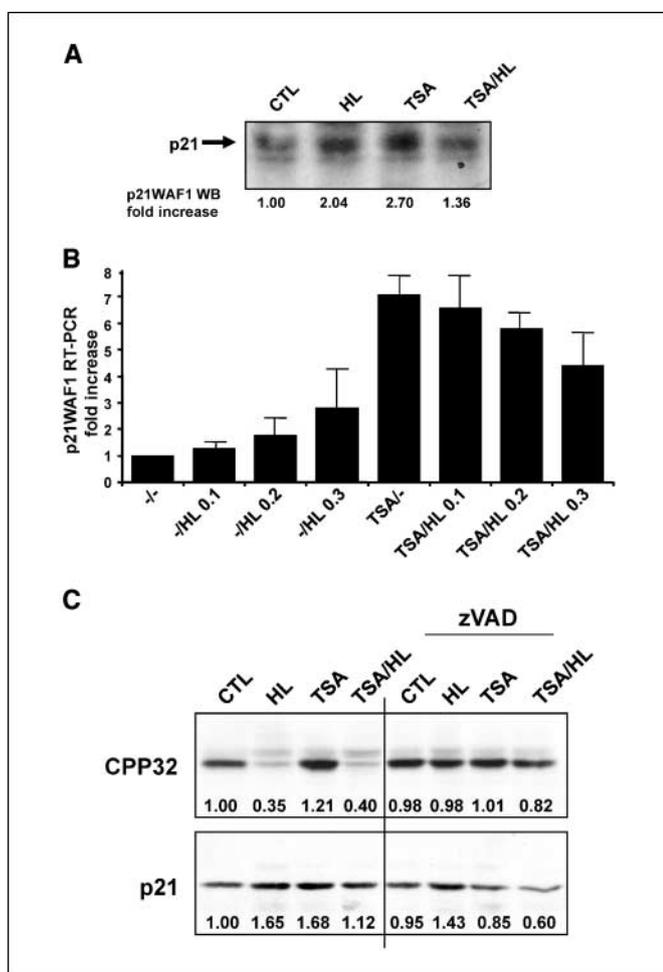


Figure 6. Effect of HDIs and HAMLET on p21WAF1 expression. *A*, enhanced p21WAF1 expression in response to TSA or HAMLET but reduced expression when both agonists were combined. Jurkat cells were pretreated with TSA (330 nmol/L, 3 h) followed by HAMLET (0.15 mg/mL, 3 h) and p21WAF1 expression was analyzed by Western blot (quantified using ImageJ software). Results are the fold increase above the control. *B*, p21waf1 expression was disrupted by the combination of both agonists (RT-PCR, p21WAF1/glyceraldehyde-3-phosphate dehydrogenase fold increase above the control; columns, mean of three independent triplicates), bars, SD. *C*, p21WAF1 expression is not rescued by caspase inhibitors. Following zVAD preincubation (50 μ mol/L, 30 min), Jurkat cells were first pretreated with TSA (330 nmol/L, 3 h) and then exposed to HAMLET (0.15 mg/mL, 3 h). The levels of p21WAF1 were decreased by TSA and HAMLET even when caspases were inhibited (one of two typical experiments).

Combination of HAMLET and HDIs disrupts p21WAF1 expression. p21WAF1 or CDKN1A acts as a regulator of cell cycle progression at the G₁ checkpoint and is mutated or epigenetically down-regulated in many cancers (23, 24). p21WAF1 is a potent cyclin-dependent kinase inhibitor, which binds to and inhibits the activity of cyclin-CDK2 or cyclin-CDK4 complexes and causes a rapid G₁-S arrest. TSA treatment has previously been shown to restore p21WAF1 expression in Jurkat cells and to limit tumor cell progression (25). In addition, the lethality of HDIs is inhibited if p21WAF1 expression is induced, possibly through a mechanism involving the inhibition of the apoptotic caspase cascade (26), which p21WAF1 has been shown to block.

To examine if HAMLET and TSA might influence p21WAF1 expression, Jurkat cells were treated with HAMLET (0.15 mg/mL, 3 h) or with TSA (330 nmol/L, 3 h) and p21WAF1 was quantified by Western blots or by RT-PCR (Fig. 6A). HAMLET induced a 2-fold

increase in p21WAF1 expression, consistent with DNA damage (27). TSA pretreatment induced a 2.7-fold increase in p21WAF1 expression, consistent with chromatin relaxation (28). The increase in p21WAF1 expression was blocked by the combined treatment with TSA followed by HAMLET, as shown by Western blot (Fig. 6A) and by RT-PCR (Fig. 6B). The results show that HAMLET and TSA each trigger a p21WAF1 response, which is inhibited in the presence of both agonists.

To examine if the reduction of p21WAF1 expression was due to caspase cleavage, Jurkat cells were pretreated with zVAD (50 μ mol/L, 30 min), incubated with TSA (330 nmol/L, 3 h) and then exposed to HAMLET (0.15 mg/mL, 3 h; Fig. 6C). HAMLET and TSA/HAMLET induced CPP32 cleavage (caspase 3 native; lines 2 and 4) and zVAD blocked this cleavage (lines 6 and 8). p21WAF1 expression was enhanced by HAMLET in the presence of the zVAD (line 6) but was reduced when both agonists were combined (line 8). The results show that the p21WAF1 response to HAMLET and TSA is caspase-independent.

Discussion

Both HDIs and HAMLET modify the structure of chromatin and trigger tumor cell death. HDIs trigger tumor cell death by restoring the expression of antitumoral genes, whereas HAMLET binds histones with high affinity and alters the chromatin structure. This study compared the effects of HDIs and HAMLET on tumor cell viability, histone acetylation, and DNA integrity when the agonists were combined. HDI pretreatment caused a marked increase in the death response to HAMLET and HAMLET a further increase in the histone hyperacetylation response to HDIs. Cell death was accompanied by increased DNA damage and decreased p21WAF1 expression when the two agonists were combined. We propose that the synergy is based on different but converging death pathways, which involve alterations of the chromatin. As HDIs and HAMLET have documented therapeutic effects, the combined use of HAMLET and HDIs might be of value in the treatment of cancer.

Tumor cell death increased markedly when HDIs and HAMLET were combined and the effect was synergistic when TSA was added before HAMLET. The molecular basis of this synergy is unclear, but several death pathways may be discussed. Caspases are essential for the execution of apoptosis and HDIs have been proposed to trigger cell death via the extrinsic, death receptor-dependent pathway and via the intrinsic mitochondrial death pathway through activation or inhibition of specific Bcl-2 family members, via the regulation of reactive oxygen species and via cell cycle arrest (for a review, see ref. 2). HAMLET acts directly on the mitochondria and triggers an effector caspase response and the nuclear caspase 2 response to HAMLET may contribute to further mitochondrial activation (12). The death of HAMLET-treated cells is caspase-independent, however (9, 12). In the present study, the DNA damage response was shown to be caspase-independent, further supporting the notion that classical apoptosis is not the key to the combined effect of HAMLET and TSA.

TSA facilitated the lethal effect but HAMLET seemed to dominate the execution of the death response. Cells subjected to the combined treatment showed rapid HAMLET-like death kinetics and the chromatin condensation pattern was characteristic of HAMLET-treated cells. HDIs have been shown facilitate the access of other compounds to the chromatin and we speculate that the synergistic effect of HAMLET and TSA might reflect the increased accessibility of histones to HAMLET binding (20, 29). We detected

an increase in nuclear size after TSA pretreatment, but a condensation after HAMLET treatment, consistent with the high-affinity interactions that occur between HAMLET and chromatin. When HAMLET was given first, there seemed to be a reduced accessibility of histone tails for the HDIs, suggesting that the changes in chromatin structure cannot be reversed by TSA. This might explain why TSA had no effect on cell death when HAMLET was added first.

Tumor cells reduce the expression of antitumoral genes by site-specific histone hypoacetylation and by hypermethylation of promoter sequences. HDIs restore gene expression by enhancing the acetylation of histones and by selective DNA demethylation of previously silent antitumoral genes (30). In this study, the histone acetylation response to TSA was confirmed, but HAMLET was found to increase acetylation even further when combined with TSA. To our knowledge, HAMLET is the first example of a compound that increases the hyperacetylation response to HDIs. In previous studies, HDIs have been combined with compounds such as cisplatin and etoposide or with UV irradiation, but no further increase in acetylation has been reported. The combined effect of HDIs and HAMLET was only observed when the cells were pretreated with TSA, but the reverse was not true, as HAMLET pretreatment was shown to partially block the acetylation response to TSA. The combined effect on acetylation cannot be explained, at present, but several mechanisms may be discussed. HAMLET might increase acetylation by activating histone acetylases or endogenous deacetylation inhibitors (like butyrate) or by inhibiting deacetylation by releasing HDACs from chromatin. HAMLET binding to the histone core rather than the tail may cause a compensatory increase in acetylation. Previous studies have shown that histone acetylation correlates with DNA damage following HDI treatment and that apoptosis follows (31). This study suggested that the chromatin was acetylated first and that DNA damage occurred just before DNA fragmentation. Finally, the increase in acetylation might reflect the selective removal by HAMLET of cells with poorly acetylated chromatin but this was not likely, as virtually all cells had hyperacetylated H4 before HAMLET exposure, due to TSA pretreatment. Further studies are needed to understand the molecular basis of hyperacetylation response to HAMLET and TSA.

DNA damage triggers cell death if the damage exceeds the threshold for repair. This might be the case in HAMLET- and TSA-treated cells (Fig. 6), as HAMLET triggered DNA fragmentation, which was enhanced by TSA. Previous studies have shown that HDIs induce phosphorylated H2AX expression (31), but in our case, the increase was small due to the short exposure times. Like *p53*, p21WAF1 is activated by DNA damage and triggers cell cycle arrest

by inducing cell differentiation (32). p21WAF1 transcription and a p53 response (data not shown), were observed, however suggesting that the cells tried to repair damaged DNA. The consequences of p21WAF1 expression in human cancer is controversial due to its antiapoptotic and proapoptotic effects (for review, see refs. 33, 34). Overexpression of p21WAF1 has been shown to enhance the apoptotic response to the chemotherapeutic agent cisplatin in glioma (35) and ovarian carcinoma (36) cell lines. However, increased p21WAF1 expression may also reduce the sensitivity to classic cancer treatments (2) and block the caspase cascade (37). Moreover, antisense expression of p21WAF1 has been shown to improve cell death in response to HDIs (38) and the disruption of p21WAF1 induction during HDI treatment was proposed to lower the apoptotic threshold (26). Previous studies showed that inhibition was due to cleavage by caspases during cell death (39), but the present study showed that the decrease was caspase-independent. The HDI-dependent increase in p21WAF1 was blocked by HAMLET, suggesting that this rescue mechanism is inactivated. In view of the relative tumor selectivity of HAMLET, this effect might be relevant for sensitivity and death.

The combined use of HDIs and HAMLET might be of interest in future cancer therapy. HDIs have shown efficacy in clinical trials, and some HDIs can be taken orally without important side effects (40). The compounds act in synergy with other antitumor treatments, and given their pleiotropic anticancer activities, HDIs will probably be used in combination with other anticancer drugs. In clinical studies, HAMLET has been shown to act topically on skin papillomas and mucosal cancers (11, 13). If combined with HDIs, the use of HAMLET might also be extended to metastatic tumors, with topical HAMLET and systemic HDI treatment, to achieve the optimal combined effect. In this way, the tumor will be attacked from two different routes. Further studies in animal models are needed to evaluate the feasibility of this approach.

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