HAMLET promotes the tumoricidal effect of HDAC inhibitors

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Running title: HAMLET and HDAC inhibitors work synergistically

Keywords: Tumor, Cell death, DNA fragmentation, Lactalbumin, Histone acetylation, HDAC, TSA, Vorinostat, Antitumor drugs, Chromatin modifications, CDKN1A

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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. HDIs pretreatment of tumor cells 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 while HDIs modify the acetylation of the histone tail. The important DNA damage response induced by HAMLET was triggered by HDIs pretreatment. While HAMLET and HDIs alone both induced p21WAF1 restoration, the combination of the two compounds was compromising this expression. 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.

Introduction

The chromatin undergoes constant structural modifications in living cells, and this dynamic process is essential to control 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, ubquitination, and sumoylation and those posttranslational modifications form the basis 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 (HATs) 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 over-expressed in tumors and promote tumor cell longevity by blocking the transcription of anti-tumoral 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 anti-tumoral 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 lipo-protein 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 *in situ,* 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 cells 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 shown 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.*

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 subG1 population were identified and defined as dead or dying by apoptosis (15). Cell viability was also assessed by trypan blue exclusion. Jurkat cells were first exposed to 330 nmol/l of TSA for 3 or 18 hours. The longer exposure time increased the subG1 population from 2.80% in the control to 26.13% in TSA treated cells ($p< 0.01$, **Fig. 1A**) but the shorter exposure time had no effect (3.7 % in TSA treated cells, n.s). By trypan blue exclusion, the longer TSA exposure was shown to kill 30.2% of the cells compared to 5.3% in the control ($p<0.01$.) but after 3 hours, TSA had no effect on cell viability (6.0% in TSA treated cells, n.s., **Supplementary Fig. 1B**).

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 hours. The short HAMLET exposure time was sufficient to increase the subG1 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. 1B**). The increase in the subG1 population was directly proportional to the decrease in the G1 peak (28.93% in the control to 22.86% in HAMLET treated cells). *The results show that the death response to HAMLET is 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 hours) and exposed to HAMLET (0.15 mg/ml, 3 hours). The combined

treatment killed a significantly higher number of cells than either agonist alone (**Fig. 1A**). The subG1 population increased from 2.8% in the control to 14.58% in the TSA/HAMLET treated cells ($p<0.01$, compared to HAMLET or TSA). The response to HAMLET was further enhanced by longer TSA pre-treatment. After18 hours' pretreatment, HAMLET killed 51.43% of Jurkat cells compared to 26.13% 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 41.3% of the cells, compared to 6% for TSA and 25% for HAMLET treatment ($p<0.01$). With trypan blue exclusion, the corresponding numbers for TSA/HAMLET treated cells were 57% compared to 19.7% for HAMLET alone and 24.9% for TSA alone ($p< 0.01$, **Supplementary Fig. 1A**).

The dose dependent cell death response is shown in Fig. 1B. In addition to flow cytometry, ATP levels were used to quantify cell death (16). TSA pretreatment increased the lethal effect of HAMLET at concentrations ranging from 0.1 to 0.3 mg/ml. By concentration effect calculation, the effect was shown to be synergistic as a lower concentration of HAMLET was required to kill TSA pretreated cells (decrease from 0.18 mg/ml to 0.12 mg/ml (660µmol/l TSA) to kill 30% of cells using subG1 quantification **Fig. 1B** and from 0.26 mg/ml to 0.18 mg/ml to kills 40% of cells using ATP levels) (**Supplementary Fig. 1C**). *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 hours, followed by TSA for 3 or 18 hours (**Fig. 1C**). TSA had no effect on cell viability at this time. HAMLET pretreatment followed by TSA treatment for 18 hours further reduced cell viability compared to HAMLET alone, however, and a lethal effect of TSA alone was observed at this time. 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 histone deacetylase activity (17). The effect of HDIs and HAMLET on histone acetylation was analyzed by flow cytometry in Jurkat cells, using histone H4 specific antibodies. Cells were pretreated with TSA (330 nmol/l, 3 or 18 hours) and exposed to HAMLET (0.15 mg/ml, 3 hours (**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 had occurred after 3 hours ($p<0.001$), with a further increase after 18 hours ($p<0.001$ compared to 3 hours, **Fig. 2A, B and Supplementary Fig. 2A**). These results were confirmed by using Vorinostat **(Supplementary Fig. 2B)**. Three different concentrations of TSA were tested (165, 330 and 660). The maximum hyperacetylation response to HDIs was reached at the lowest TSA concentration (165, 3 hours, **Fig. 2C**). The results confirmed the known hyper-acetylation 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 to TSA alone). Hyperacetylation increased further when HAMLET was combined with increasing TSA concentrations (Fig, $p<0.001$). *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 hyper-acetylation, intact and dying Jurkat cells were compared. Apoptotic cells in the subG1 population were identified by propidium iodide staining of nuclear DNA and H4 acetylation by specific antibody staining and examined by 2D flow cytometry (**Fig. 2B)**. The subG1 control cells showed a lower degree of acetylation than living cells ($p<0.001$) but TSA pretreatment increased the acetylation of both the subG1 population and the intact cells (**Fig. 2A and B**). This effect was especially pronounced after 18 hours.

HAMLET treatment increased hyper-acetylation in TSA treated, intact cells ($p<0.001$, compared to TSA alone) (**Supplementary Fig. 2A**). When dying cells were included, there was an effect of HAMLET/TSA on acetylation after 3 but not after 18 hours (**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 subG1 accumulation (data not shown). *The results show that living and dying cells share the hyper-acetylation 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 nM of TSA for 3 hours. There was no increase in acetylation after 3 hours but HAMLET was shown to antagonize TSA hyper-acetylation (**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, while 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 hours) caused a slight increase in nuclear size ($p<0.05$) while 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 to TSA alone or control). *The results suggested that TSA treatment opens the chromatin, while HAMLET causes chromatin condensation. These observations are compatible with the known ability of HDIs to open up the chromatin (17) 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 chromatin in tumor cells. HDIs bind to the histone deacetylases, 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 N-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 assembled into octamers (18), (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.*

Phospho-H2AX and DNA fragmentation

DNA damage was quantified as the phosphorylation of ser139 of Histone H2A.X (19) by 2D flow cytometry, as previously described. Phospho-H2AX staining was rare in control cells (4.04%) and in TSA-treated cells (330nM, 3h, 6.60%). In contrast, HAMLET induced an increased in Phospho-H2AX staining (21.30%, $p<0.01$). The increase was mainly seen in cells with low DNA content, corresponding to the subG1 population (**Fig. 5A**). When cells were pretreated with TSA for 3 hours, a further increased of phospho-H2AX (29.21%, $p<0.05$) was observed in subG1 cells but also in G1.

DNA damage was further examined by FIGE as described (20). The accumulation of high molecular weight DNA fragments was recorded (294kb and 48kb) (Fig. 5B). HAMLET treatment (0.15 ng/ml, 3 hours) increased the formation of 294kb fragments 2.8 fold compared to the control. A 7-fold increase was detected after TSA pretreatment (330 nmol/l, 3 hours) and a 12-fold increase compared to control occurred after Vorinostat pretreatment (2.5 µmol/l, 3 hours). The 48kb 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.*

Combination of HAMLET and HDIs disrupts p21WAF1 expression

p21WAF1 or CDKN1A acts as a regulator of cell cycle progression at the G1 check point and is mutated or epigenetically down-regulated in many cancers (21, 22). *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 (23).

In addition, the lethality of HDIs is inhibited if p21WAF1 expression is induced, possibly through a mechanism involving the restoration of the apoptotic caspase cascade (24), 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 hours) or with TSA (330 nmol/l, 3 hours) and *p21WAF1* was quantified by Western blots or by RT-PCR (**Fig. 6A**). HAMLET induced a two-fold increase in p21WAF1 expression, consistent with DNA damage (25). TSA pre-treatment induced a 2.7 fold increase in p21WAF1 expression, consistent with chromatin relaxation (26). 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 µmol/l, 30 min), incubated with TSA (330 nmol/l, 3 hours) and then exposed to HAMLET (0.15 mg/ml, 3 hours) (**Fig 6C**). HAMLET and TSA/HAMLET induced CPP32 cleavage (caspase 3 native) (line 2 and 4) and zVAD blocked this cleavage (line 6 and 8). p21WAF1 expression was enhanced by HAMLET in the presence of zVAD line but reduced when both agonists were combined (line 8). *The results show that the p21WAF1 response to HAMLET and TSA is caspase independent.*

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Discussion

Both HDIs and HAMLET modify the structure of chromatin and trigger tumor cell death. The HDIs trigger tumor cell death by restoring the expression of anti-tumoral genes, while 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 a further increase in thhe 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 review see (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, DNA damage response was shown to be caspase independent, further supporting the notion that classical apoptosis is not the key to the effects of HAMLET and TSA in this study.

TSA facilitated the lethal effect but HAMLET appeared 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 access of other compounds to the chromatin and we speculate that the synergistic effect of HAMLET and TSA may reflect the increased accessibility of histones for HAMLET binding (17, 27). 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 appeared to be reduced accessibility of histone tails for the HDIs, suggesting that the changes in chromatin structure cannot be reversed by TSA. This may explain why TSA had no effect on cell death when HAMLET was added first.

Tumor cells reduce the expression of anti-tumoral genes by site-specific histone hypoacetylation and by hypermethylation of promotor sequences. HDIs restore gene expression by enhancing acetylation of histones and by selective DNA demethylation of previously silent anti-tumoral genes (28). 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 like Cysplatin 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 and instead, 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 additional

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 (29). This study suggested that cells were 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 phospho-H2A.X expression (29) but in our case, the increase was small due to the short time. Like *p53*, p21WAF1 is activated by DNA damage and triggers cell cycle arrest by inducing cell differentiation (30) and p21WAF transcription and a P53 response were observed, however (data not shown), suggesting that the cells tried to repair damaged DNA. The consequences of of p21WAF1 expression in human cancer is controversial, due to its antiapoptotic and proapoptotic effects (for review see (31, 32)). While overexpression of p21 has been shown to enhance the apoptotic response to the chemotherapeutic agent cisplatin in glioma (33) and ovarian carcinoma (34) cell lines, increased p21WAF1 expression may also reduce the sensitivity to classic cancer treatments (2) and block the caspase cascade (35). Moreover, anti-sense expression of p21WAF1 has been shown to improve cell death in response to HDIs (36) and the disruption of p21WAF1 induction during HDIs treatment was

proposed to lower the apoptotic threshold (24). Previous studies showed that inhibition was due to cleavage by caspases during cell death (37), but the present study showed that the decrease was caspase independent. The HDIs 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 (38). The compounds act in synergy with other anti-tumor treatments and given their pleiotropic anti-cancer activities, HDIs will probably be used in combination with other anti-cancer drugs. In clinical studies, HAMLET has been shown to act topically, on skin papillomas and mucosal cancers. If combined with HDIs, the use of HAMLET might be extended also to metastatic tumors, with topical HAMLET and systemic HDIs 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.

Materials and Methods

Reagents—HDAC inhibitors Trichostatin A (TSA) and Vorinostat (Suberoylanilide hydroxamic acid or SAHA) were from Upstate (Dundee, UK) or Alexis (Lausen, Switzerland) respectively. ATP monitoring ViaLight™ HS kit was from Cambrex (Wokingham, U.K.). zVAD-fmk was from Alexis and added 30 min prior the experiment at 50µmol/l . Protease inhibitors (Roche Applied Science). PVDF membrane (Immobilon-P, Millipore). Deoxyribonuclease I, Amplification Grade (Invitrogen). Primers were purchased from SuperArray Bioscience Corporation (www.medprobe.com). HAMLET was produced from native purified from 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 described (9). HeLa cells were grown in Dulbecco's modified Eagle's medium with glutamax supplemented with penicillin (100 units/ml)-streptomycin (100 µg/ml), sodium pyruvate (1 mM) (Invitrogen), 10% fetal calf serum, and for cells expressing green fluorescent protein (GFP)-tagged histones, 2 µg/ml blasticidin S (Invitrogen).

C**onfocal microscopy**- Hela cells were grown in Lab-Tek Chamber slides and exposed to HAMLET or TSA as described (14). Cells were analysed in an LSM 510 META confocal microscope (Carl Zeiss, Germany, x63). The frequency of each chromatin patterns is shown is given after counting a minimum of 30 cells.

Flow cytometry- Harvested cells were fixed in 75% PBS-ice-cold ethanol for 2 hours, centrifuged, washed with PBS, treated with 0.25% triton X-100 for 10 minutes, rt, incubated 30 min in swine serum (1% in PBS) and for 3 hours with anti-acetyl Histone H4 or anti-

phospho ser139 Histone H2aX (clone JBW301) antibody (1/200, rt), Cells were incubated with FITC anti-rabbit secondary antibodies (1/20 in PBS, 1% BSA for 2 hours), washed, resuspended in 2.5 µg/ml of PI and 250 µg/ml Rnase A in PBS, and incubated at 4° overnight. Fluorescence intensity values FL2-A and FL2-W were quantified in a Facscalibur (Becton Dickinson, San Jose, CA). Red and green emissions from each cell were separated and quantified using standard optics.

Histones- Tailless Drosophila melanogaster histones were expressed in *E. coli,* purified and assembled into octamers (18). 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 (39) was gel-purified from an EcoR1 or Nci1 digest of plasmid pLV405–10 (40). The DNA was end-labeled with [γ-32P] ATP (Amersham Pharmacia biotech, UK). Mixtures were analysed 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 mM Tris, 1.25 mM EDTA, 45 mM boric acid, pH 8.0), at 12°C, with the ramping rate changing from 0.8 s 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 mM Tris-Cl (pH 7.5), 100 mM NaCl, 5 mM MgCl2, 0.5% Nonidet P-40] supplemented with protease inhibitors. 50 µg of protein extracts were separated, electrotransferred onto a PVDF membrane, and

incubated with anti-p21waf1 polyclonal antibodies (1/2000) or anti-CPP32 (1/1000) overnight at 4°C. Goat anti-rabbit antibodies (hrp-conjugated 1:10,000) (Dako) were then applied for 1 hour at room temperature. Immunoreactive bands were revealed by enhanced chemiluminescence (ECL, Amersham).

RT-PCR- RNA was DNase treated with Deoxyribonuclease 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. Real-time semi-quantitative PCR (sqRT-PCR) used RT^2 Real-time SYBR Green technology from SuperArray Bioscience Corporation (www.medprobe.com) and SmartCycler II apparatus (Cepheid, Sunnyvale, CA). Expression of target genes was measured after normalization against GAPDH, and values were expressed as the fold increased using the C_T method.

Acknowledgments

We thank L Gustafsson, C Moreilhon and S Aits for comments and help with confocal microscopy and R J Tallarida for isobologram calculations. This study was supported by the American Cancer Society (the Sharon D. Lund foundation grant), the Swedish Cancer Society, the Swedish Pediatric Cancer Foundation, the Medical Faculty (Lund University), the Segerfalk Foundation, The Fondation de la Recherche Médicale (FRM, Paris), The Gunnar Nilsson Cancer Foundation, the Anna-Lisa and Sven-Erik Lundgren Foundation for Medicl Research, the Knut and Alice Wallenberg Foundation, the Lund City Jubileumsfond, the John and Augusta Persson Foundation for Medical Research, the Maggie Stephens Foundation, the Inga-Britt and Arne Lundbergs foundation, the HJ Forssman Foundation for Medical Research and the Royal Physiographic Society.

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Figure Legends

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 hours) and then exposed to HAMLET (HL, 0.15 mg/ml, 3 hours). FACS analysis of the enhanced cell death response to HAMLET and TSA. The proportion of dying cells (subG1) and living cells (G1, S, G2) is indicated above the superscript line. One typical experiment out of three.

B. Concentration dependent increase in Jurkat cell death. TSA pretreatment (165, 330 or 660 nM, 3 hours) followed by HAMLET (0.1, 0.2 and 0.3 mg/ml, 3 hours). The sub-G1 population was quantified by flow cytometry (left panel). Concentration-effect calculations (right panel) shown as the concentration of HAMLET required to kill 30% of the cells at different TSA concentration.

C. Effect of HAMLET pretreatment on the response to TSA. Jurkat cells were pretreated with HAMLET for 3 hours, followed by TSA for 3 or 18 hours. The sub-G1 population was quantified by flow cytometry (n=3). After 3 hours, cell death was only caused by HAMLET. After 18 hours of TSA treatment the effect on cell death was additive.

Figure 2. HAMLET and HDIs stimulate histone H4 acetylation

Jurkat cells were pretreated with TSA (330 nmol/l, 3 or 18 hours) and then exposed to HAMLET (0.15 mg/ml, 3 hours). The increase in histone H4 acetylation was quantified by flow cytometry after staining with specific antibodies. Results represent one of four typical experiments. **A**. Histone H4 acetylation levels increase synergistically after TSA pretreatment followed by HAMLET. **B**. Acetylation in dying (subG1) or intact cells (G1, S, G2). 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 660nm/l, 3 hours) followed by HAMLET (0.15 mg/ml, 3 hours). Results are the means of at least 5000 cells in three independent experiments.

D. HAMLET pretreatment prevents the hyperacetylation response to TSA. Jurkat cells were pretreated with HAMLET (0.15 mg/ml, 3 hours) and then exposed to TSA (330 nmol/l, 3 hours, means± SDs of at least 5000 cells, three independent experiments.

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 hours) and exposed to HAMLET (0.3 mg/ml, 2 hours. Panel shows the influence of these treatments on the morphology of the nuclei as revealed by GFP-tagged histone H4. Quantification the modification in nuclear size (μm^2). Results are the means of at least 30 cells in each experiment.

Figure 4. HAMLET binds to tailless histones

HAMLET was added to a mixture of radio labeled 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, designated N (lane 2). The band intensity increased with higher HAMLET concentrations, and HAMLET associated with them to form a second band (lanes 3 – 7). HAMLET also dissolved the unspecific histone-DNA aggregates (A). Free probe is denoted by B.

Figure 5. Effect of HDIs and HAMLET on DNA damage

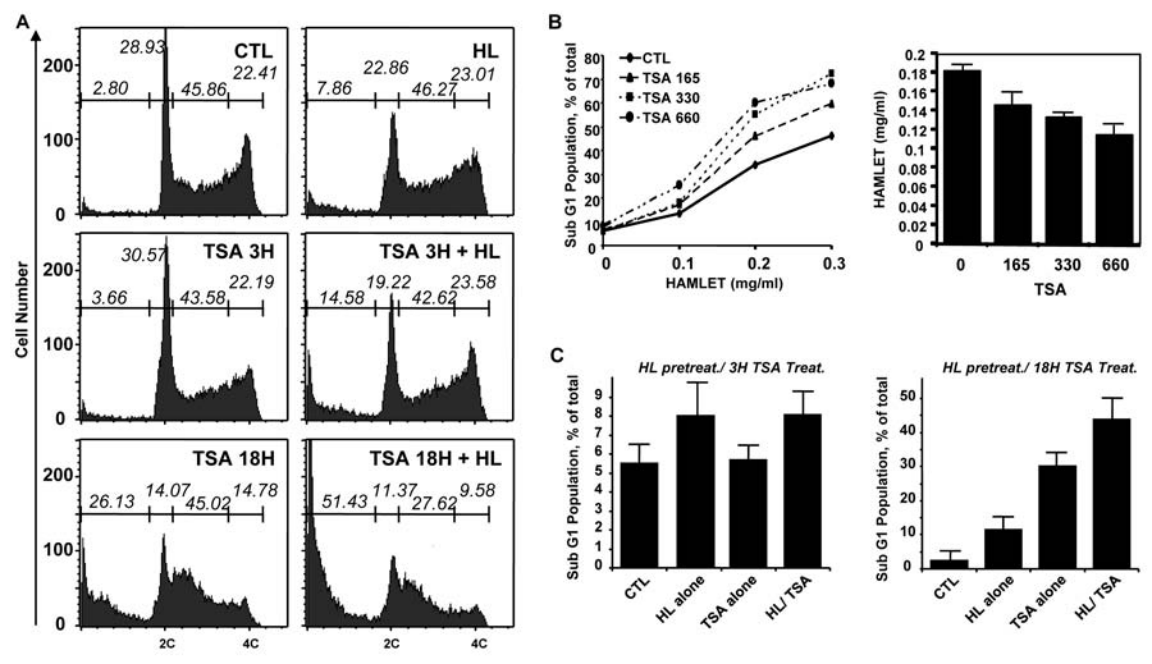
A. Increase in the number of cells with DNA damage after HAMLET treatment of TSA pretreated cells. Jurkat cells were pretreated with TSA (330 nmol/l, 3 or 18 hours) and then

exposed to HAMLET (0.15 mg/ml, 3 hours). The increase in phophorylation of ser139 of histone H2A.X was quantified by flow cytometry after staining with specific antibodies. Cells were defined as dying (subG1) or intact (G1, S, G2), as determined by a density plot with the DNA content on the Y-axis and the phosphor-histone H2A.X on the X-axis. Results represent one typical experiment out of three.

B. Accumulation of large DNA fragments in Jurkat cells pretreated with HDIs (TSA 330 nmol/l or Vorinostat 2.5 µmol/l, 3 hours) followed by HAMLET (0.15 mg/ml, 3 hours). Large DNA fragment were resolved by field-inversion gel electrophoresis (FIGE) (left panel): Results represent one typical experiment out of three. Bands were quantified using ImageJ software (histograms, right panel).

Figure 6. Effect of HDIs and HAMLET 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 hours) followed by HAMLET (0.15 mg/ml, 3 hours) 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/GAPDH fold increase above the control, mean of three independent triplicates). C. p21WAF1 expression is not blocked by caspase inhibitors. Following zVAD pre-incubation (50µmol/l, 30 min), Jurkat cells were first pretreated with TSA (330 nmol/l, 3 hours) and then exposed to HAMLET (0.15 mg/ml, 3 hours). The levels of p21WAF1 were decreased by TSA and HAMLET even when caspases were inhibited (one of two typical experiments).


A
Cell Number
CTL
28.93
2.80
45.86
22.41
HL
22.86
7.86
46.27
23.01
TSA 3H
30.57
3.66
43.58
22.19
TSA 3H + HL
19.22
14.58
42.62
23.58
TSA 18H
26.13
14.07
45.02
14.78
TSA 18H + HL
51.43
11.37
27.62
9.58
2C
4C
B
Sub G1 Population, % of total
CTL
TSA 165
TSA 330
TSA 660
HAMLET (mg/ml)
HAMLET (mg/ml)
TSA
C
HL pretreat./ 3H TSA Treat.
HL pretreat./ 18H TSA Treat.
Sub G1 Population, % of total
CTL
HL alone
TSA alone
HL/ TSA

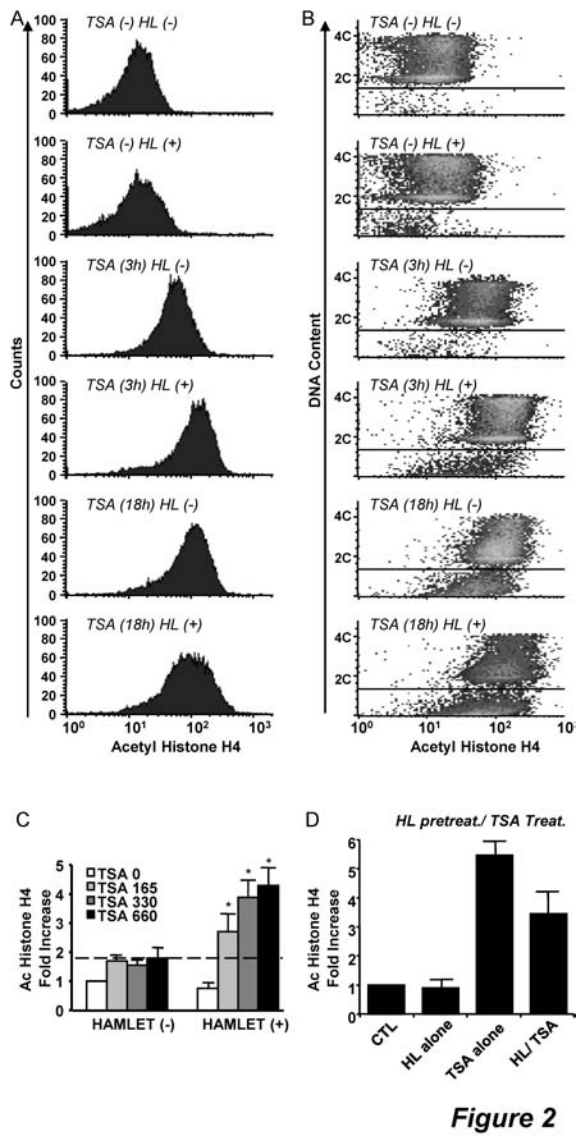
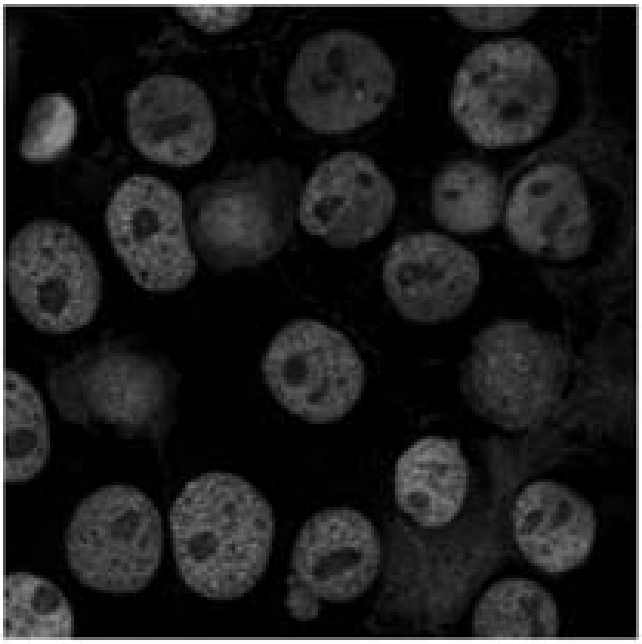

A
TSA (-) HL (-)
TSA (-) HL (+)
TSA (3h) HL (-)
TSA (3h) HL (+)
TSA (18h) HL (-)
TSA (18h) HL (+)
Counts
Acetyl Histone H4
B
DNA Content
4C
2C
C
Ac Histone H4 Fold Increase
TSA 0
TSA 165
TSA 330
TSA 660
HAMLET (-)
HAMLET (+)
D
HL pretreat./ TSA Treat.
CTL
HL alone
TSA alone
HL/ TSA

Figure 2

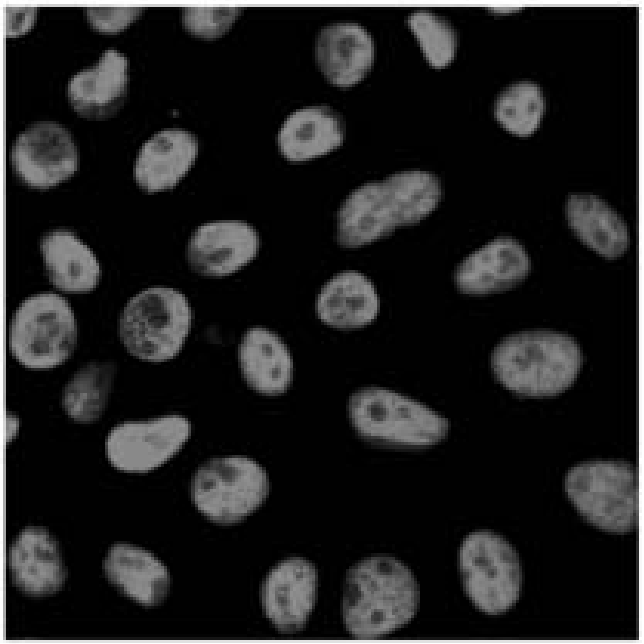
- / -

Size Mean 127 µm² ± 37



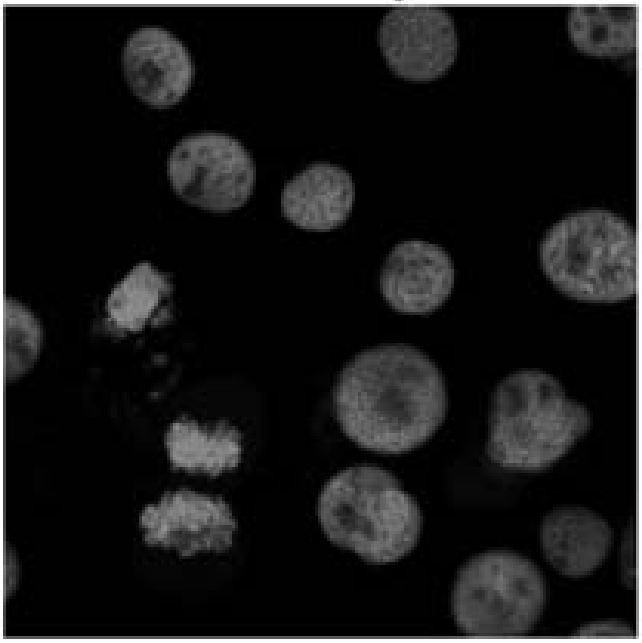
- / HL

Size Mean 54 µm² ± 21



TSA / -

Size Mean 152 µm² ± 39



TSA / HL

Size Mean 77 µm² ± 24

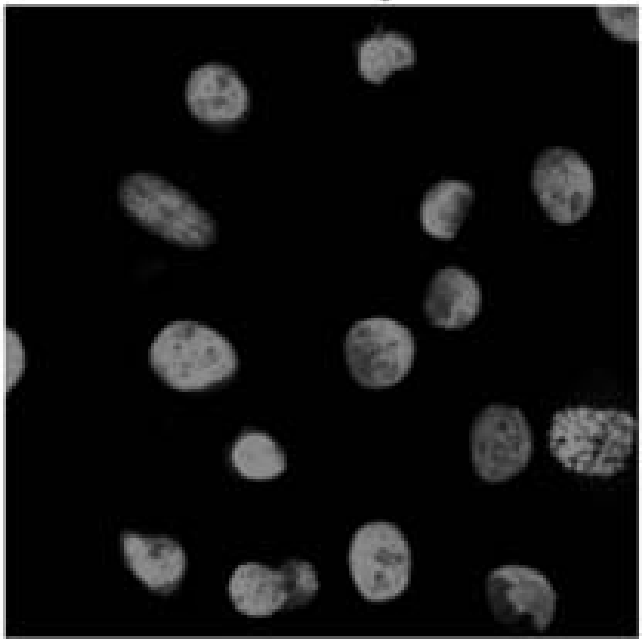
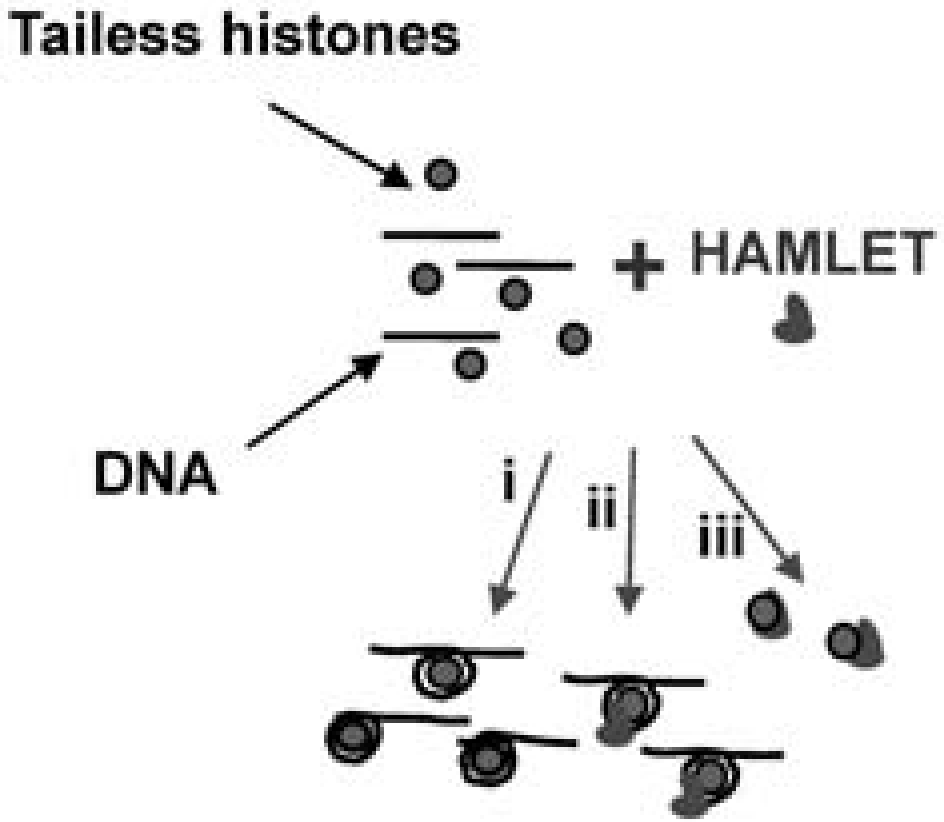


Figure 3


Tailess histones
+ HAMLET
DNA
i
ii
iii

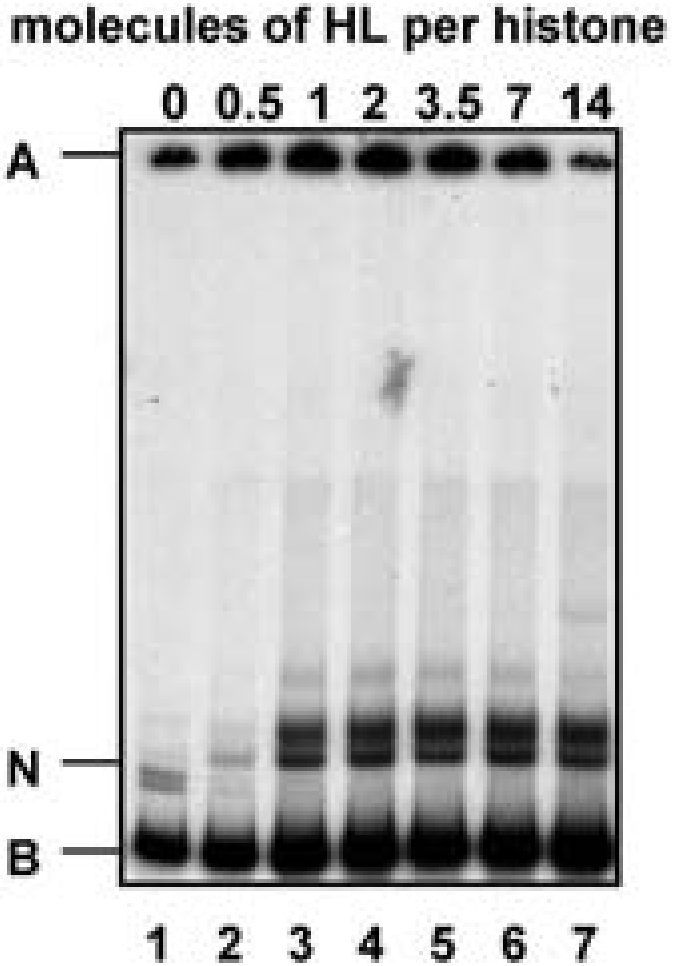

molecules of HL per histone
0 0.5 1 2 3.5 7 14
A
N
B
1 2 3 4 5 6 7

Figure 4

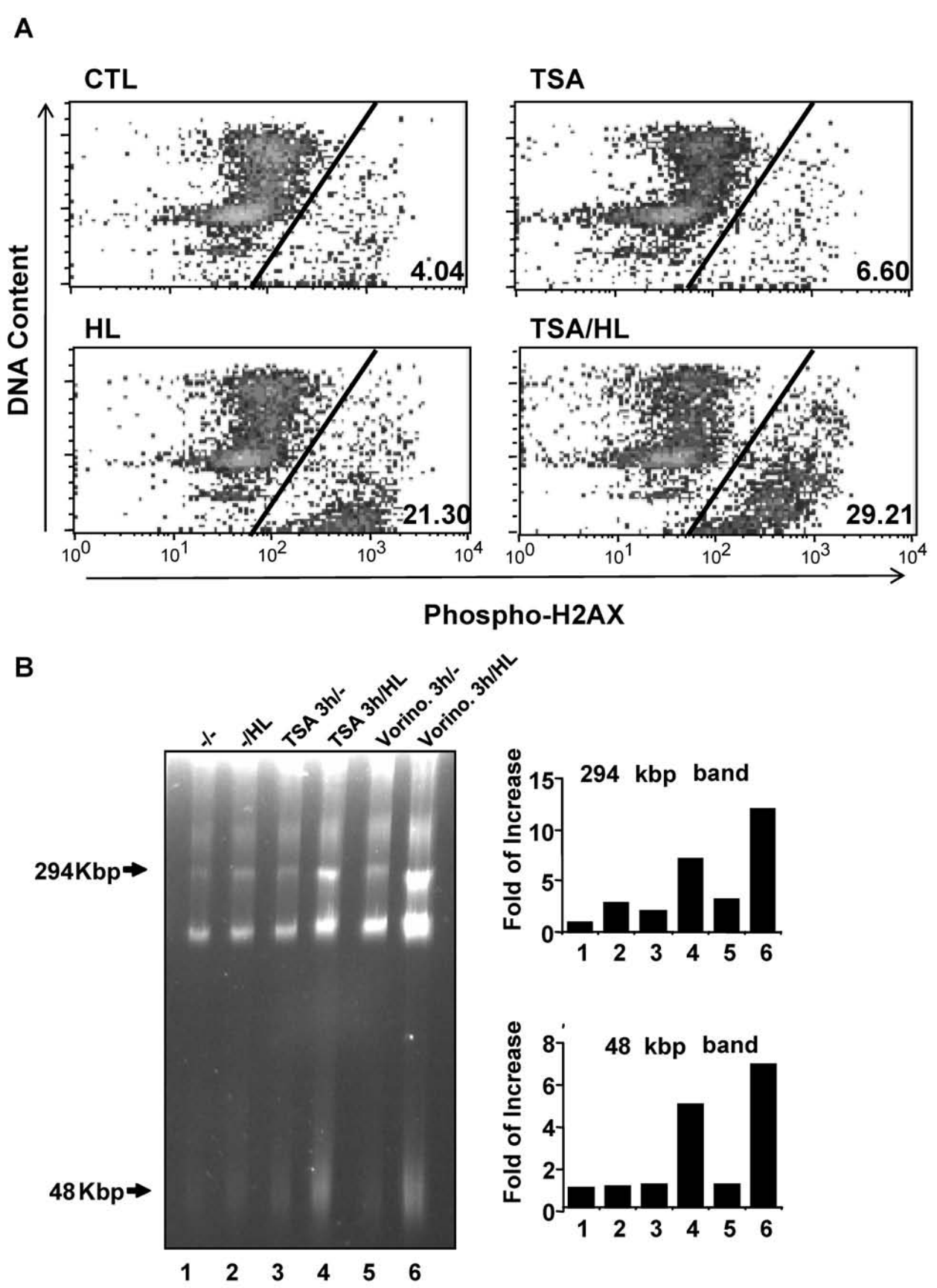

A
CTL
TSA
HL
TSA/HL
4.04
6.60
21.30
29.21
DNA Content
Phospho-H2AX
B
-/-
-/HL
TSA 3h/-
TSA 3h/HL
Vorino. 3h/-
Vorino. 3h/HL
294Kbp
48Kbp
1 2 3 4 5 6
294 kbp band
Fold of Increase
48 kbp band
Fold of Increase

Figure 5

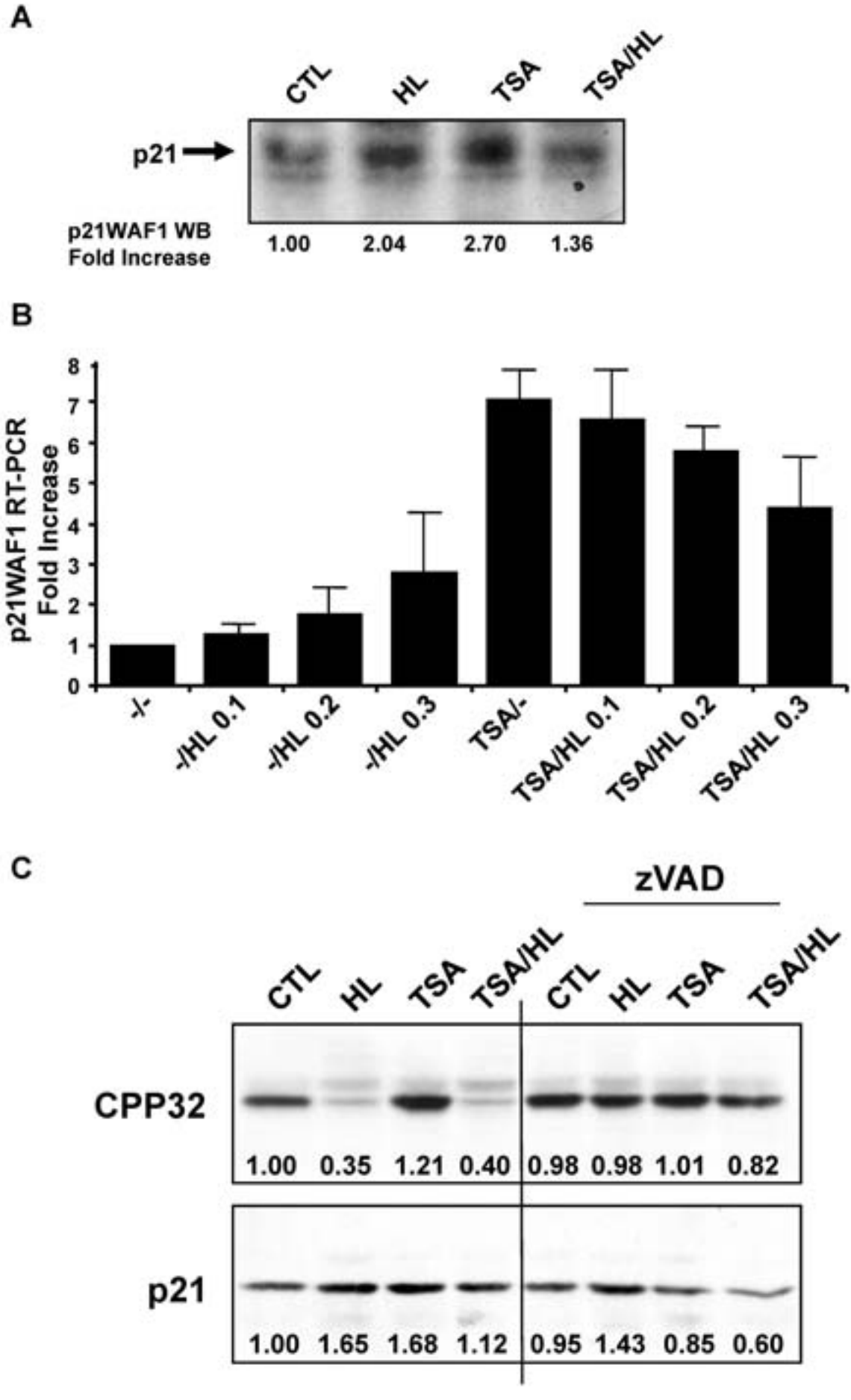

A
CTL
HL
TSA
TSA/HL
p21
p21WAF1 WB
Fold Increase
1.00
2.04
2.70
1.36
B
p21WAF1 RT-PCR
Fold Increase
0
1
2
3
4
5
6
7
8
-/-
-/HL 0.1
-/HL 0.2
-/HL 0.3
TSA/-
TSA/HL 0.1
TSA/HL 0.2
TSA/HL 0.3
C
zVAD
CTL
HL
TSA
TSA/HL
CTL
HL
TSA
TSA/HL
CPP32
1.00 0.35 1.21 0.40 0.98 0.98 1.01 0.82
p21
1.00 1.65 1.68 1.12 0.95 1.43 0.85 0.60

Figure 6