Histone deacetylase inhibitors induce cell death and enhance the susceptibility to ionizing radiation, etoposide, and TRAIL in medulloblastoma cells

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Received September 23, 2005; Accepted November 16, 2005

Abstract. Histone deacetylase inhibitors (HDIs) are a promising new class of antineoplastic agents with the ability to induce apoptosis and growth arrest of cancer cells. In addition, HDIs have been suggested to enhance the anticancer efficacy of other therapeutic regimens, such as ionizing radiation (IR) or chemotherapy. The objective of this study was to evaluate the activity of HDIs against medulloblastoma cells when applied either as single agents or in combination with IR, cytostatics, or TRAIL. The HDIs, suberoyl anilide hydroxamic acid (SAHA), sodium butyrate, and trichostatin A, were examined for their effects on the medulloblastoma cell lines, DAOY and UW228-2. We found that treatment with HDIs induced the dissipation of mitochondrial membrane potential, activation of caspase-9 and -3 and, consequently, apoptotic cell death. Moreover, all three HDIs significantly enhanced the cytotoxic effects of IR in DAOY cells. Likewise, treatment with SAHA markedly augmented the cytotoxicity of etoposide, while it had no effect on vincristine-mediated cell death. HDIs also potently increased the killing efficiency of TRAIL. TRAIL-induced, but not SAHA-induced, cell killing could be prevented by the caspase-8 inhibitor, z-IEDT-fmk. We conclude that HDIs may be useful for the treatment of medulloblastoma as monotherapy and particularly when given in combination with IR, appropriate cytostatics, or TRAIL.

Introduction

Medulloblastoma is the most common brain tumor of childhood. It is a highly malignant neoplasia and, despite aggressive multimodality treatment with surgery, ionizing radiation (IR), and chemotherapy, more than a third of children with medulloblastoma die from the disease within 5 years of diagnosis (1). Moreover, survivors of medulloblastoma commonly have severe treatment-induced neurocognitive sequelae (2). Hence, more effective treatment strategies aimed both at improving the chance of survival and at reducing therapy-related long-term side-effects are urgently needed for this disease.

Currently, considerable attention focuses on histone deacetylase inhibitors (HDIs) as antineoplastic agents. HDIs are a novel class of relatively specific anticancer drugs which were originally identified by their capacity to reverse the transformed phenotype (3). Subsequently, they have been shown to prevent proliferation, activate differentiation, and/or induce apoptosis of tumor cells. HDIs function by inhibiting histone deacetylases, resulting in the accumulation of acetylated histones, in turn leading to an increase in transcriptionally active chromatin (4). In so doing, they reactivate the gene expression of dormant tumor suppressor genes, such as *CDKN1A* (p21) (5). However, in spite of the growing interest in these agents, the molecular basis underlying their anticancer action is not fully understood.

Recently, HDIs have been introduced as chemotherapeutic compounds. As such, their antitumor activity was observed in cell lines originating from different types of human cancer, including neuroblastoma (6), breast cancer (7), prostate cancer (8), and renal cancer (9). In addition, studies on mice xenograft models of human neoplasia have demonstrated anticancer effects of HDIs on colorectal, lung, pancreatic and ovarian cancer (10) as well as prostate cancer (8). Most notably, they have been shown both *in vitro* and *in vivo* to affect cancer cells while leaving normal cells comparatively unscathed (8,11). The clinical potential of these agents has been documented by several Phase I trials of different HDIs in patients with solid tumors or leukemias (12-17). Various HDIs have been evaluated in brain tumors in a few studies (18-22) but, with the exception of MS-275 (23) and phenylbutyrate and phenylacetate (24), not in pediatric medulloblastoma.

In the present study, we assessed the effects of three different HDIs belonging to two structural classes, the hydroxamic acids, suberoylanilide hydroxamic acid (SAHA) and

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Key words: etoposide, histone deacetylase inhibitors, medulloblastoma, radiotherapy, TRAIL

trichostatin A (TSA), and the carboxylic acid, sodium butyrate (NaB), in the medulloblastoma cell lines, DAOY and UW228-2. Our results show that all three compounds elicit cell death in both cell lines. Even more importantly, we demonstrate that HDIs are capable of enhancing the cytotoxic activity of IR, etoposide, or TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) in medulloblastoma cells.

Materials and methods

Reagents. SAHA, TSA, and the pan-caspase inhibitor, z-VAD-fmk, were purchased from Alexis (Grünberg, Germany). NaB, etoposide, and vincristine were purchased from Sigma (Deisenhofen, Germany). TRAIL was purchased from Peprotech (Rocky Hill, NJ, USA). The caspase-8 inhibitor, z-IETD-fmk, was purchased from Merck Biosciences (Schwalbach, Germany).

Cell culture. DAOY and UW228-2 medulloblastoma cells were a gift from Dr M. Grotzer (Zurich, Switzerland) and were maintained in Improved MEM Zinc Option (Invitrogen, Karlsruhe, Germany) or DMEM medium, respectively, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (DMEM and supplements were purchased from Biochrom, Berlin, Germany). Cells were cultivated at 37˚C in a humidified 5% CO_2 incubator and routinely passaged when 90-95% confluent. Cell viability was determined by the trypan blue exclusion test. Cells were regularly inspected to be free of mycoplasma with mycoplasma detection reagents from Roche (Mannheim, Germany). To inhibit the activation of caspases, z-VAD-fmk was applied 1 h before treatment with HDIs.

Ionizing radiation. Cells were exposed to IR using a Siemens Mevatron MXE linear accelerator (Siemens, Concord, CA, USA) delivering photon beams of 6 MV.

Cytofluorometric analysis of cell death. To determine cell death, cells were harvested after 24- to 72-h cultivation in the presence of HDIs, followed by a 5-min incubation in 2 μg/ml propidium iodide (PI) (Sigma) in PBS at 4˚C in the dark. PI uptake was assessed by flow cytometry analysis on a Becton Dickinson (Heidelberg, Germany) FACScalibur using CellQuest software. In each sample, 10,000 cells were analyzed; data were gated to exclude debris.

Cytofluorometric analysis of DNA content. To measure the DNA content, cells were analyzed for PI incorporation into DNA. Cells were harvested 24-72 h after treatment with HDIs, washed twice with PBS and fixed in 70% ethanol at -20˚C for at least 30 min. After centrifugation, cells were resuspended in PBS containing 1% glucose, 50 μg/ml RNase A (Roche) and 50 μg/ml PI and incubated in the dark at room temperature for 30 min. Flow cytometry analysis was performed on a FACScalibur using CellQuest software. In each sample, 20,000 cells were analyzed; data were gated to exclude debris. Sub-G_1, G_1 and G_2/M phase cells were calculated from the DNA content histograms.

Cytofluorometric analysis of mitochondrial transmembrane potential ($\Delta\Psi_m$). $\Delta\Psi_m$ was determined by assessing the accumulation of the cationic lipophilic fluorochrome, 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], in the mitochondrial matrix. Twenty-four hours after treatment with HDIs, cells were incubated with 50 nM $DiOC_6(3)$ (Molecular Probes, Eugene, OR, USA) at 37˚C for 30 min. After washing, 10,000 cells were analyzed using a FACScalibur and CellQuest software. Data were gated to exclude debris.

Caspase-3 and -9 activity. Caspase activity was measured 24 h after treatment with HDIs using the synthetic fluorogenic substrates, Ac-DEVD-AFC or Ac-LEHD-AFC (Bachem, Heidelberg, Germany), for determining caspase-3 and -9 activity, respectively. Cells were lysed in 10 mM Tris-HCl, 10 mM NaH_2PO_4/$NaHPO_4$ (pH 7.5), 130 mM NaCl, 1% Triton-X-100, and 10 mM $Na_4P_2O_7$ and then incubated with 20 mM HEPES (pH 7.5), 10% glycerol, 2 mM DTT and 25 μg/ml Ac-DEVD-AFC or 25 μg/ml Ac-LEHD-AFC at 37˚C for 2 h. The release of trifluoromethylcoumarin (AFC) was analyzed on a Wallac Victor fluorometer (Perkin-Elmer, Rodgau-Jügesheim, Germany) using an excitation/emission wavelength of 390/510 nm. Relative caspase activity was calculated as a ratio of emission of treated cells to untreated cells.

Western blot analysis. Cells were lysed on ice for 15 min in 40 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS supplemented with a protease inhibitor cocktail (Roche) followed by brief sonification. Protein concentration was assayed using bicinchoninic acid (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. For immunoblotting, 30 μg of total cellular protein per lane was separated by standard SDS-PAGE on 10% gels and electrophoretically transferred to PVDF membranes (Millipore, Eschborn, Germany). After blocking in PBS containing 5% dry milk and 0.05% Tween-20, acetylated histone H3 was immunodetected using rabbit anti-acetylated histone H3 polyclonal antibody (dilution 1:40,000; Upstate Biotechnology, Lake Placid, NY, USA). Even loading of protein was verified by detection of GAPDH using mouse anti-GAPDH monoclonal antibody (dilution 1:10,000; Bio-Design International, Saco, ME, USA). Peroxidase-conjugated goat anti-rabbit or anti-mouse IgGs (dilution 1:25,000; Dianova, Hamburg, Germany) followed by enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany) were used for detection.

Statistical analysis. Statistical significance of differences between experimental groups was determined using the paired two-tailed Student's t-test.

Results

HDIs induce cell death in medulloblastoma cells. To investigate possible cytotoxic effects of HDIs on medulloblastoma cells, we initially monitored cell killing by assessing the integrity of the cell membrane by cytofluorometric analysis of PI uptake. DAOY and UW228-2 cells were cultured without or with SAHA (2-20 μM), NaB (1-10 mM), or TSA (0.2-2 μM) for

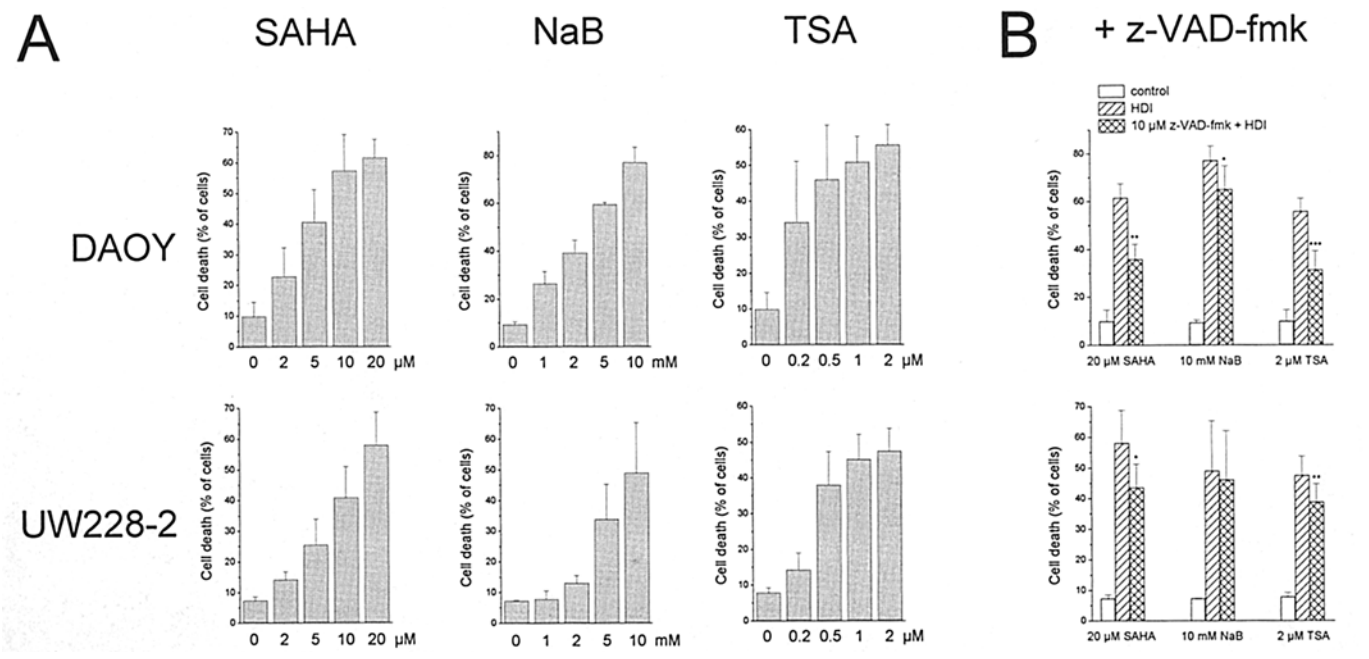

Figure 1. HDIs induce cell death. (A) DAOY cells were incubated with SAHA and TSA for 24 h, and with NaB for 48 h; UW228-2 cells were incubated with SAHA and TSA for 48 h, and with NaB for 72 h. (B) z-VAD-fmk was applied 1 h before treatment with HDIs, cells were then incubated for 24-72 h. Cell death was determined by cytofluorometric analysis of PI uptake. Means ± SD of 4 separate experiments are shown ($^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.001$).

24-72 h (DAOY: SAHA and TSA, 24 h; NaB, 48 h; UW228-2: SAHA and TSA, 48 h; NaB, 72 h). For both cell lines, we observed a concentration-dependent increase in dead cells with increasing doses of the three compounds (Fig. 1A). After treatment with 20 μM SAHA, approximately 60% of DAOY or UW228-2 cells were non-viable; similar results were obtained with 10 mM NaB and 2 μM TSA.

HDI-induced cell death in medulloblastoma cells involves apoptosis

z-VAD-fmk. Subsequently, we explored whether SAHA-, NaB-, and TSA-induced cell death in DAOY and UW228-2 cells could be induced by apoptosis. Apoptosis was evaluated by using the broad-spectrum irreversible caspase inhibitor, z-VAD-fmk, by assessing DNA fragmentation with cytofluorometric cell cycle analysis and by measuring caspase-3 activity. First, the effect of z-VAD-fmk on HDI-mediated cell death was examined by determining PI uptake. z-VAD-fmk (10 μM) was applied 1 h before administration of SAHA, NaB, or TSA, and cells were cultured for 24-72 h. In DAOY cells, the pan-caspase inhibitor significantly, albeit partially, prevented HDI-induced cell killing (Fig. 1B). In contrast, only a tenuous protective effect of z-VAD-fmk was observed in UW228-2 cells.

DNA fragmentation. Second, cells were assessed for apoptosis by cell-cycle analysis: the sub-G_1 (hypodiploid) subpopulation of cells is indicative of apoptosis. As shown in Fig. 2A, there was a concentration-dependent increase in sub-G_1 cells, consistent with DNA fragmentation and apoptosis, with increasing doses of SAHA, NaB, or TSA in both DAOY and UW228-2 cells. The control (no HDIs) samples showed sub-G_1 fractions of approximately 5%, whereas samples cultured with HDIs for 24 h showed sub-G_1 fractions of 32-43% (DAOY) or 12-20% (UW228-2), concomitant with reduced G_1 fractions. In DAOY cells, pretreatment with 10 μM z-VAD-fmk significantly antagonized HDI-induced DNA fragmentation, while no protection of z-VAD-fmk against HDI-induced DNA fragmentation was detected in UW228-2 cells (Fig. 2B). Interestingly, with the inhibition of caspases, a significant G_2/M arrest, largely concealed after exposure to HDIs alone, became evident in DAOY cells.

Caspase-3. The activation of caspase-3 is a hallmark of apoptosis in many cell types (25). Thus, we assessed whether HDIs would induce caspase-3 activity in DAOY and UW228-2 cells. As illustrated in Fig. 3, a 24-h incubation with SAHA, NaB, or TSA triggered caspase-3 activity in a dose-dependent manner in both cell lines. In DAOY cells, HDIs provoked an up to 18-fold increase in caspase-3 activity; in UW228-2 cells, an up to 5.5-fold increase in caspase-3 activity was observed.

HDIs induce the mitochondrial pathway of apoptosis in medulloblastoma cells. Mitochondrial transmembrane potential ($\Delta\Psi_m$). HDIs have been reported to harness the mitochondrial pathway of apoptosis in different cancer types (26-28). To evaluate whether HDI-induced apoptosis would involve mitochondria in medulloblastoma cells, we examined mitochondrial depolarization by determining $\Delta\Psi_m$ and measured caspase-9 activity. A concentration-dependent decline of $\Delta\Psi_m$ was found for all the three HDIs in both cell lines (Fig. 4A). A 24-h treatment with HDIs caused a $\Delta\Psi_m$ dissipation in 30-46% of DAOY cells and 12-25% in UW228-2 cells. In DAOY cells, the decay of $\Delta\Psi_m$ was significantly inhibited by pre-incubation with 10 μM z-VAD-fmk (Fig. 4B). In contrast, z-VAD-fmk did not inhibit HDI-triggered loss of $\Delta\Psi_m$ in UW228-2 cells.

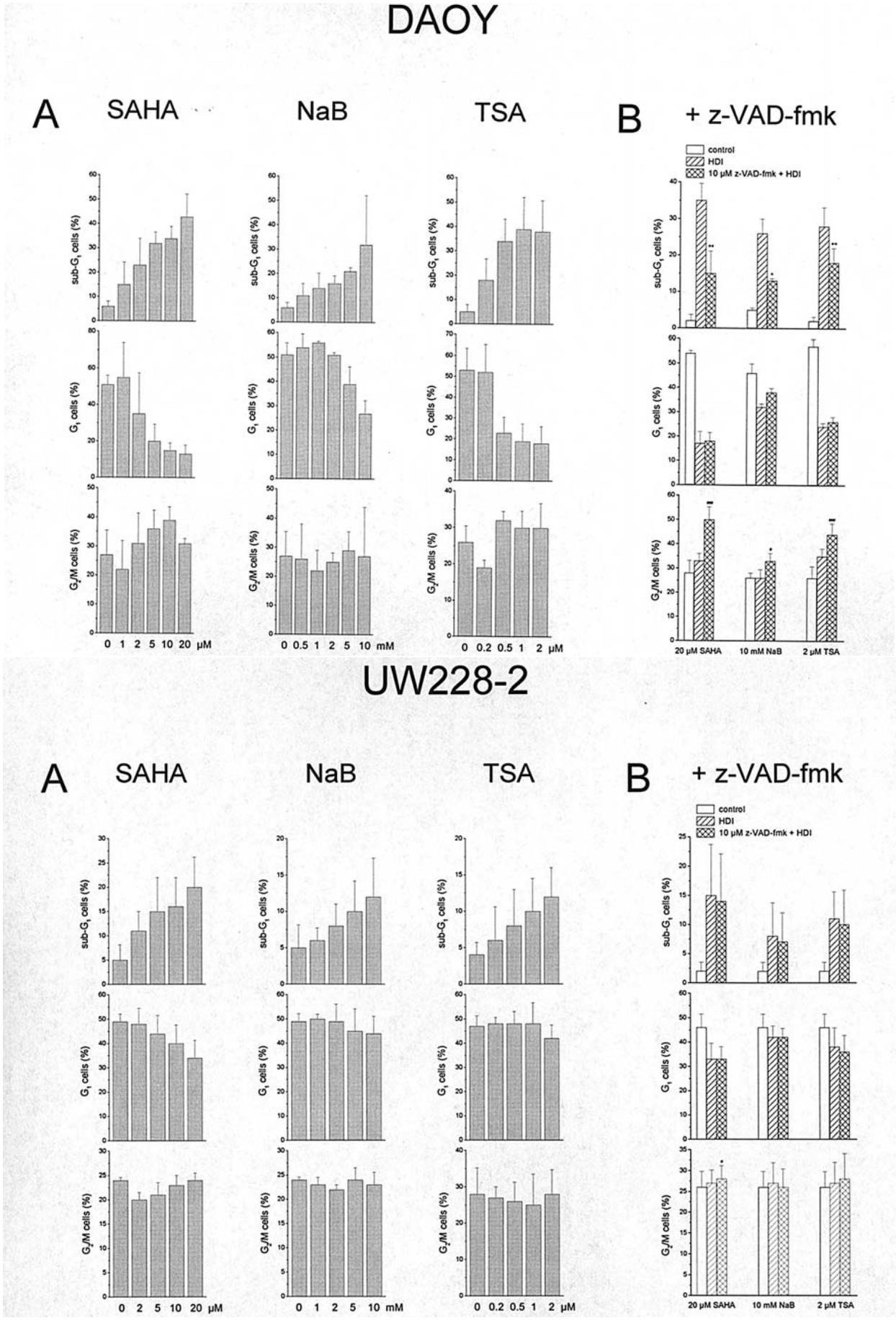

Figure 2. HDIs alter cell-cycle profile. (A) DAOY and UW228-2 cells were incubated with SAHA, NaB, or TSA for 24 h. (B) z-VAD-fmk was applied 1 h before treatment with HDIs, cells were then incubated for 24 h. Cell cycle profiles were analyzed by flow cytometry. Means ± SD of 3 separate experiments are shown (z-VAD-fmk versus HDI: $^{*}p<0.05$, $^{**}p<0.005$; z-VAD-fmk versus control: $^{\#}p<0.05$, $^{\#\#\#}p<0.001$).

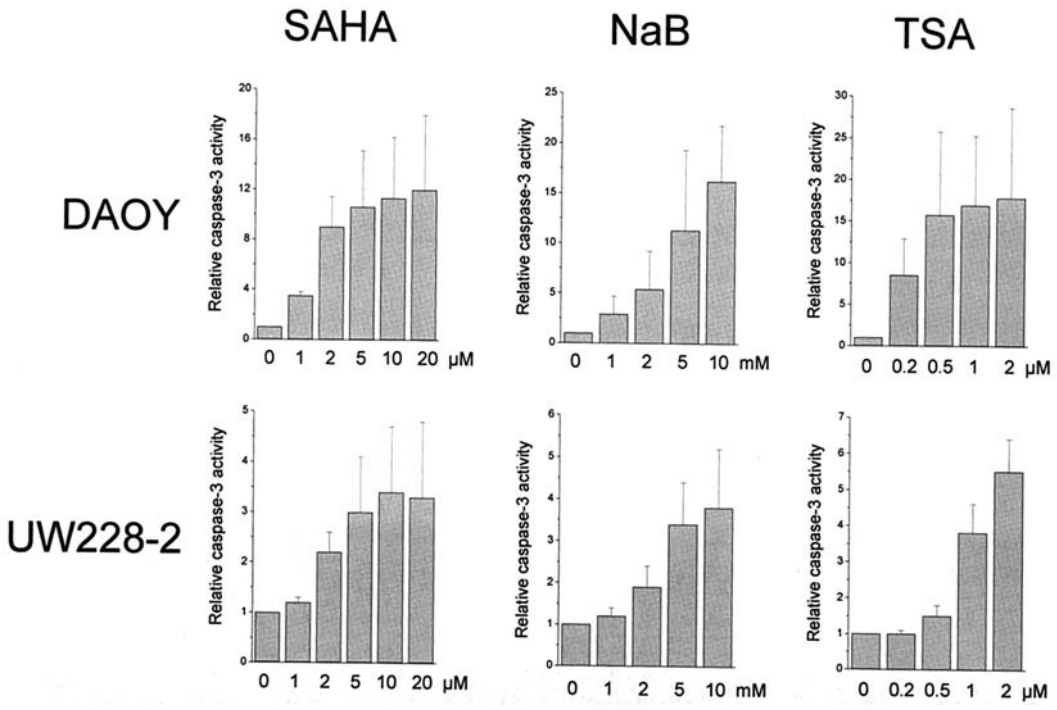

Figure 3. HDIs induce caspase-3 activity. DAOY and UW228-2 cells were incubated with SAHA, NaB, or TSA for 24 h. Caspase-3 activity was measured using the fluorogenic substrate, Ac-DEVD-AFC. Relative caspase-3 activity is the ratio of treated cells to untreated cells. Means ± SD of 3 separate experiments are shown.

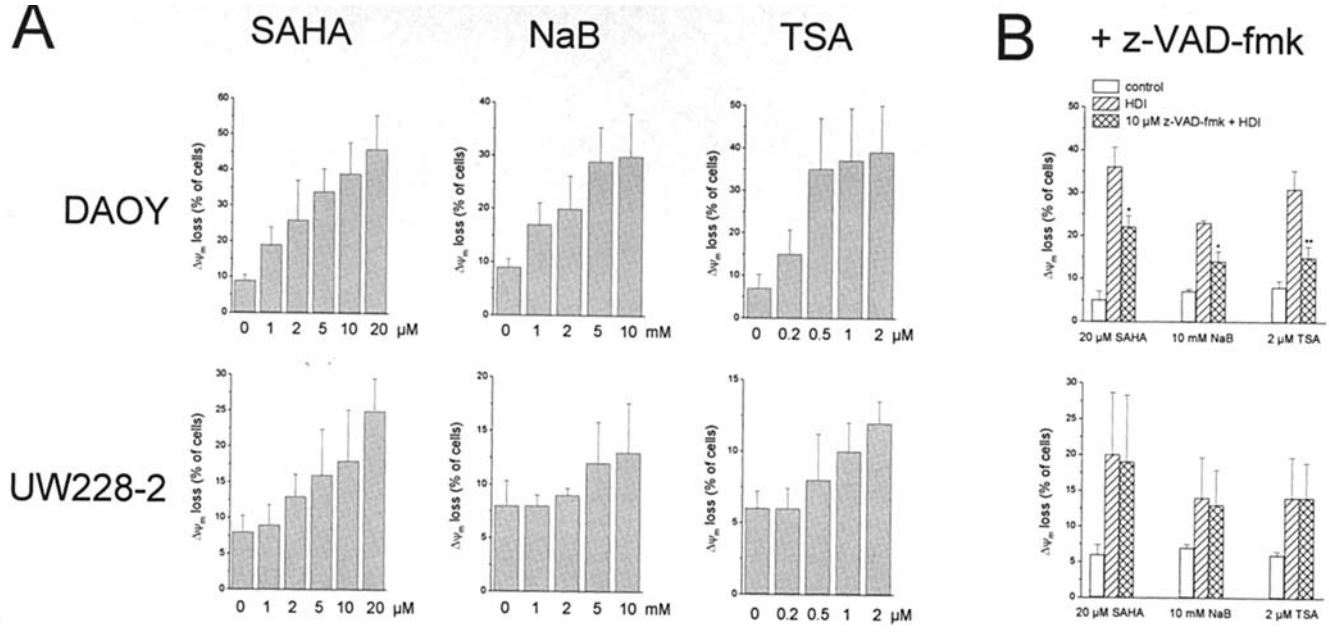

Figure 4. HDIs induce $\Delta\Psi_m$ dissipation. (A) DAOY and UW228-2 cells were incubated with SAHA, NaB, or TSA for 24 h. (B) z-VAD-fmk was applied 1 h before treatment with HDIs, cells were then incubated for 24 h. $\Delta\Psi_m$ was assessed by cytofluorometric analysis of $DiOC_6(3)$ staining. Means ± SD of 3 separate experiments are shown ($^*p<0.05$, $^{**}p<0.005$).

Caspase-9. Typically, the permeabilization of mitochondria is accompanied by the release of cytochrome *c* into the cytosol concomitant with the activation of caspase-9. We thus tested whether HDIs could stimulate caspase-9 activity in DAOY and UW228-2 cells. As demonstrated in Fig. 5, a 24-h exposure to SAHA, NaB, or TSA resulted in a concentration-dependent activation of caspase-9 in both cell lines: we detected an up to 3- and 4.5-fold increase in caspase-9 activity in DAOY and UW228-2 cells, respectively.

HDIs sensitize DAOY cells to ionizing radiation. Based on established action mechanisms of HDIs (4), we hypothesized that HDIs might sensitize cells to IR. Four hours after treatment with HDIs, DAOY cells were exposed to single doses of IR,

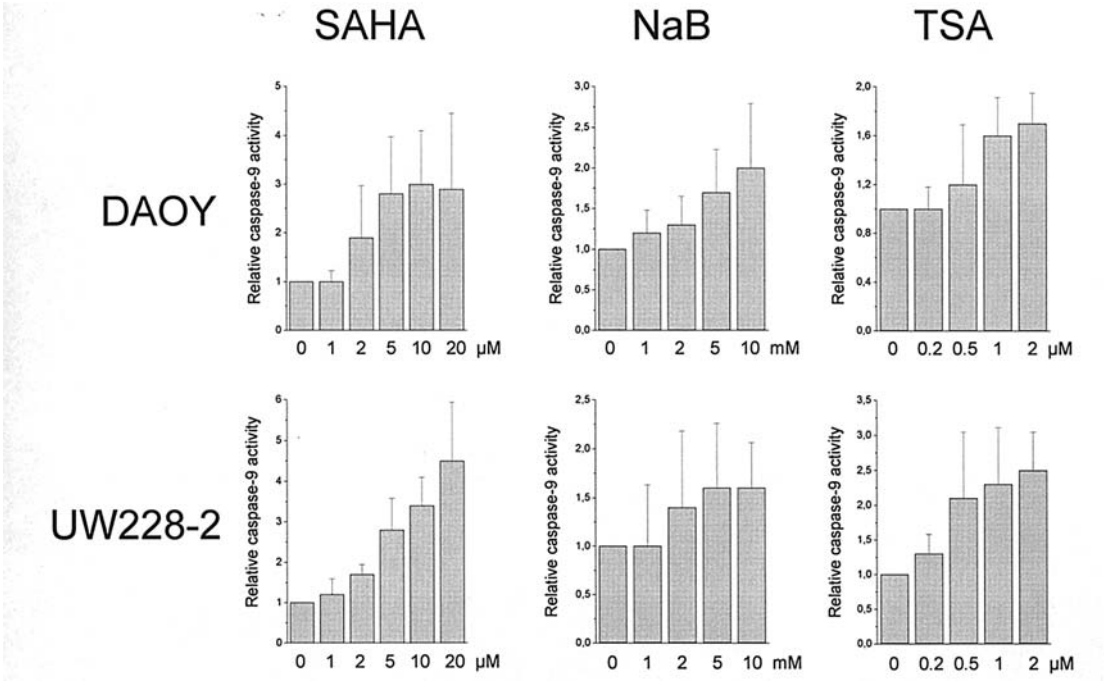

Figure 5. HDIs induce caspase-9 activity. DAOY and UW228-2 cells were incubated with SAHA, NaB, or TSA for 24 h. Caspase-9 activity was measured using the fluorogenic substrate, Ac-LEHD-AFC. Relative caspase-9 activity is the ratio of treated cells to untreated cells. Means ± SD of 3 separate experiments are shown.

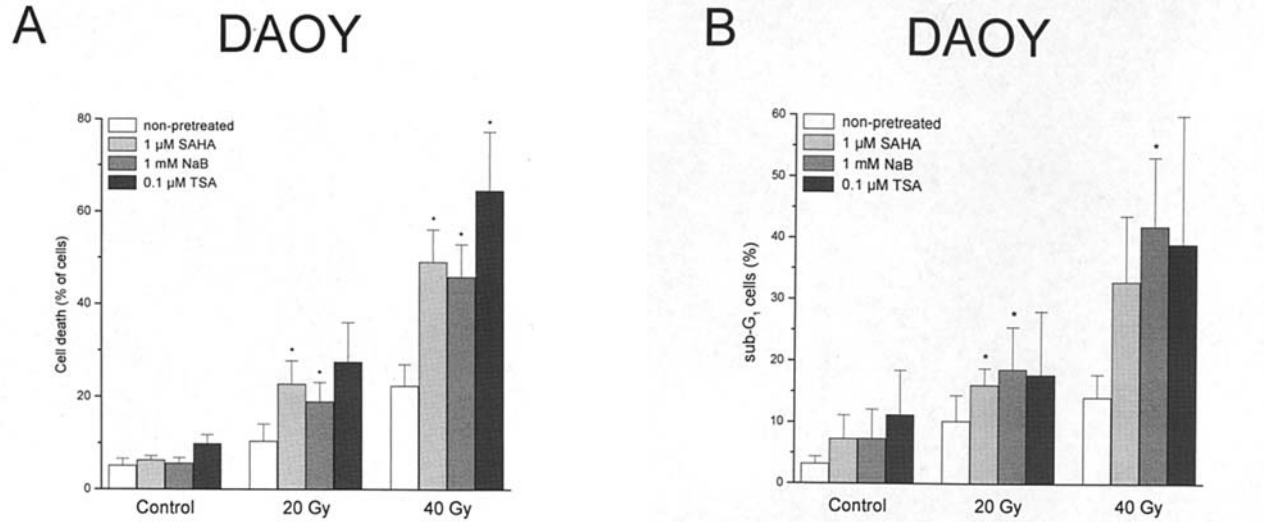

Figure 6. HDIs induce radiosensitization. DAOY cells were irradiated 4 h after treatment with HDIs and cultured for a further 24 h. (A) Cell death was determined by cytofluorometric analysis of PI uptake. (B) DNA fragmentation was determined by cytofluorometric cell cycle analysis. Means ± SD of 4 (PI uptake) or 3 (DNA fragmentation) experiments are shown (*$p<0.05$).

and PI uptake was determined after a further 24-h incubation. As shown in Fig. 6A, the modest doses of HDIs (1 μM SAHA, 1 mM NaB, or 0.1 μM TSA) applied in these experiments had little effect on cell viability. Likewise, cells displayed only moderate responsiveness to IR alone: doses of 20 or 40 Gy elicited 10.5 and 22% cell death, respectively. However, combined treatment with HDIs and IR resulted in supra-additive cytotoxicity with 19-27.5% cell killing at 20 Gy and 46-65% cell killing at 40 Gy. This potentiation of cell death with a combined treatment of HDIs and IR was further confirmed using cell-cycle analysis to assess DNA fragmentation. As illustrated in Fig. 6B, treatment with IR alone caused only a weak increase in sub-G_1 cells (10% at 20 Gy and 14% at 40 Gy). In contrast, in cells pretreated with SAHA, NaB, or TSA for 4 h, IR at 20 or 40 Gy provoked DNA fragmentation in 16-19 and 33-42%, respectively, of cells.

SAHA sensitizes DAOY cells to etoposide, but not to vincristine. We also evaluated whether SAHA could increase the efficiency of drugs used for the management of medulloblastoma, such as etoposide and vincristine. DAOY cells were pretreated

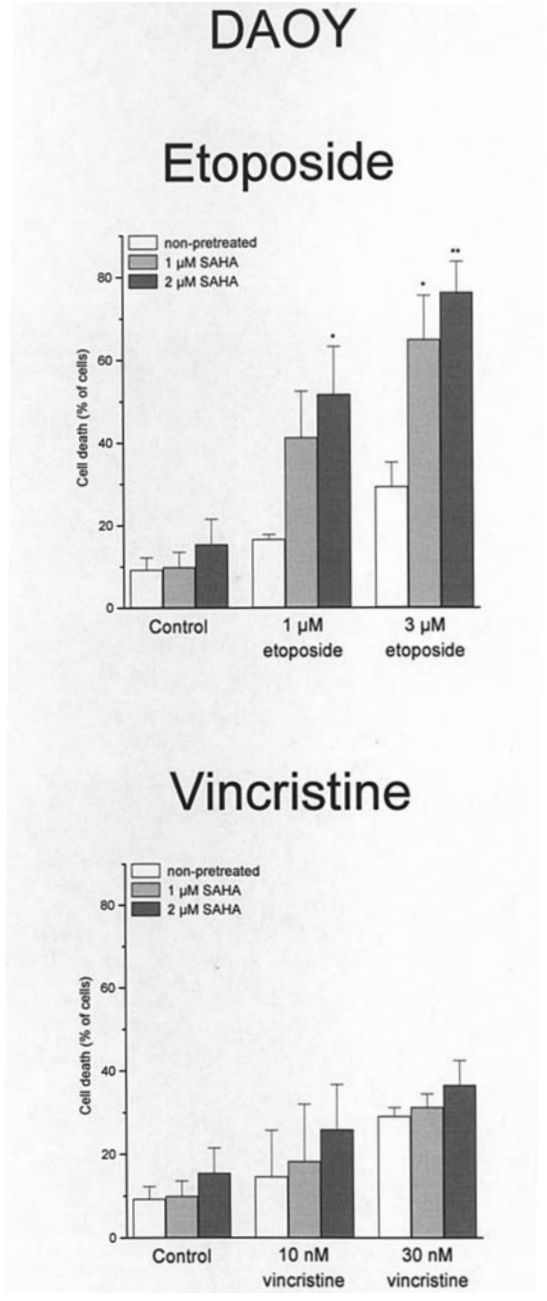

Figure 7. HDIs induce sensitization to etoposide, but not to vincistine. Four hours after treatment with SAHA, DAOY cells were exposed to etoposide or vincristine for further 24 h. Cell death was determined by cytofluorometric analysis of PI uptake. Means ± SD of each 3 experiments are shown ($^{*}p<0.05$, $^{**}p<0.005$).

with 1 or 2 μM SAHA for 4 h, and cultured with the cytostatics for a further 24 h. At the highest doses applied (3 μM etoposide, 30 nM vincristine), the cytostatics alone evoked cell death in 29% of cells. Pretreatment with SAHA potently increased etoposide killing efficiency, resulting in up to 76% non-viable cells (Fig. 7). In contrast, the combined treatment of SAHA and vincristine led to a mere additive effect. This discrepancy of the impact of SAHA on either etoposide or vincristine treatment may be attributable to the different modes of action of these cytostatics (see Discussion).

HDIs sensitize medulloblastoma cells to TRAIL. Finally, we investigated whether HDIs would sensitize medulloblastoma cells also to TRAIL-mediated apoptosis. First, we monitored cell death in DAOY and UW228-2 cells by PI uptake. Twenty-four hours after treatment with HDIs, cells were exposed to varying concentrations of TRAIL for another 24 h. As presented in Fig. 8A, non-pretreated cells showed some responsiveness at the highest dose (100 ng/ml) of TRAIL applied (18 or 10% cell death in DAOY and UW228-2 cells, respectively). In cells pretreated with 2 μM SAHA, 2 mM NaB, or 0.2 μM TSA, however, TRAIL evoked a pronounced cytotoxic effect, with up to 94% cell killing in DAOY cells and up to 60% cell killing in UW228-2 cells. Second, cells were assessed for DNA fragmentation by cell-cycle analysis. Treatment with TRAIL alone for 24 h resulted in DNA fragmentation in a maximum of 19 or 17% in DAOY and UW228-2 cells, respectively (Fig. 8B). When the same experiment was carried out after pretreatment with HDIs for 24 h, a highly significant increase of apoptotic cells was observed, with sub-G_1 fractions amounting to up to 78% in DAOY cells and up to 52% in UW228-2 cells. Third, we determined the effect of HDIs on TRAIL-induced caspase-3 activity. In DAOY cells afore treated with HDIs for 24 h, 100 ng/ml TRAIL activated caspase-3 by at least 22-fold whereas, in non-pretreated cells, only an 11.5-fold increase in caspase-3 activity was observed (Fig. 8C). In UW228-2 cells, treatment with 100 ng/ ml TRAIL resulted in a 7-fold caspase-3 activation and an at least 14.5-fold activation in cells pretreated with HDIs for 24 h.

TRAIL-mediated apoptosis has been shown to involve $\Delta\Psi_m$ dissipation (29). We thus analyzed whether HDIs and TRAIL would interact at the mitochondrial level to induce apoptosis. DAOY cells were cultured with 2 μM SAHA for 24 h, and exposed to 100 ng/ml TRAIL for a further 24 h. As shown in Fig. 8D, treatment with SAHA or TRAIL alone led to $\Delta\Psi_m$ loss in 25 or 48% of cells, respectively. After combined treatment of SAHA and TRAIL, decay of $\Delta\Psi_m$ occurred in 92% of cells. In addition, we asked whether the observed activation of mitochondria could depend on the activation of caspase-8, an initiator caspase employed by active death receptors (30). TRAIL-triggered decline of $\Delta\Psi_m$ could be prevented by the caspase-8 inhibitor, z-IETD-fmk. In contrast, z-IETD-fmk had no effect on SAHA-induced $\Delta\Psi_m$ dissipation. These results show that active caspase-8 is required for TRAIL-induced, but not for SAHA-induced, apoptosis via the mitochondrial pathway.

HDI treatment leads to histone acetylation in medulloblastoma cells. The effect of SAHA, NaB and TSA on histone acetylation was examined in order to confirm that HDIs effectively inhibit the enzymatic activity of histone deacetylases. DAOY and UW228-2 cells were incubated with the indicated concentrations of HDIs for 24 h, and the acetylation status of histone H3 was analyzed by Western blotting using an acetylated H3-specific antibody. As presented in Fig. 9, treatment with all three HDIs induced pronounced histone H3 hyperacetylation in both cell lines.

Discussion

HDIs induce growth arrest, activate differentiation, and/or elicit apoptosis in many epithelial and hematological malignancies

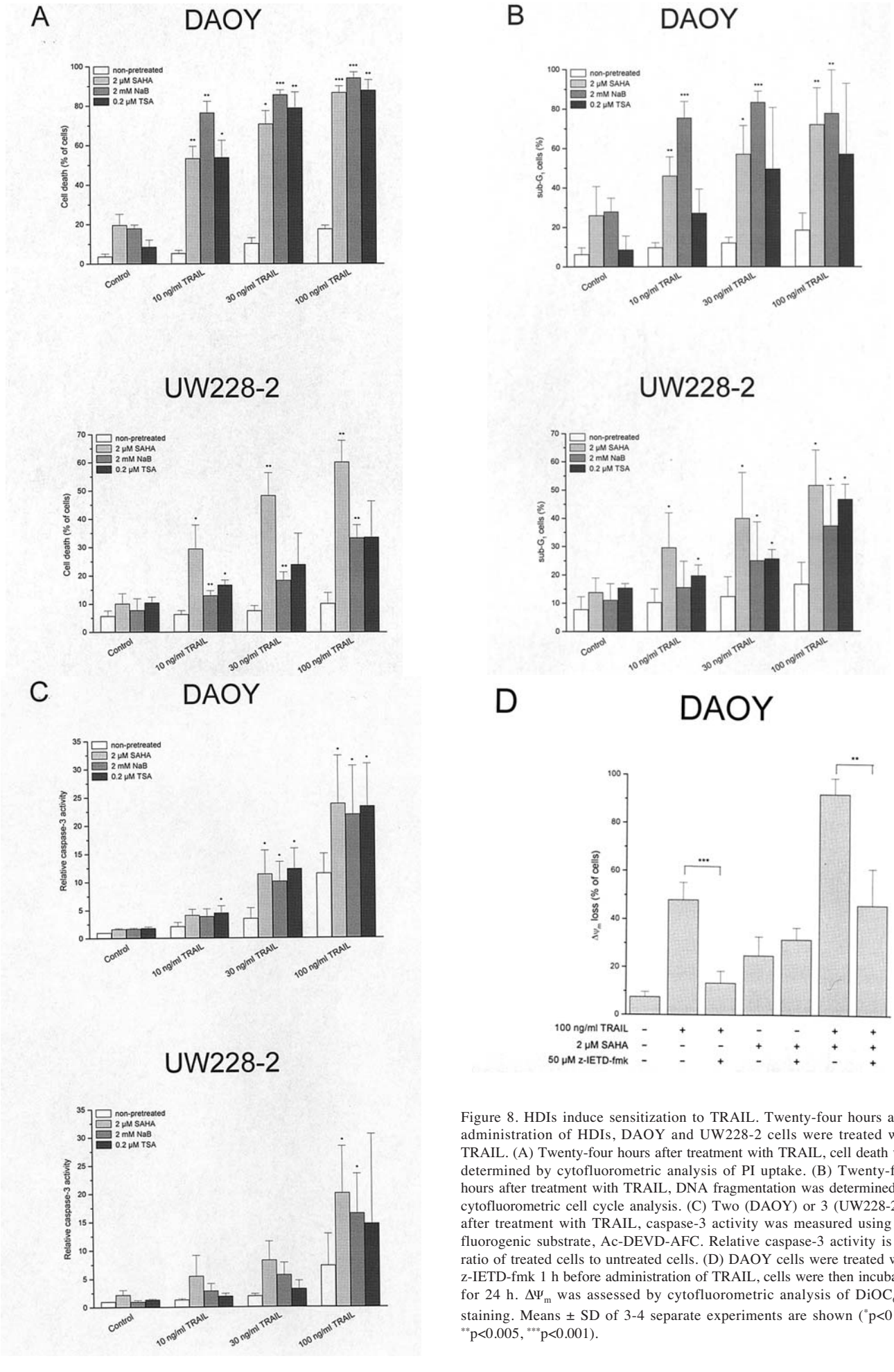

Figure 8. HDIs induce sensitization to TRAIL. Twenty-four hours after administration of HDIs, DAOY and UW228-2 cells were treated with TRAIL. (A) Twenty-four hours after treatment with TRAIL, cell death was determined by cytofluorometric analysis of PI uptake. (B) Twenty-four hours after treatment with TRAIL, DNA fragmentation was determined by cytofluorometric cell cycle analysis. (C) Two (DAOY) or 3 (UW228-2) h after treatment with TRAIL, caspase-3 activity was measured using the fluorogenic substrate, Ac-DEVD-AFC. Relative caspase-3 activity is the ratio of treated cells to untreated cells. (D) DAOY cells were treated with z-IETD-fmk 1 h before administration of TRAIL, cells were then incubated for 24 h. $\Delta\Psi_m$ was assessed by cytofluorometric analysis of $DiOC_6(3)$ staining. Means ± SD of 3-4 separate experiments are shown ($^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.001$).

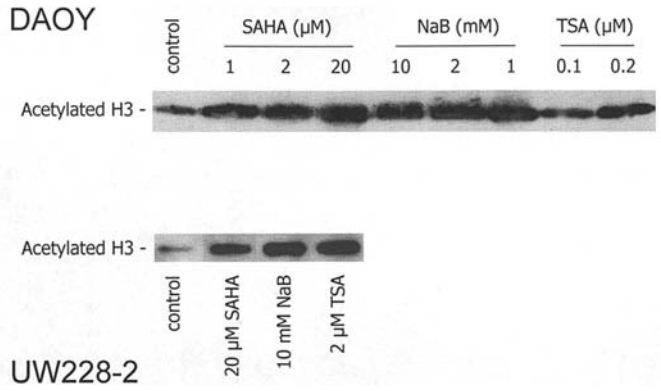

Figure 9. HDIs increase acetylation of histone H3. DAOY and UW228-2 cells were treated for 24 h with the indicated concentrations of SAHA, NaB or TSA. Total protein (30 μg) was separated by a 10% polyacrylamide gel. Western blot analyses were performed with antibodies against acetylated histone H3. GAPDH was used as a loading control.

(31). However, the effects of these compounds on medulloblastoma have not been fully investigated. Recent studies have shown that the benzamide derivative, MS-275, inhibits DNA synthesis in DAOY and D283-Med medulloblastoma cells (23), and that the carboxylic acids, phenylbutyrate and phenylacetate, induce differentiation and inhibit proliferation in these cell lines *in vitro* and *in vivo* (24). Here, we demonstrate that the HDIs SAHA, NaB, and TSA were effective in inducing cell death in DAOY and UW228-2 medulloblastoma cells. This is evidenced by a number of different read-outs. As judged by PI uptake, we observed that all three HDIs acted very similarly in initiating death in both the cell lines; however, DAOY cells were more sensitive to HDIs, which might be related to their higher growth rate. Several lines of evidence indicate that HDIs triggered cell death in DAOY and UW228-2 through the induction of apoptosis. Apoptosis is initiated in response to a number of stimuli, including antineoplastic agents, and is usually mediated through the activation of caspases, eventually resulting in typical morphological changes such as DNA fragmentation (30). We found that exposure to HDIs led to the accumulation of sub-G_1 cells, a finding that is consistent with DNA fragmentation and apoptosis. We also found that HDIs potently activated capase-3, another feature characteristic of apoptosis. Apoptosis can proceed via two pathways, the death receptor (extrinsic) and the mitochondrial (intrinsic) pathway; typically, chemotherapeutic drugs harness the mitochondrial pathway of apoptosis. SAHA, NaB, and TSA induced loss of $\Delta\Psi_m$, which was paralleled by the activation of caspase-9, demonstrating the functionality of the intrinsic pathway in HDI-triggered apoptosis in medulloblastoma cells.

The importance of caspases for HDI-induced apoptosis is a controversial issue. For example, SAHA has been reported to elicit cell death in leukemia and colon carcinoma cells independent of the activation of caspase-8 or -3 (26,32,33). On the other hand, several reports point to a critical role of the caspase system for HDI-mediated apoptosis (28,34-36). Hence, the significance of caspase activation in the apoptotic pathway induced by HDIs is uncertain. However, chemoresistance in various malignant tumors has been related to defects in the caspase system (37,38). Thus, the definition of the role of caspases in HDI-induced apoptosis may have implications for developing strategies especially for the treatment of chemoresistant cancers. We demonstrate that the three HDIs investigated were strong inducers of caspase-3 and -9 activity in DAOY and UW228-2 cells. However, the activation of a caspase does not necessarily imply its requirement for the apoptotic process. Consequently, additional analyses were performed to dissect whether activated caspases are indispensable components in the HDI-triggered apoptotic machinery. Experiments using the polycaspase inhibitor, z-VAD-fmk, revealed that activated caspases were indeed required for HDI-provoked cell death in DAOY cells, as judged by measuring PI uptake, by quantifying DNA fragmentation, and by determining $\Delta\Psi_m$. In contrast, HDI-induced cell death was only slightly affected by z-VAD-fmk in UW228-2 cells, indicating that the activity of caspases which are blocked by z-VAD-fmk is less critical for the cytotoxic activity of HDIs in UW228-2 cells. Hence, the contradictory findings regarding the role of caspases for HDI-triggered apoptosis might be explicable by the different cell lines employed in different studies.

As an accessory finding of the cell-cycle analyses, an HDI-induced G_2/M arrest was observed when cells were protected from apoptosis by z-VAD-fmk, consistent with previous reports (39,40). Inhibition of caspases did not only prevent HDI-induced apoptosis in DAOY cells but significantly and systematically increased the percentage of G_2/M cells (on expense of G_1 cells) even above the levels of G_2/M cells in the controls. The mechanism by which HDIs arrest cells in G_2/M has not yet been clearly defined (41). HDI-induced G_2/M arrest has been found to associate with the reduced expression of cyclins A and B as well as CDK1, key components for G_2/M transition, and the increased expression of the CDK inhibitors, p21 and p27 (42,43). In addition, it has been reported that HDIs induce a G_2/M block by activating the p38 MAPK checkpoint (44). In any case, our observation shows that HDIs are still capable of eliciting growth arrest when their apoptosis-inducing activity is blocked. Therefore, HDIs might be useful for the treatment of cancers with curtailed apoptotic responsiveness due to defects in the caspase system.

The greatest potential of HDIs may lie in their capability to enhance the antitumor efficiency of other therapeutic regimens, such as IR or chemotherapy. With respect to medulloblastoma, an improvement of the efficacy of IR is especially desirable: although IR is a very efficient treatment for this disease, it is associated with severe neurological toxic side-effects (2). Thus, in order to reduce morbidity with IR, in the USA, the standard craniospinal radiation dose has been lowered from 36 Gy to 23.4 Gy by the introduction of adjuvant chemotherapy (45). Nonetheless, a dose of 23.4 Gy is still associated with neurocognitive sequelae, particularly in children younger than 8 years (46). A further reduction in radiation dose bears the chance of decreasing IR-related morbidity, but also bears the risk of reducing disease control. Our data demonstrate the capacity of HDIs to enhance IR-induced cytotoxicity in DAOY cells. In fact, a dose of 20 Gy after pretreatment with HDIs was as effective in cell killing as a dose of 40 Gy without pretreatment. Similar favorable interactions between HDIs and IR have been described in a few studies. Pretreatment or coadministration of different HDIs has been shown to augment radiosensivity in prostate carcinoma

(20,47,48), glioma (22), gastrointestinal carcinoma (49), squamous carcinoma (50), and melanoma (51). However, the mechanism responsible for enhanced IR response after inhibition of histone deacetylases has not been clearly defined. One explanation for HDI-mediated radiosensitization lies in HDI impact on chromatin structure: HDIs facilitate decondensation of chromatin; relaxed chromatin has been reported to be generally more sensitive to IR (52,53).

If the looser chromatin structure was responsible for elevated radiosensitivity, an HDI-induced increase in chromatin accessibility might also render cells more sensitive to antineoplastic drugs that target DNA or enzymes acting on the DNA. To address this possibility, we combined SAHA with etoposide, a drug frequently employed in the management of medulloblastoma. Etoposide functions by locking topoisomerase II to the DNA, resulting in irreversible DNA damage during the replication process. Our results demonstrate that pretreatment with SAHA indeed led to a strongly increased killing efficiency of etoposide. We compared this to the combination of SAHA with vincristine, another agent commonly used in medulloblastoma treatment, which does not target the DNA but exerts its cytotoxicity by acting on mitotic spindle microtubules. Our data show that pretreatment with SAHA had no significant effect on vincristine-mediated cell death, indicating that HDIs do not unspecifically increase anticancer drug cytotoxicity irrespective of the drug's mode of action. These observations are in concordance with a recent study which shows that pretreatment with HDIs enhances the cytotoxicity of DNA-targeting agents but not that of the antimetabolite 5-fluorouracil (19).

However, chemotherapy activates the apoptotic machinery only indirectly; more effective outcomes might be accomplished by direct induction of apoptosis. In this respect, TRAIL is a highly promising candidate, as it provokes cell death in the majority of cancer cells without inflicting collateral damage on most normal cells (29). Nevertheless, some cancers fail to respond to TRAIL's cytotoxic effects, suggesting that treatment with TRAIL alone may be insufficient for cancer therapy. In several tumor types, TRAIL activity could be amplified by cotreatment with HDIs. For example, coadministration of HDIs increased the killing efficiency of TRAIL in leukemia cells (54), colon carcinoma cells (55), mesothelioma cells (56), breast cancer cells (57), and lung and prostate cancer cells (58). In this study, we demonstrate that HDIs and TRAIL synergized to induce apoptosis in medulloblastoma cells, as evidenced by measuring PI uptake, quantifying DNA fragmentation, and determining caspase-3 activity. Notably, HDIs and TRAIL also synergistically affected mitochondrial function, indicating that the activities of HDIs and TRAIL converge upstream of mitochondria. TRAIL-triggered decline of $\Delta\Psi_m$ could be abolished by the caspase-8-specific inhibitor, z-IETD-fmk, showing that caspase-8 is essential for the TRAIL-mediated mitochondrial death pathway in DAOY cells. In contrast, z-IETD-fmk had no effect on SAHA-induced $\Delta\Psi_m$ loss, ruling out the possible involvement of caspase-8 in SAHA-triggered apoptosis. In leukemia cells, HDIs have been shown to elicit apoptosis via the TRAIL receptor pathway (59,60). If this was also the case, however, in medulloblastoma cells, the inhibition of caspase-8 should have resulted in the inhibition of SAHA-induced mitochondrial depolarization. Therefore, the data presented here provide evidence that the activation of the death receptor pathway does not account for HDI-mediated cell death in medulloblastoma cells.

In conclusion, our results suggest that HDIs may be useful for the treatment of medulloblastoma as monotherapy and particularly when given in combination with IR, appropriate cytostatics, or TRAIL. Given the importance of IR for the treatment of medulloblastoma, it now will be of special interest to disclose the mechanisms that account for the sensitization of medulloblastoma cells to IR by HDIs on a molecular level, as such findings may lead to the development of improved treatment tools for medulloblastoma.

Acknowledgements

We thank J. Gänge, U. Glawe, and A. Plath for their excellent technical assistance. This work was supported by a grant from the Deutsche Krebshilfe.

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