Histone deacetylase inhibitors induce cell death in supratentorial primitive neuroectodermal tumor cells

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Abstract. Histone deacetylase inhibitors (HDIs) are a promising new class of antineoplastic agents with the capacity to induce differentiation and/or apoptosis of cancer cells. The objective of this study was to evaluate the activity of HDIs against supratentorial primitive neuroectodermal tumor (sPNET) cells. We show that the HDIs, suberoylanilide hydroxamic acid, sodium butyrate, and trichostatin A, induced cell death, and activated caspase-3 and -9 in a sPNET cell line, PFSK. The poly-caspase inhibitor z-VAD-fmk partially prevented the action of HDIs, as judged by determining the mitochondrial membrane potential and by quantifying internucleosomal DNA fragmentation. In conclusion, the HDIs explored possess potent activity against sPNET cells, suggesting that HDIs may be effective in the treatment of sPNET.

Introduction

The supratentorial primitive neuroectodermal tumors (sPNETs) are embryonal central nervous system tumors primarily affecting young children. Together with medulloblastoma, sPNETs are among the most malignant childhood brain tumors. However, although sPNETs are histologically similar to medulloblastoma, they show different clinical behavior and are generally considered to be more aggressive than medulloblastoma. The contemporary treatment standard for sPNETs includes post-operative ionizing radiation and multiagent chemotherapy (1). However, the outcome for children with sPNET treated with radiation and chemotherapy appears worse than for children with medulloblastoma treated with identical therapy (2). Moreover, there are no effective therapies for most sPNETs that recur after previous radiation and chemotherapy.

Thus, there is an imperative for new approaches that improve survival.

Inhibitors of histone deacetylases (HDIs) are presently emerging as a promising class of antineoplastic agents. HDIs are relatively specific anticancer drugs, which were originally identified by their capacity to reverse the transformed phenotype (3). Subsequently, they have been shown to reactivate gene expression and prevent proliferation, activate differentiation, and/or induce apoptosis of tumor cells (4). HDIs function by inhibiting histone deacetylases, resulting in the accumulation of acetylated histones, in turn leading to an increase in transcriptionally active chromatin. In so doing, they e.g. reactivate gene expression of dormant tumor suppressor genes, such as CDKN1A (p21) (5).

Recently, HDIs have been introduced as chemotherapeutic compounds. Their potent antitumor activity has been observed in cell lines originating from different types of human cancers as well as in studies on mice xenograft models of human neoplasia (4). Importantly, HDIs have been shown both *in vitro* and *in vivo* to affect cancer cells while leaving normal cells comparatively unscathed (6,7). In addition, the clinical potential of these agents has been documented by several Phase I trials of different HDIs in patients with solid tumors or leukemias (8). With respect to sPNET, only the HDIs valproic acid (9) and depsipeptide (10) have been evaluated for their antitumor efficacy. A case report has demonstrated that valproic acid induced differentiation in a relapsed sPNET (11).

In a recent study, we have shown that HDIs were capable of effectively eliciting cell death in medulloblastoma cells (12). These findings prompted us to extend our studies to another brain neoplasm, sPNET. Herein, we report that the viability of sPNET PFSK cells could be potently reduced by treatment with three different HDIs belonging to two structural classes, the hydroxamic acids, suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA), and the short chain fatty acid sodium butyrate (NaB). Our results show that all three compounds triggered cell death in PFSK cells. In addition, we show that all three compounds activated caspase-3 and -9, and that the induction of cell death was partially caspase-dependent.

Materials and methods

Reagents. SAHA, TSA, and the pan-caspase inhibitor z-VAD-fmk were purchased from Alexis (Grünberg, Germany). NaB was purchased from Sigma (Deisenhofen, Germany).

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Cell culture. PFSK cells were a gift from Dr M. Grotzer (Zurich, Switzerland) and maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (medium 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.

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

Cytofluorometric analysis of DNA content. To measure DNA content, cells were analyzed for PI incorporation into DNA. Cells were harvested 24 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. Cells (20,000) were analyzed in each sample; 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 activities. Caspase activities were 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 activities, 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 activities were calculated as a ratio of emission of treated cells to untreated cells.
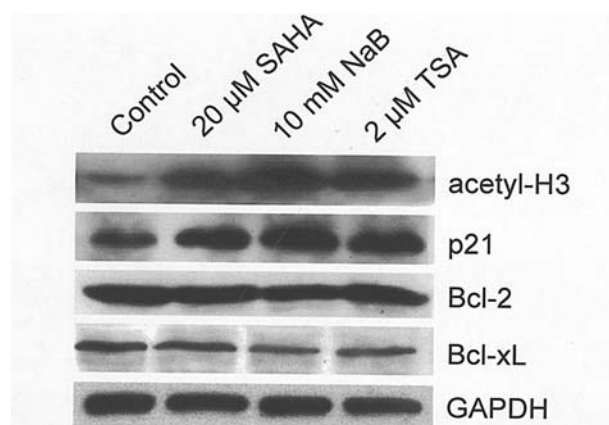

Figure 1. HDIs increase acetylation of histone H3. PFSK cells were treated for 24 h with the indicated concentrations of SAHA, NaB or TSA. Total protein (30 μg) was separated by a 15% polyacrylamid gel. Western blot analyses were performed with antibodies against acetylated histone H3, p21, Bcl-2, and Bcl-xL. GAPDH was used as a loading control.

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 sonication. 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 were separated by standard SDS-PAGE on 15% 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 1:25,000; Upstate Biotechnology, Lake Placid, NY, USA). p21, Bcl-2, and Bcl-xL were immunodetected using mouse anti-p21 (dilution 1:500), anti-Bcl-2 (dilution 1:500), and anti-Bcl-xL (dilution 1:1000) monoclonal antibodies (Santa Cruz Biotechnology, Heidelberg, Germany), respectively. Even loading of protein was verified by detection of GAPDH using mouse anti-GAPDH monoclonal antibody (dilution 1:10,000; Biodesign 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

HDI treatment leads to histone hyperacetylation and upregulation of p21 in PFSK cells. Initially, to determine whether HDIs would induce accumulation of acetylated histone H3 in PFSK cells, cells were treated with 20 μM SAHA, 10 mM NaB, or 2 μM TSA 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. 1, treatment with all three HDIs induced pronounced histone H3 hyperacetylation in PFSK cells. In addition, we
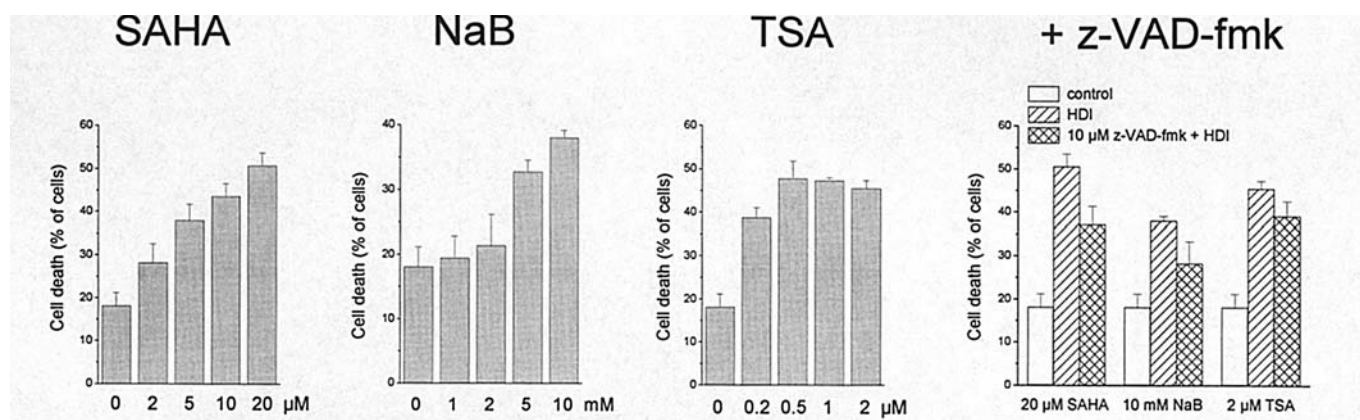
Figure 2. HDIs induce cell death. PFSK cells were incubated with SAHA, NaB, or TSA for 24 h. z-VAD-fmk was applied 1 h before treatment with HDIs, cells were then incubated for 24 h. Cell death was determined by cytofluorometric analysis of PI uptake. Means ± SD of each 3 experiments are shown.
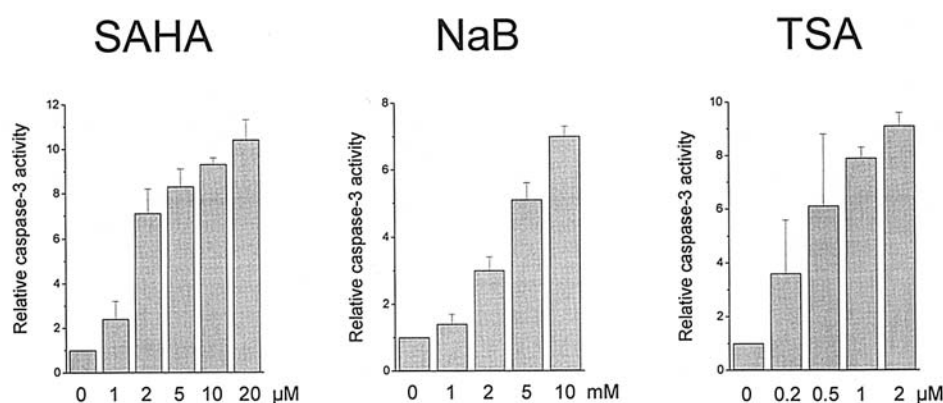

Figure 3. HDIs induce caspase-3 activity. PFSK cells were incubated with SAHA, NaB, or TSA for 24 h. Caspase-3 activity was measured using the fluorogenic substrate Ac-DEVD-AFC. Relative caspase-3 activities are the ratio of treated cells to untreated cells. Means ± SD of each 3 experiments are shown.

evaluated the level of p21 by immunoblot analysis: the three HDIs markedly increased p21 protein levels in PFSK cells.

HDIs induce cell death in PFSK cells. To assess possible cytotoxic effect of HDIs on sPNET cells, we monitored cell killing by determining the integrity of the cell membrane by cytofluorometric analysis of PI uptake. PFSK cell were cultured without or with varying doses of SAHA, NaB, or TSA for 24 h. We observed a concentration-dependent increase in dead cells with increasing doses of the three compounds (Fig. 2). After treatment with 20 μM SAHA, 10 mM NaB, or 2 μM TSA, 40-50% of cells underwent cell death. The pan-caspase inhibitor partially - albeit not significantly - prevented cell killing induced by the three agents.

HDIs induce caspase-3 activity and DNA fragmentation in PFSK cells. Next, we assessed whether HDI-induced cell death in PFSK cells would involve apoptosis. Induction of the caspase pathway is one of the hallmarks of apoptosis (13). Accordingly, its activity is considered as a suitable measure of apoptotic responsiveness. We determined the dose-response relationship of HDI-induced caspase-3 activity after a 24-h incubation of PFSK cells with increasing concentrations of HDIs. SAHA, NaB, and TSA triggered caspase-3 activity in a

dose-dependent manner, with 10-, 7-, or 9-fold activation of caspase-3, respectively, being observed at the highest concentrations applied (Fig 3).

Apoptosis was determined by cell cycle analysis; the sub-G_1 population of cells is indicative of apoptosis. Cell cycle distribution was assessed by staining the nuclei of ethanol-fixed cells with PI and quantifying the percentage of each cell cycle phase by cytofluorometry. The analysis revealed a progressive accumulation of the sub-G_1 fraction of cells over increasing concentrations of HDIs, concomitant with a decrease of cells within G_1 and G_2/M (Fig. 4). At low concentrations, HDIs caused an accumulation of cells with 4n DNA content, consistent with a G_2/M cell cycle arrest. z-VAD-fmk was used to assess whether HDI-induced DNA fragmentation was caspase dependent. Cells were cultured with HDIs in the presence or absence of 10 μM z-VAD-fmk. Cytofluorometric analysis revealed a significant reduction of sub-G_1 cells in the presence of the caspase inhibitor.

HDIs induce dissipation of the mitochondrial membrane potential ($\Delta\psi_m$) and caspase-9 activity in PFSK cells. HDIs have been reported to harness the mitochondrial pathway of apoptosis (14-16). To evaluate the participation of mito-
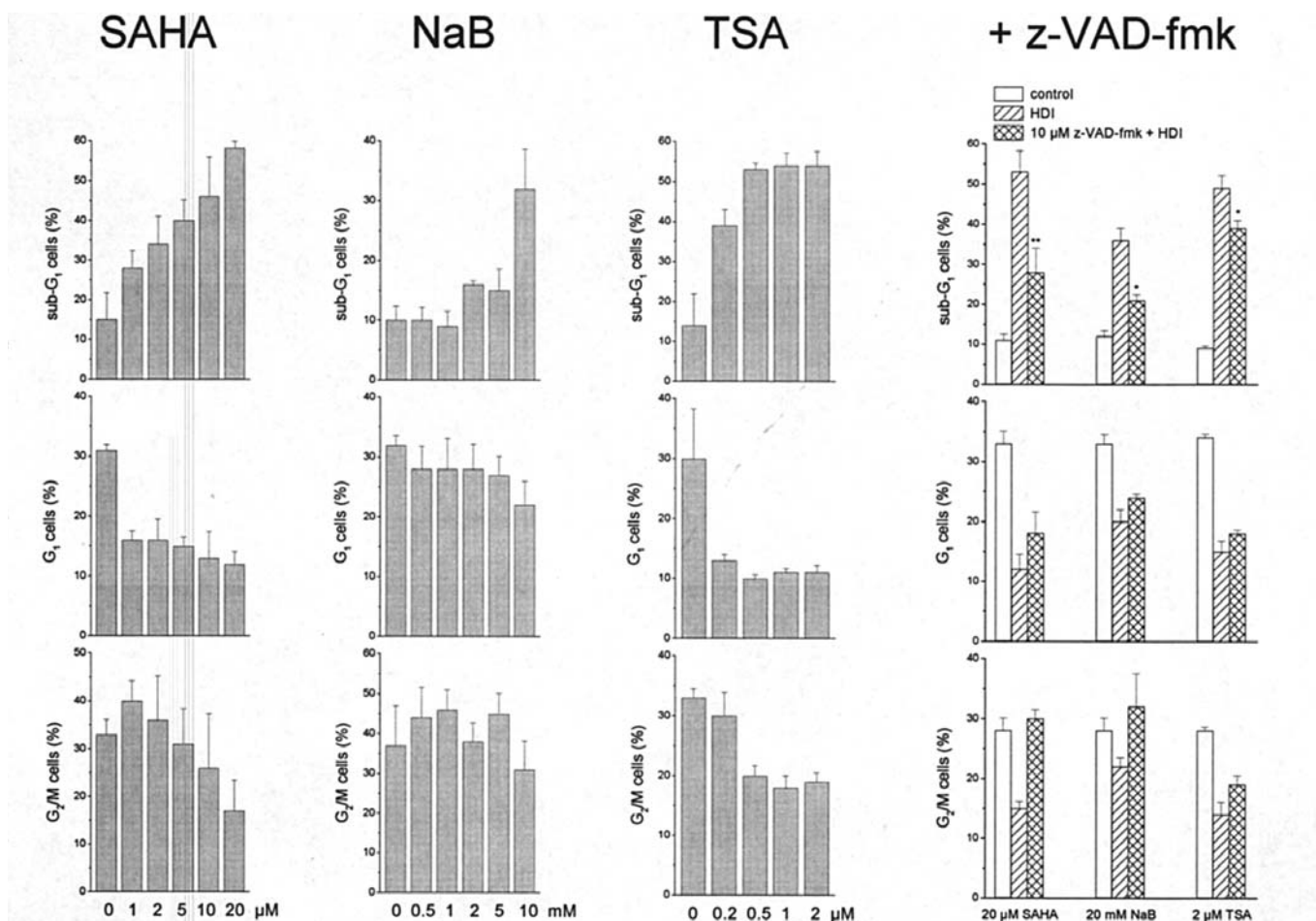
Figure 4. HDIs alter cell cycle profile. PFSK cells were incubated with SAHA, NaB, or TSA for 24 h. z-VAD-fmk was applied 1 h before treatment with HDIs, cells were then incubated for 24 h. Cell cycle profiles were analyzed by flow cytometry. Means ± SD of each 3 experiments are shown (*p<0.05, **p<0.005).

chondria in the apoptotic process triggered by HDIs in PFSK cells, we assessed the effects of SAHA, NaB, or TSA on mitochondria by determining $\Delta\psi_m$. As depicted in Fig. 5A, a 24-h incubation with increasing concentrations of HDIs caused a significant decay of $\Delta\psi_m$ in PFSK cells in a dose-dependent manner. The $\Delta\psi_m$ loss was reduced by pretreatment with 10 μM z-VAD-fmk, demonstrating that the mitochondrial function in HDI-mediated apoptosis is at least partially caspase-dependent.

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 would stimulate caspase-9 activity in PFSK cells. Fig. 5B shows that SAHA, NaB, and TSA activated caspase-9 in a dose-dependent manner, with a 12-, 3.5-, and 10-fold activation of caspase-9, respectively, being measured at the highest doses administered.

The mitochondrial pathway of apoptosis is controlled by proteins of the Bcl-2 family (17). Likewise, HDI-induced mitochondria-dependent apoptosis has been reported to be associated with the downregulation of the antiapoptotic factors, Bcl-2 and Bcl-xL (15,16). On the other hand, NaB has been shown to upregulate Bcl-2 and Bcl-xL in hepatocarcinoma cells (18). To clarify whether HDIs would also affect the expression of these proteins in PFSK cells,

their expression levels were analyzed by Western blotting. As illustrated in Fig. 1, treatment with SAHA, NaB or TSA did not result in significant changes of Bcl-2 or Bcl-xL levels in PFSK cells.

Discussion

Medulloblastoma and sPNET are highly malignant childhood brain tumors with poor prognosis. In a recent study, we have demonstrated that HDIs are effective in inducing cell death in medulloblastoma cells and may thus be useful for the treatment of medulloblastoma (12). In the present study, we demonstrate that the HDIs explored, SAHA, NaB, and TSA, also possess potent antineoplastic activity against sPNET cells. All three agents were effective in inducing cell death in PFSK sPNET cells, as evidenced by cytofluorometric PI uptake analysis. Cell killing was paralleled by the accumulation of acetylated histone H3 after culture of PFSK cells with HDIs, demonstrating the histone deacetylase inhibitory activity of SAHA, NaB, and TSA in sPNET cells. p21 has repeatedly been shown to be upregulated by various HDIs (5,19,20); its induction, thus, serves as an additional marker for the activity of these compounds. Exposure of PFSK cells to SAHA, NaB, or TSA resulted in the upregulation of p21, providing further evidence for the action of these HDIs in sPNET cells.
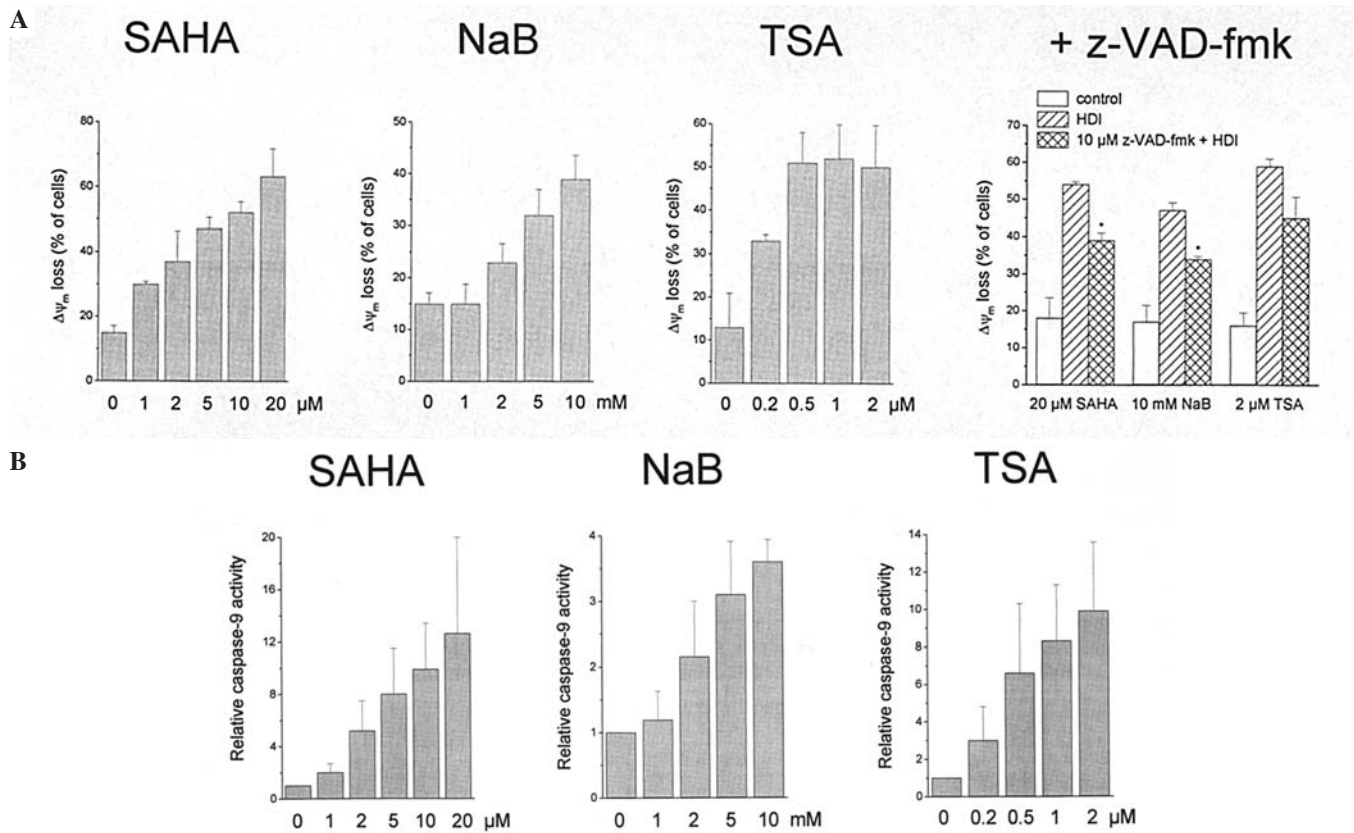
Figure 5. HDIs induce $\Delta\psi_m$ dissipation and caspase-9 activity. (A) $\Delta\psi_m$ dissipation. PFSK cells were incubated with SAHA, NaB, or TSA for 24 h. z-VAD-fmk was applied 1 h before treatment with HDIs, cells were then incubated for 24 h. $\Delta\psi_m$ was assessed by cytofluorometric analysis of DiOC$_6$(3) staining. Means ± SD of each 3 experiments are shown (*p<0.05). (B) Caspase-9 activity. PFSK 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 activities are the ratio of treated cells to untreated cells. Means ± SD of each 3 experiments are shown.

HDIs have been reported to exert their cytotoxic effects by inducing apoptosis (21) as well as by different other mechanisms (22-24). Herein, we present evidence that SAHA, NaB, and TSA triggered cell death in PFSK cells through the induction of apoptosis. We found that exposure to these agents led to the accumulation of sub-G$_1$ cells, a finding that is consistent with DNA fragmentation and apoptosis. We also found that these HDIs potently activated caspase-3, another feature characteristic for apoptosis. However, whether activation of caspases is required for HDI-induced cell death is uncertain. Some studies point to a critical role of caspases for HDI-mediated cell death (12,25,26), whereas others suggest a caspase-independent mechanism (14,27). In this study, experiments using the polycaspase inhibitor z-VAD-fmk revealed that activated caspases were indeed required for HDI-induced cytotoxic effects in PFSK cells, as judged by assessing DNA fragmentation, and by measuring $\Delta\psi_m$. The only incomplete protection against HDI-triggered activities by z-VAD-fmk may be explicable by the existence of caspase-independent cell death mechanisms or, alternatively, by residual caspase activities due to the relatively low dose of z-VAD-fmk applied. As with other chemotherapeutic drugs, the intrinsic pathway of apoptosis is central to HDI-induced cell death (21). In concordance, in our study, SAHA, NaB, and TSA induced loss of mitochondrial membrane potential paralleled by the activation of caspase-9, demonstrating the functionality of the mitochondrial pathway in HDI-triggered apoptosis in sPNET cells. Typically, this pathway is regulated by proteins of the Bcl-2 family (17). However, in the sPNET cell line examined, treatment with HDIs did not cause significant expression changes of the antiapoptotic factors, Bcl-2 and Bcl-xL. Thus, these proteins do not appear to have a substantial role in HDI-induced cell death in sPNET cells.

In conclusion, our study provides evidence that HDIs possess anticancer activity in sPNET cells. This finding is consistent with our previous work demonstrating that SAHA, NaB, and TSA elicit cell death in medulloblastoma cells (12). Taken together, these studies support the notion that HDIs warrant a more in-depth investigation for their potential use in the therapy of childhood brain cancers.

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