Inhibitors of Histone Deacetylases Enhance Neurotoxicity of DNA Damage

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Abstract The nonselective inhibitors of class I/II histone deacetylases (HDACs) including trichostatin A and the clinically used suberoylanilide hydroxamic acid (SAHA, vorinostat) are neuroprotective in several models of neuronal injury. Here, we report that in cultured cortical neurons from newborn rats and in the cerebral cortex of whole neonate rats, these HDAC inhibitors exacerbated cytotoxicity of the DNA double-strand break (DSB)-inducing anticancer drug etoposide by enhancing apoptosis. Similar neurotoxic interactions were also observed in neurons that were treated with other DNA damaging drugs including cisplatin and camptothecin. In addition, in rat neonates, SAHA increased cortical neuron apoptosis that was induced by a single injection of the NMDA receptor antagonist dizocilpine (MK801). In etoposide-treated neurons, the nonselective HDAC inhibition resulted in more DSBs. It also potentiated etoposide-induced accumulation and phosphorylation of the pro-apoptotic transcription factor p53. Moreover, nonselective HDAC inhibition exacerbated neuronal apoptosis that was induced by the overexpressed p53. Importantly, such effects cannot be fully explained by inhibition of HDAC1, which is known to play a role in DSB repair and regulation of p53. The specific HDAC1 inhibitor MS275 only moderately enhanced etoposide-induced neuronal death. Although in etoposide-treated neurons MS275 increased DSBs, it did not affect

activation of p53. Our findings suggest that besides HDAC1, there are other class I/II HDACs that participate in neuronal DNA damage response attenuating neurotoxic consequences of genotoxic insults to the developing brain.

Keywords DNA damage · Apoptosis · Histone deacetylases · Topoisomerase inhibitors · Neuroprotection · Neurotoxicity

Abbreviations

β-gal	β-Galactosidase
BH3	Bcl-2 homology domain-3
BME	Basal medium Eagle
DIV	Day in vitro
DSB	Double-strand break
E2F1	E2 promoter binding factor-1
HAT	Histone acetyltransferase
HDACi	HDAC inhibitor
HDAC	Histone deacetylase
SAHA	Suberoylanilide hydroxamic acid
SBHA	Suberoyl bis-hydroxamic acid
SSB	Single-strand break
TSA	Trichostatin A
γH2Ax	Histone H2Ax variant containing the phosphorylated serine-139 residue

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Introduction

While role of DNA damage in pathogenesis of cancer is well established, DNA damage may also be a contributing factor to neuronal dysfunction and death in various neurodegenerative diseases. In the brain, increased DNA

damage has been documented in age-related neurodegenerative diseases including Alzheimer's disease, during normal aging and after acute insults such as stroke (Markesbery and Lovell 2006; Brasnjevic et al. 2008; Brooks 2008). Moreover, genetic defects in repair of various DNA lesions including single-strand breaks (SSBs), double-strand breaks (DSBs) and adducts have been associated with neurodegeneration (Niedernhofer 2008; Katyal and McKinnon 2008; Caldecott 2008). Recently, induction of DSBs has been demonstrated in response to physiological stimuli increasing neuronal activity (Suberbielle et al. 2013). In addition, the Alzheimer's disease-associated amyloidosis enhanced the frequency of such activity-related lesions (Suberbielle et al. 2013). Therefore, the response to DNA damage including DSBs may be critical for the maintenance of the functional nervous system and may contribute to neurodegenerative diseases.

The DNA topoisomerase II inhibitor etoposide is an anticancer drug that preferentially induces DSBs in a wide range of cells (Nitiss 2009). Therefore, etoposide has been widely used as a research tool to study the neuronal response to DNA damage including neurotoxicity of DSBs (Enokido et al. 1996; Keramaris et al. 2003; Kruman et al. 2004; Martin et al. 2009; Pietrzak et al. 2011; Brochier et al. 2013). Intriguingly, multidrug anticancer chemotherapy protocols containing etoposide treatment have been associated with mild cognitive impairment in children and adults (Kaasa et al. 1988; Mok et al. 2005; Riva et al. 2009; Whitney et al. 2008). Hence, etoposide neurotoxicity may be relevant to at least some aspects of the chemotherapy-induced brain damage and/or dysfunction. In addition to etoposide, other DNA-damaging anticancer drugs including cisplatin were also implicated in neurotoxic complications of chemotherapy (Kannarkat et al. 2007; Ahles and Saykin 2007; Robaey et al. 2008).

Transcriptional inhibition has been proposed as a direct cause of DNA damage neurotoxicity (Hetman et al. 2010). In human brain, oxidative DNA damage of gene promoters has been implicated in aging-associated downregulation of neuronal gene expression affecting synapse function and intracellular signaling (Lu et al. 2004). In cultured rat cortical neurons, transcriptional inhibition of nucleolar rRNA genes such as that induced by the SSB inducing drug camptothecin has been sufficient to activate the p53-dependent apoptosis (Kalita et al. 2008). Therefore, counteracting DNA damage-induced transcriptional inhibition may be neuroprotective.

Posttranslational modifications of histones regulate chromatin structure and affect various processes that involve nuclear DNA including transcription. A prominent pro-transcriptional modification of histones is acetylation that is carried out by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs) (Graff et al.

2011). Hence, stimulation of HATs or inhibition of HDACs increases histone acetylation relaxing chromatin structure and increasing transcription. In humans, there are at least 11 Zn^{2+} -dependent (class I/II) HDACs with distinct expression patterns and substrate specificity (Chuang et al. 2009; Graff et al. 2011). Nonselective inhibitors targeting all but one of the class I/II HDACs include trichostatin A (TSA), suberoyl bis-hydroxamic acid (SBHA) and suberoylanilide hydroxamic acid (SAHA, also known as vorinostat) that is FDA-approved for use against cutaneous T cell lymphoma (Chuang et al. 2009). These drugs have been found to be neuroprotective in several mouse models of neuronal injury/neurodegeneration including stroke, Huntington's disease and Alzheimer's disease (Chuang et al. 2009; Graff et al. 2011). Conversely, the nonselective HDACs are cytostatic and pro-apoptotic in cancer cells (Marks and Xu 2009). Pro-apoptotic effects of HDACs have been also reported in cultured cerebellar granule-, cortical- and sympathetic neurons as well as retinal explants (Salminen et al. 1998; Boutillier et al. 2003; Liu et al. 2005; Wallace and Cotter 2009; Wright et al. 2007). Finally, selective inhibition of HDAC1 has been associated with DNA double-strand breaks and neuronal apoptosis (Kim et al. 2008).

This study has been initiated to examine consequences of HDAC inhibition on neuronal response to DSBs. Effects of the nonselective HDACs TSA, SBHA or SAHA as well as the HDAC1-selective inhibitor MS275 were investigated in cultured rat neurons and whole rat pups that were challenged with genotoxic drugs including etoposide.

Materials and Methods

Animals

Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN, USA). All animal experiments strictly followed the protocols that were approved by the Institutional Animal Care and Use Committee of the University of Louisville and NIH guidelines.

Materials

The following plasmids have been described previously: temperature-sensitive p53 mutant (p53 Val135; TS-p53) (Michalovitz et al. 1990) and the β -galactosidase (β -gal) expression plasmid pON260 (Cherrington and Mocarski 1989). SAHA was purchased from Cayman Chemical company; Trichostatin A was from Sigma; MS275 was from Selleck Chemical; and SBHA was from Enzo Life Sciences. Other reagents were obtained from Sigma, VWR or Invitrogen.

Cell Culture and Transfection

Dissociated cultures of cortical neurons were prepared from newborn rats as described previously (Habas et al. 2006). Cells were seeded in poly-D-lysine/laminin-coated 96-well plates (5×10^4 cells/well) or on poly-D-lysine/laminin-coated plastic coverslips that were placed in 24-well plates (5×10^5 cells/well) or in poly-D-lysine-coated 35-mm dishes (2×10^6 cells/dish) for MTT survival assay or morphological analysis or protein extraction, respectively. Cells were cultured in basal medium Eagle (BME) supplemented with 10 % heat-inactivated bovine calf serum (Hyclone, Logan, UT, USA), 35 mM glucose, 1 mM L-glutamine, 100 U/mL of penicillin and 0.1 mg/mL streptomycin. On day in vitro 2 (DIV2), 2.5 μ M cytosine arabinoside was added to cultures to inhibit the proliferation of non-neuronal cells. Cells were used for experiments on DIV5–6. Transient transfections were performed on DIV4 using Lipofectamine 2000.

Drug Treatments of Cultured Neurons

Etoposide, camptothecin, cisplatin, suberoyl bishydroxamic acid (SBHA), suberoylanilide hydroxamic acid (SAHA), MS275 and trichostatin A (TSA) were dissolved in DMSO and added to the cell culture medium. The final media concentration of DMSO did not exceed 0.4 %.

Systemic Drug Treatments of P7 Rats

SAHA was dissolved in DMSO (100 mg/ml) and then diluted in sterile saline (1:2 v/v , 33 mg/ml). As at lower DMSO concentrations, SAHA precipitated out of solution, treatments were done with such a concentrated stock to avoid excessive in vivo use of DMSO. SAHA was administered subcutaneously 30 min prior to intracerebroventricular injections of etoposide or immediately before *i.p.* injections of dizocilpine. Dizocilpine (MK801) was dissolved in ethanol (10 mg/ml). For *i.p.* injection, it was further diluted with sterile saline to 1 mg/ml.

Intracerebroventricular Injections of Etoposide

Rats received intracerebroventricular injections at postnatal day 7 (P7) as previously described (Pietrzak et al. 2011). Briefly, the injections of 10 nmoles etoposide in 5 μ L 20 % v/v DMSO in artificial cerebro-spinal fluid were made into the left lateral ventricle at the following coordinates: 1.5 mm rostral and 1.5 mm lateral to lambda 2 mm deep from the skull surface.

Quantitation of Neuronal Survival by MTT Assay

The MTT assay was performed in 96-well plates as described (Hetman et al. 1999).

Quantitation of Apoptosis

To visualize nuclear morphology, cells were stained with 2.5 μ g/mL of the DNA dye Hoechst 33258 (bis-benzimide) (Hetman et al. 1999). Nuclear morphology was evaluated using fluorescent microscopy. Cells with uniformly stained nuclei were scored as viable; cells with condensed and/or fragmented nuclei were scored as apoptotic. At least 150 transfected (i.e., positive for the transfection marker β -galactosidase) or 300 non-transfected cells were analyzed for each condition in each experiment.

Immunofluorescence

Immunofluorescence for β -gal was performed using a rabbit anti- β -gal antibody (MP Biomedicals) as described previously (Hetman et al. 1999). Immunofluorescence for γ H2AX was done using a standard immunofluorescence protocol with some modifications. Briefly, cells were fixed with 4 % paraformaldehyde and incubated in 0.5 % NP-40 for 10 min at room temperature followed by blocking in 10 % goat serum/PBS/0.2 % Triton X-100 for 1 h at room temperature. The rabbit anti- γ H2AX antibody (Abcam) was applied overnight at 4 °C (1:200 dilution in 5 % goat serum/PBS/0.2 % Triton X-100) followed by 1-h incubation with the Alexa-488-coupled anti-rabbit IgG antibody (Invitrogen, 1:200) at RT. Images were captured using the Zeiss AxioObserver inverted microscope that was powered by the AxioVision software.

Immunoblotting analysis was performed using standard procedures. For preparation of lysates for histone proteins analysis, cells were lysed in NTEN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8.0, 0.5 % NP-40 and protease inhibitor) for 10 min at 4 °C. The lysate was centrifuged at 13,000 rpm for 15 min. The supernatants were removed, and the pellets were dissolved in 1 $\times$ SDS-PAGE sample buffer followed by boiling for 10 min before loading on gel. The primary antibodies were as follows: anti-p53 (Santa Cruz Biotechnology, dilution 1:500), anti-phospho-Ser15-p53 (Cell Signaling Technology, dilution 1:1,000), anti- β -actin (Sigma, dilution 1:2,000), anti- γ H2Ax (Abcam, dilution 1:1,000), anti-cleaved caspase-3 (Cell signaling Technology, dilution 1:1,000), anti-acetylated N-terminus histone H3 including the acetylated K14 residue (Millipore, dilution 1:2,000) and anti-histone H3 (Upstate, dilution 1:1,000). Secondary antibodies were horseradish peroxidase-conjugated. For quantifications, non-saturated exposures of the blots were used. After image acquisition, densitometry analysis of the bands was performed using Image-J.

Statistical Analysis

Statistical analysis of the data was performed using the nonparametric Kruskal–Wallis ANOVA; comparisons

between pairs of conditions were performed using the nonparametric *u* test.

Results

The Nonselective HDACis Enhance Neurotoxicity of DNA Damaging Drugs

To evaluate effects of pan-HDACis on DNA damage neurotoxicity, primary rat cortical neurons were treated with the DSB inducer etoposide with or without TSA or SBHA or SAHA. After 90 min, HDACis increased acetylation of histone H3 (Fig. 1a, b). Conversely, etoposide reduced levels of acetylated H3 under basal conditions without affecting the response to the treatment with HDACis (Fig. 1a, b). After 24 h and/or 48 h of treatment, TSA and SAHA but not SBHA moderately reduced neuronal survival (Fig. 1c–f). While under similar conditions 1 or 2 μM etoposide did not lower survival by more than 16 % (1 μM at 48 h), its combination with pan-HDACis produced a strong anti-survival effect (Fig. 1c–f). In contrast, higher etoposide concentrations resulted in a pronounced decrease of neuronal viability that was unaffected by HDACis (Fig. 1e, f). Importantly, at such higher concentrations, etoposide appeared to reach the maximum anti-survival activity (Fig. 1e). Hence, pan-HDACis potentiate etoposide-induced neurotoxicity when the cell death response to the latter drug is not saturated. In addition, the neurotoxic interaction between 1 μM etoposide and TSA reached its maximum at the low TSA concentration of 0.1 μM (Fig. 1f).

Effects of pan-HDACis on the neurotoxic response to other anticancer drugs that induce DNA damage were also examined. Thus, after the 24 h co-treatment with 0.1 μM TSA and 1 μM camptothecin, neuronal survival declined to 60 % (Fig. 2a). Conversely, at such low concentration, camptothecin did not affect survival by itself consistently with previously published results from a similar cell culture system (Hetman et al. 1999). Similar effects were also observed when 0.1 μM TSA or 10 μM SAHA was so-applied with 5 $\mu\text{g}/\text{ml}$ cisplatin (Fig. 2a, b).

Non-selective HDACis Enhance Apoptotic Response to DNA Damaging Drugs

Etoposide, camptothecin and cisplatin induce apoptosis in cultured neurons (Gozdz et al. 2003; Morris and Geller 1996; Enokido et al. 1996; Pietrzak et al. 2011). Therefore, we have investigated whether the cytotoxic interactions between the DNA damaging drugs and pan-HDACis involve enhancement of apoptosis. After 16-h treatment with TSA or etoposide or TSA + etoposide, apoptotic

changes of nuclear chromatin were observed in 11 ± 1.8 or 20 ± 5.4 or 44 ± 4.7 % neurons, respectively (Fig. 3a, b). In addition, in etoposide-treated neurons, proteolytic activation of the apoptotic protease caspase-3 was enhanced by TSA or SBHA or SAHA (Fig. 3c, d). Therefore, exacerbated apoptosis contributes to the neurotoxic interactions between etoposide and pan-HDACis.

To investigate whether co-treatment with pan-HDACis and etoposide results in enhanced neuronal apoptosis *in vivo*, rat neonates at postnatal day 7 (P7) received subcutaneous injections of SAHA. Under these conditions, SAHA increased acetylation of histone H3 in the neocortex (Fig. 4a). In addition, 10 nmoles etoposides were injected into the lateral ventricle as such treatment was shown before to induce cortical neuron apoptosis in the ipsilateral neocortex (Pietrzak et al. 2011). Consistent with these observations, activated caspase-3 was observed in the ipsilateral neocortex of etoposide-treated rats (Fig. 4b, d). No activated caspase-3 was detected in animals that were treated with SAHA without etoposide (Fig. 4b, c). Conversely, enhanced activation of caspase-3 was present after co-treatment with etoposide and SAHA (Fig. 4b, d). To determine whether the pro-apoptotic effect of SAHA was specific for etoposide, the NMDA receptor antagonist dizocilpine was applied. Its single dose is sufficient to induce neuronal apoptosis and activate caspase-3 in the neocortex of P7 rodents (Ikonomidou et al. 1999). When applied at a dose of 1 mg/kg, dizocilpine activated caspase-3 in the neocortex (Fig. 4c, d). Interestingly, SAHA treatment enhanced dizocilpine-induced activation of caspase-3, albeit to a lesser extent than in etoposide-treated rats. These findings suggest that pan-HDACis promote apoptosis in response to either etoposide or NMDA receptor blockade. In the case of etoposide, DSB induction and/or the DSB response is a likely target of the neurotoxic activity of pan-HDACis. A similar mechanism may underlie the neurotoxic interactions between pan-HDACis and the NMDA receptor blockers as in rodent neonates dizocilpine has been shown to induce oxidative stress that could potentially lead to DNA damage including DSBs (Papadia et al. 2008).

Increased apoptosis in neurons that were co-treated with etoposide and pan-HDACis may be a result of conditions favoring more DSBs. Such conditions are expected as HDACis elevate acetylation of histones resulting in chromatin opening. Indeed, we observed increased apoptosis in neurons that were first treated with TSA and then exposed to etoposide (Vashishta and Hetman, unpublished observations). Another interesting possibility is that HDACis influence apoptotic outcome of DSBs by modulating DNA damage response including DSB repair and activation of DNA damage signaling pathways. Therefore, neurons were treated with etoposide for 2 h and then were let to recover

Fig. 1 Nonselective HDACis enhance neurotoxicity of etoposide. Rat cortical neurons were treated with etoposide in the presence or absence of pan-HDACis as indicated (vehicle, 0.2 % DMSO, V; 0.1 μ M TSA, TS; 10 μ M SBHA, SB; 10 μ M SAHA, SA). **a**, **b** Immunoblot analysis of histone H3 acetylation. Total H3 levels were determined by analyzing the same samples on a separate blot; quantitations were performed by densitometry; numbers under the blot indicate optical density ratios of the bands for the acetylated- and total H3 (fold control). After the 90-min treatment, etoposide reduced acetylation of H3 suggesting HDAC activation. Conversely, pan-

HDACis increased acetylated-H3 levels suggesting effective HDAC inhibition. **c–f** Neuronal survival analysis. MTT assay was performed at 24 h after starting co-treatments unless indicated otherwise. Pan-HDACis synergized with sub-toxic concentrations of etoposide to reduce neuronal survival. Data represent means $\pm$ SD from three independent experiments except **e**, where two experiments are depicted. In **a**, **b** or **c–f** single or six sister cultures were used per each condition, respectively; * p < 0.05; *** p < 0.001; NS p > 0.05 (u test); in **e–f**, u test analysis compared effects of each dose of etoposide in TSA-untreated versus TSA-treated cultures

Fig. 2 Nonselective HDACis enhance neurotoxicity of cisplatin and camptothecin. Rat cortical neurons were treated as indicated. MTT assay was performed at 24 h after starting co-treatments. Combinations of the pan-HDACis TSA or SAHA with the DNA damaging anticancer drugs camptothecin or cisplatin resulted in pronounced reductions of neuronal survival. Data represent means $\pm$ SD from five (a), or two (b) independent experiments; in each experiment, six sister cultures were used per each condition; $**p < 0.01$; $***p < 0.001$; NS $p > 0.05$ (*u* test)

for the next 24 h (Fig. 5). Under such conditions, no apoptosis was observed unless pan-HDACis were added during the recovery period (Fig. 5b). Hence, pan-HDACis exacerbate neurotoxicity of etoposide at least in part by modulating the DNA damage response.

Non-selective HDACis Increase Etoposide-Induced DSBs and the Pro-apoptotic Activation of p53

To further examine mechanisms behind the neurotoxic interactions of the nonselective HDACis and etoposide, accumulation of the Ser-139-phosphorylated H2Ax variant (γ H2Ax) was investigated using immunoblotting. γ H2Ax is a specific and quantitative marker of DSBs

(Kinner et al. 2008). Etoposide increased levels of γ H2Ax in cultured cortical neurons (Fig. 6a, b). The effect was enhanced by pan-HDACis (Fig. 6a, b). Similar enhancement was observed when analyzing γ H2Ax by immunofluorescence (Fig. 6c). These observations suggest that pan-HDACis increased DSBs in response to etoposide. Such an effect may be due to pan-HDACis-induced deficiency in DSB repair as HDAC1 and HDAC2 are required for that process in non-neuronal cells (Miller et al. 2010; Lahue and Frizzell 2012). Alternatively, pan-HDACis may increase chromatin sensitivity to etoposide (Kim et al. 2003).

The transcription factor p53 is a key mediator of the DNA damage-induced neuronal apoptosis (Morrison and Kinoshita 2000; Jacobs et al. 2006). Activation of p53 occurs by its stabilization as well as posttranslational modifications including phosphorylation at the Ser-15 residue. Etoposide treatment elevated levels of the pSer15-p53 and the total p53 (Fig. 7a, b). The co-treatment with TSA or SBHA or SAHA led to further enhancement of the p53 response (Fig. 7a, b).

To determine whether pan-HDACis directly affect the pro-apoptotic activity of p53, neurons were transfected with an expression plasmid for a temperature-sensitive mutant of p53 (TS-p53). For the next 24 h, cells were kept at the nonpermissive temperature of 37 °C, which favored the dominant-negative conformation of TS-p53 (Michalovitz et al. 1990). Then, the temperature was lowered to 32 °C restoring the wild-type activity of the overexpressed TS-p53 (Michalovitz et al. 1990). In a pilot study, we determined that at 0.1 μg plasmid DNA/5 $\times$ 10⁵ neurons, TS-p53 only moderately affected apoptosis after 24 h at 32 °C (data not shown). Consistently with our previously published results, higher doses of TS-p53 were strongly pro-apoptotic under such conditions (data not shown) (Kalita et al. 2008). Hence, TS-p53 was transfected at 0.1 μg plasmid DNA/5 $\times$ 10⁵ neurons for the subsequent experiments to determine its pro-apoptotic effects in the presence of the pan-HDACi TSA. In vehicle-treated neurons that received the empty vector or TS-p53 and were shifted to 32 °C for 24 h, there were 21 or 27 % apoptosis, respectively (Fig. 7c, $p > 0.05$). TSA treatment increased apoptosis in TS-p53 transfected neurons to 38 % (Fig. 7c, $p < 0.01$). Conversely, apoptosis was unaffected by TSA in empty vector transfected neurons (21 or 20 % apoptosis in vehicle- or TSA-treated cells, respectively, Fig. 7c, $p > 0.05$). These observations suggest that in neurons, pan-HDACis potentiate the pro-apoptotic activation of p53. Besides their effects on DSBs, such a modulation of the DNA damage response may contribute to the observed neurotoxic interactions between pan-HDACis and etoposide.

Fig. 3 Nonselective HDACis enhance apoptotic response to etoposide. Neurons were treated with etoposide and/or pan-HDACis as indicated (vehicle, 0.2 % DMSO, V; 0.1 μM TSA, TS; 10 μM SBHA, SB; 10 μM SAHA, SA). **a, b** After 16 h, apoptotic changes of nuclear chromatin including condensation and fragmentation were visualized by staining with Hoechst-33258. Representative photomicrographs of Hoechst-33258 stained cells are shown in **a**; arrows point apoptotic cells. Quantification of apoptotic cells from three independent

experiments is shown in **b, c, d** Immunoblot analysis of the activated apoptotic protease caspase-3 after 6 h of treatment (cleaved caspase-3). Equal protein loading was verified by reprobing the blot for β -actin; quantitations were performed by densitometry; numbers under the blot indicate optical density ratios of the bands for the active caspase-3 and β -actin (fold control). Pan-HDACis enhanced etoposide-induced activation of caspase-3. Graphs depict means $\pm$ SD of three independent experiments; * $p < 0.05$ (u test)

Selective Inhibition of HDAC1 Only Partially Reproduces Pan-HDACis Effects on Etoposide-Challenged Neurons

Selective inhibition of HDAC1 has been previously implicated in impaired DSB repair and neurotoxicity (Kim et al. 2008; Miller et al. 2010). Hence, a question can be raised whether the neurotoxic interactions between pan-HDACis and etoposide are mediated by loss of HDAC1 activity. To address that question a selective HDAC1 inhibitor, MS275 was used. Consistent with the results presented in Fig. 1a, b, 1 μM etoposide reduced levels of acetylated H3. Conversely, treatment with 1 μM MS275 increased H3 acetylation regardless of etoposide presence suggesting that HDAC1 is the major histone H3 deacetylase in rat cortical neurons (Fig. 8a). A 24-h treatment with

either 1 μM MS275 or 1 μM etoposide did not affect neuronal survival (Fig. 8b). When these drugs were combined, neuronal viability declined to 73 % of vehicle-treated controls (Fig. 8b). However, such an anti-survival effect was significantly smaller than that observed for the combination of etoposide with TSA suggesting nonoverlapping contributions by HDAC1 and other HDACs to the neuronal resistance against the DNA damage (Fig. 8b).

Similar to pan-HDACis, MS275 enhanced etoposide-induced accumulation of γH2Ax (Fig. 8c). Unlike pan-HDACis, MS275 did not potentiate etoposide-mediated activation of p53 as revealed by similar increases of pSer15-p53- and total p53 levels in etoposide- or etoposide + MS275-treated neurons (Fig. 8d, e). Hence, HDACs other than HDAC1 regulate p53 activation in etoposide-challenged neurons. These observations also

Fig. 4 The nonselective HDACi SAHA enhances apoptotic response to etoposide or dizocilpine in the neocortex of whole rats. At postnatal day 7 (P7), rats received subcutaneous injections of SAHA that were followed by other treatments as indicated. The neocortex was dissected and analyzed by immunoblotting with the anti-active caspase-3 antibody (cleaved caspase-3) followed by reprobing with the anti- β -actin antibody to ensure equal protein content. Each lane represents an individual animal; quantitations were performed by densitometry. **a** Increased levels of acetylated histone H3 in SAHA-treated rats. Equal levels of H3 were verified by immunoblotting analysis of the same samples for total H3. Numbers under the blots indicate optical density ratios of the bands for the acetylated- and the total H3 (fold control). **b** Thirty minutes after SAHA administration, rats received injections of etoposide into the left lateral ventricle. In the ipsilateral neocortex, caspase-3 activation was observed 4 h later; the increase was enhanced in the SAHA-treated group. **c** Immediately after SAHA administration, rats received the NMDA receptor antagonist dizocilpine (MK801, *i.p.*). After 24 h, NMDA receptor blockade activated caspase-3; the activation was enhanced by SAHA. **d** The graph illustrates effects of SAHA on etoposide- and dizocilpine-induced activation of caspase-3; means $\pm$ SD of three (etoposide experiment) or six (dizocilpine experiment) animals are depicted; ** $p < 0.01$; * $p < 0.05$ (*u* test)

suggest that the effects of pan-HDACis on p53 activation are not just a simple consequence of more DSBs. Instead, pan-HDACis may affect DSB levels and p53 activation through nonoverlapping mechanisms (Fig. 8f).

Fig. 5 Neuronal apoptosis induced by treatment with nonselective HDACis during recovery after etoposide exposure. **a** Experimental design. Neurons were treated with etoposide followed by placement in etoposide-free conditioned media that was supplemented with vehicle (0.2 % DMSO, V) or 0.1 μ M TSA (TS) or 10 μ M SBHA (SB). Apoptosis was analyzed using Hoechst-33258 staining. **b** Quantification of apoptotic response. After 24 h of recovery following transient exposure to etoposide, apoptosis did not increase unless cells were treated with pan-HDACis during the recovery period. Data represent means $\pm$ SD of three independent experiments (2 sister cultures/experiment/condition); * $p < 0.05$; NS $p > 0.05$ (*u* test)

Discussion

The presented data indicate that pan-HDACis potentiate neurotoxicity of DNA damaging anticancer drugs including the DSB inducer etoposide. These neurotoxic interactions may be due to enhanced induction and/or removal of DSBs and modulation of the DNA damage response. While in etoposide-challenged neurons selective inhibition of HDAC1 results in more DSBs, pan-HDACis also enhance activation of the pro-apoptotic transcription factor p53 (Fig. 8f). These findings suggest that in neurons, HDACs influence outcome of the DNA damage by promoting genomic integrity and suppressing DNA damage-induced apoptosis.

Several reports have suggested pro-apoptotic neurotoxicity of HDACis (Salminen et al. 1998; Boutillier et al. 2003; Liu et al. 2005; Kim et al. 2008; Wallace and Cotter 2009). Consistent with these reports, we have observed moderate but significant reduction of cultured cortical neuron survival after 24- or 48-h exposure to TSA or SAHA. However, no toxicity has been observed with SBHA or MS275, for at least 24 h posttreatment. The

Fig. 6 Nonselective HDACis increase etoposide-induced DSBs. Neurons were treated with etoposide in the presence or absence of pan-HDACis for 90 min as indicated (vehicle, 0.2 % DMSO, V; 0.1 μM TSA, TS; 10 μM SBHA, SB; 10 μM SAHA, SA). **a**, **b** Levels of the Ser139-phosphorylated histone H2Ax (γH2Ax) that is a specific marker of DSBs were determined by immunoblotting; equal content of chromatin proteins was verified by reprobing the blot for the total histone H3. *Numbers* under the *blots* indicate optical density

ratios of the bands for the γH2Ax and the total H3 (fold control). Pan-HDACis potentiated etoposide-mediated induction of DSBs. **c** Such an effect was also obvious when γH2Ax levels were determined by immunofluorescence (γH2Ax-positive foci in neuronal nuclei are indicated by *arrows*). In **b**, data represent means $\pm$ SD of three independent experiments; $*p < 0.05$ (*u* test). In **c**, representative photomicrographs are shown; similar effects were observed in three independent experiments

reasons for such an attenuated neurotoxic response to these HDACis may include the relative maturity of cortical cultures that were used here as compared to embryonic cortical neurons that were used by others (Liu et al. 2005; Kim et al. 2008; Langley et al. 2008).

We observed enhanced apoptosis when cultured cortical neurons or rat neonates were challenged with etoposide in the presence of HDACis. That interaction was maximal when pan-HDACis were used together with sub-toxic concentrations of etoposide. In addition, the enhanced neurotoxic response was observed when HDACis were applied during recovery from a transient etoposide exposure. Finally, enhanced neurotoxicity was also present when pan-HDACis were combined with the SSB inducer camptothecin or the DNA bulky adduct inducer cisplatin. To the best of our knowledge, such an HDACi-induced enhancement of neurotoxicity of DNA damaging drugs has not yet been reported. Conversely, in cancer cells, cytotoxic synergy between DNA damaging anticancer drugs

and HDACis including etoposide, TSA, SAHA and MS275 is well documented (Hajji et al. 2008; Kim et al. 2003; Chen et al. 2007). Hence, the role of HDACs in the DNA damage response may be similar in postmitotic and rapidly proliferating cells.

Our data indicate that HDACis increased DSBs in response to etoposide. Such effects were observed with pan-HDACis or the HDAC1 preferring inhibitor MS-275 indicating a critical role of HDAC1 in maintenance of the neuronal genome. This conclusion is in agreement with findings that in HDAC1-inhibited neurons, neuronal death has been preceded by increased accumulation of DSBs (Kim et al. 2008). While it is not clear how HDACs may regulate neuronal DSB repair, in non-neuronal cells, HDAC1 and HDAC2 have been shown to be rapidly recruited to DSBs to promote their repair by local deacetylation of the H3 Lys-56 and H4 Lys-16 residues (Miller et al. 2010). Interestingly, such a scenario may take place in etoposide-treated neurons as they show reduced

Fig. 7 The pro-apoptotic activation of p53 is enhanced by nonselective HDACis. **a, b** Neurons were treated with etoposide in the presence or absence of the pan-HDACis for 6 h as indicated (vehicle, 0.2 % DMSO, V; 0.1 μM TSA, TS; 10 μM SBHA, SB; 10 μM SAHA, SA). Levels of the Ser15-phosphorylated (P-p53) p53 and the total p53 (T-p53) were determined by immunoblotting; the membranes were reprobed for β-actin to ensure equal protein loading in each lane. **a** Blots from representative experiments; numbers under the blots indicate optical density ratios of the bands for the P-p53 and the β-actin or the T-p53 and the β-actin (fold control). **b** Quantification of phospho-p53 levels as determined in three independent experiments. Pan-HDACis increased p53 activation in response to etoposide. In addition, TSA or SBHA weakly increased p53 phosphorylation/accumulation without etoposide. **c** Apoptosis in response to the overexpressed p53 is enhanced by the pan-HDACi TSA. Neurons were co-transfected with expression plasmids for β-gal

and either a temperature-sensitive mutant of p53 (TS-p53) or an empty expression vector (pCMV, vector; 0.2 + 0.1 μg of plasmid DNAs/5 × 10⁵ neurons, respectively). For next 24 h, neurons were kept at 37 °C. At this temperature, TS-p53 remained in an inactive conformation. Then, neurons were placed at 32 °C restoring wild-type p53 conformation of TS-p53. In addition, cells were treated with TSA as indicated. At 24 h after the temperature shift, TS-p53 induced apoptosis in TSA-treated neurons. Representative micrographs of transfected (i.e., β-gal-positive) neurons are shown; a surviving neuron with uniformly stained nucleus is indicated by an arrow; an arrowhead identifies an apoptotic neuron with fragmentation of nuclear chromatin. The graph presents quantitation of the apoptosis response from three independent experiments (2 sister cultures/experiment/condition). In **b, c**, error bars represent SD; NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$ (u test)

levels of acetylated H3 (Figs. 1a, b, 8a). In addition, HDACs including HDAC1 may stimulate DNA repair by acting on nonhistone substrates such as DNA repair enzymes themselves (Chen et al. 2007; Robert et al. 2011). Finally, HDACs may also reduce initial vulnerability of neuronal DNA to DNA-damaging drugs by acting on histones to promote closed chromatin conformation as has been suggested in cancer cells (Kim et al. 2003). Indeed, our preliminary data suggest that prior exposure to TSA

sensitizes neurons to a subsequent etoposide treatment (Vashishta and Hetman, unpublished observations). Future studies are needed to determine mechanisms of HDAC effects on integrity of the neuronal genome.

In DSB-challenged neurons, the nonselective HDACis but not the HDAC1 inhibitor MS275 enhanced the pro-apoptotic activation of p53. Such observations are consistent with the greater enhancement of etoposide neurotoxicity by pan-HDACis than MS275. As p53 plays a critical

Fig. 8 Enhancement of etoposide induced neuronal death and DSBs but not the p53 activation by the selective HDAC1 inhibitor MS275. Neurons were treated with etoposide in the presence or absence of TSA or MS275 as indicated (vehicle, 0.2 % DMSO, V; 0.1 μM TSA, TS; 1 μM MS275, MS). **a** Immunoblotting for acetylated histone H3. Total H3 levels were determined by analyzing the same samples on a separate blot. After 90-min treatment, etoposide decreased levels of the acetylated histone H3 while MS275 had an opposite effect regardless of etoposide presence. The numbers below the blots indicate ratios of acetylated H3 to total H3 (fold control). **b** MTT survival assay after 24-h treatment. While MS275 did not appear to induce neuronal death by itself, it was neurotoxic when combined with etoposide. However, the latter effect was weaker than that of the pan-HDACi TSA. **c** After 90-min treatment, immunoblotting for the DSB marker γH2Ax suggested increased DSB induction when etoposide and MS275 were combined. **d** After 6-h treatment, immunoblotting for the Ser15-phosphorylated p53 revealed no effects

of MS275 on etoposide-induced p53 activation. **e** Likewise, MS275 did not affect the etoposide-induced increase of the total p53 levels. Numbers under the blots indicate optical density ratios of the bands for the total p53 (T-p53) and the β-actin (fold control). In **b**, data represent means of thirty sister cultures from five independent experiments (6 sister cultures/experiment/condition); in **c–e**, representative blots and/or quantitations of three independent experiments are shown; in **d**, **e**, equal protein loading was verified by reprobing for β-actin. Error bars represent SD; * $p < 0.05$; *** $p < 0.001$; NS $p > 0.05$ (u test). **f** A hypothetical model of interactions between HDAC1 and the neurotoxic cascade that is activated by etoposide. The selective inhibition of HDAC1 enhances etoposide-induced cell death by increasing the number of etoposide-induced DSBs either through opening chromatin for more damage or by reducing DSB repair. When the nonselective inhibition of HDACs is applied, the pro-apoptotic activation of p53 is also augmented in a direct manner

role in neuronal apoptosis in response to various DNA damaging agents including etoposide, camptothecin or irradiation (Enokido et al. 1996; Xiang et al. 1998; Herzog et al. 1998), pan-HDACis may enhance apoptosis that is induced by those various genotoxic factors. Our findings are in good agreement with observations from non-neuronal systems in which inhibition of HDACs potentiated pro-apoptotic activity of p53 (Luo et al. 2000; Terui et al. 2003). Such an effect has been proposed to be due to a direct interference with p53 deacetylation by HDAC1 (Luo et al. 2000). Acetylation of p53 is critical for its activation including protein stabilization-mediated increase in p53 levels as well as increased ability to bind DNA and transactivate p53-dependent genes (Gu and Roeder 1997; Juan et al. 2000; Tang et al. 2008). HDAC2 and HDAC3 also deacetylate p53 (Juan et al. 2000; Zhang et al. 2011). HDAC2 has recently been shown to be a critical negative regulator of p53 and a key target for the pro-apoptotic effects of pan-HDACis (Zhang et al. 2011). Increased acetylation of p53 has been demonstrated in TSA-treated cultured rat cerebellar granule neurons and in hippocampal neurons of whole rats that were subjected to global brain ischemia (Gaub et al. 2011; Raz et al. 2011). Hence, it is likely that pan-HDACis enhanced the neurotoxicity of DNA-damaging drugs at least in part by promoting p53 acetylation. MS275 has EC₅₀ of 0.18-, or 1.1-, or 2.3 μ M for HDAC1, or HDAC2, or HDAC3, respectively (Khan et al. 2008). Conversely, MS275 is ineffective against class II HDACs (Khan et al. 2008). In our experiments, MS275 was used at 1 μ M; in another cell culture study, such a concentration has been relatively ineffective against HDAC2 and HDAC3 (Pufahl et al. 2012). Therefore, inability of MS275 to modify p53 activation in DSB-challenged neurons suggests involvement of HDAC2 and/or HDAC3 and/or class II HDACs. Future studies are needed to identify which HDACs regulate neuronal p53.

Our findings that HDACis enhance neurotoxicity of DNA damaging agents are at odds with observations reported by Uo et al. (2009) and Brochier et al. (2013). In those studies, the pan-HDACis TSA and SAHA protected mouse cortical neurons against topoisomerase inhibitors including camptothecin and etoposide. The anti-apoptotic neuroprotection was despite ability of pan-HDACis to enhance DNA damage-induced accumulation of neuronal p53 and increase camptothecin-induced apoptosis of human neuroblastoma cells (Uo et al. 2009). It has been proposed that the mechanism of the pan-HDACis-mediated neuroprotection involves reduced ability of p53 to induce expression of the BH3-only protein Puma as well as direct interference with Bax. Paradoxically, p53 acetylation at lysine residues K381/382, which in cancer cells stimulated apoptosis, was contributing to TSA-mediated protection in neurons (Brochier et al. 2013). Hence, the cell-specific

regulation of p53 is a likely reason for differential effects of HDACis on apoptosis in various cells. Importantly, the pan-HDACi-mediated neuroprotection against DNA damaging agents was demonstrated in mouse cortical neurons that were isolated from either newborn or embryonic mice, maintained in serum-free media and used for experiments at DIV3-4 or DIV1, respectively (Uo et al. 2009; Brochier et al. 2013). In contrast, we used cortical neurons from newborn rats that were maintained in serum-containing medium and used for experiments at DIV5. The interspecies differences as well as the more advanced differentiation, which is expected in our case, might have affected acetylation-mediated regulation of DNA damage response contributing to different outcomes of HDACi treatments.

Besides the p53 pathway, HDACis may affect additional branches of the pro-apoptotic DNA damage response. For instance, in mouse sympathetic neurons, HDACis increase sensitivity to apoptotic stimuli by enhancing the activity of the DNA damage response transcription factor E2F1, which increases expression of *Apaf1* (Wright et al. 2007). E2F1 has also been proposed as a pro-apoptotic target for HDACis in mouse cerebellar granule neurons and mouse retina (Boutillier et al. 2003; Wallace and Cotter 2009). Moreover, in rat PC12 cells and rat cortical neurons, E2F-driven transcription of *B-myb* may be activated by HDACis leading to apoptosis (Liu et al. 2005). Reduced HDAC-mediated silencing of *B-myb* has also been proposed to play a role in DNA damage-induced apoptosis in these systems (Liu et al. 2005). Therefore, pleiotropic effects of pan-HDACis on multiple HDAC substrates may account for various effects on DNA damage-exposed cells dependent on such factors as cell type, differentiation stage, species and type of DNA damage.

As different HDACs may target distinct effector mechanisms, specific patterns of HDAC expression could determine responses to pan-HDACis. For instance, high-grade human neuroblastomas express elevated levels of HDAC8 that is required for their growth (Oehme et al. 2009). Thus, the relative ability of pan-HDACis to inhibit HDAC8 may determine their cytostatic potential against neuroblastoma cells. Hence, in normal brain cells, heterogeneous expression patterns of various HDAC isoforms may produce cell-type-specific and/or species-specific and/or differentiation stage-specific outcomes of pan-HDACi treatments.

Pan-HDACis are emerging as promising anticancer drugs (Marks and Xu 2009). Several ongoing clinical trials are evaluating their effectiveness when combined with classical chemotherapeutic drugs such as a cisplatin relative, carboplatin or etoposide (ClinicalTrials.gov). However, there is an increasing concern that such established chemotherapy agents negatively affect the nervous system including cognition (Kannarkat et al. 2007; Ahles and

Saykin 2007). For instance, in children, persistent cognitive deficits were reported after chemotherapy (Robaey et al. 2008). Our findings indicate that even a relatively transient exposure to pan-HDACis including the clinically used SAHA is sufficient to enhance neurotoxicity of the commonly used anticancer drugs. In our study, we used SAHA at the concentration of 10 μ M that is below its maximal plasma levels in humans, which received therapeutic doses of this drug (Kelly et al. 2005). We also observed similar enhancement of etoposide neurotoxicity with SAHA concentrations as low as 1 μ M (data not shown). However, at least in mice, nearly 10 times lower maximal brain concentration was reported following administration of therapeutically relevant doses of SAHA (Hanson et al. 2013). Therefore, the clinical relevance of our cell culture data is unclear.

Importantly, we also observed enhanced neurotoxicity of etoposide when SAHA was applied to whole neonate rats in a single dose of 40 mg/kg *s.c.* At least in adult mice, such a dose is expected to produce similar plasma levels of SAHA as those observed in humans that received the recommended clinical daily dose of 400 mg *p.o.* (Hanson et al. 2013; Kelly et al. 2005). Indeed, 40 mg/kg is a therapeutically relevant dose in rats as in this species, 50 mg/kg was administered daily over several days to demonstrate the beneficial effects of SAHA as an anti-inflammatory agent and/or anticancer agent (Lin et al. 2007; Ganslmayer et al. 2012). Thus, one should be aware that combining pan-HDACis including SAHA with the classical anticancer chemotherapeutics may potentially produce more neurotoxic side effects.

In rat neonates, a single injection of SAHA (40 mg/kg, *s.c.*) enhanced apoptosis that was induced by the NMDA receptor blocker dizocilpine. In the developing brain, blocking neuronal activity in general and the NMDA receptor in particular induces neuronal apoptosis (Hetman and Kharebava 2006; Hardingham 2006). This effect has been proposed to underlie long-term neurological deficits in children that were repeatedly exposed to general anesthetics (McCann and Soriano 2012). Hence, HDACis may potentially enhance the risk of neurodevelopmental toxicity of general anesthesia.

In summary, our results suggest that at least in developing rat cortical neurons HDACis enhance apoptotic consequences of DNA-damaging drugs. This neurotoxic interaction is likely due to both increased numbers of DSBs and hyperactivation of the cytotoxic arm of the DNA damage response. Hence, HDACs appear to be essential regulators of neuronal response to DNA damage. Specifically, our findings indicate that HDACs prevent accumulation of DSBs and blunt the p53-mediated apoptotic response that is induced by such genomic lesions. Thus, neurons may have extra time to restore genomic integrity

before apoptosis is initiated. The key question to be answered by future studies is which HDACs and HDAC substrates contribute to neuronal defenses against DNA damage.

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