Preclinical Development

Histone Deacetylase Inhibition Attenuates Cell Growth with Associated Telomerase Inhibition in High-Grade Childhood Brain Tumor Cells

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Abstract

Aberrant epigenetic regulation of gene expression contributes to tumor initiation and progression. Studies from a plethora of hematologic and solid tumors support the use of histone deacetylase inhibitors (HDACi) as potent anticancer agents. However, the mechanism of HDACi action with respect to the temporal order of induced cellular events is unclear. The present study investigates the anticancer effects of the HDACi trichostatin A in high-grade childhood brain tumor cells. Acute exposure to trichostatin A resulted in marked inhibition of cell proliferation, an increase in the proportion of G2-M cells, activation of H2A.X, and subsequent induction of apoptosis in the majority of cell lines. These phenotypic effects were associated with abrogation of telomerase activity and human telomerase reverse transcriptase downregulation in the majority of cell lines. In contrast, no cytotoxicity was observed in primary ependymal cells with respect to cilia function. Thus, inhibition of histone deacetylases leads to antiproliferative and proapoptotic effects in childhood brain tumor cells, likely to involve altered chromatin regulation at the human telomerase reverse transcriptase promoter.

Introduction

Of all childhood cancers, brain tumors have the poorest survival and highest morbidity rates. Although frequently considered collectively, brain tumors represent a diverse range of tumor types. Survival rates for high-grade tumors of glial [glioblastoma multiforme (GBM), ependymoma] and neuroectodermal [high-risk medulloblastoma and central nervous system neuroectodermal tumors (CNS PNET)] origin remain extremely poor. Despite treatment improvements in multimodal management, only 50% of children diagnosed with tumors of the brain and spine are cured (1–3), with a substantial proportion left with severe neurologic disability. There is, therefore, the need to develop more effective and specific chemotherapeutic regimens that can successfully eradicate residual tumor cells commonly spared by current therapies.

Histone deacetylases (HDAC) are known to regulate gene transcription and oncogenesis through remodeling of chromatin structure, canonically resulting in the repression of target genes (4, 5). Inhibition of HDACs increases acetylation of histone and nonhistone proteins at the ε-amo group of lysine residues within the peptide chain, leading to an increase in transcriptionally active, open chromatin. This conformational change can lead to restoration of transcriptionally silenced tumor-suppressor pathways and reexpression of proteins that repress tumor-propagating genes. As HDAC substrates include a vast array of nonhistone proteins such as transcription factors (6), these HDACs may more descriptively be referred to as “lysine tail deacetylases” (7, 8). Aberrant HDAC expression has been linked conceptually and mechanistically to the pathogenesis of cancer due to perturbation of acetylation-deacetylation homeostasis. As such, a number of HDAC inhibitors (HDACi) are under preclinical and clinical investigation as viable anticancer agents (9–16). Small-molecule HDACi have achieved significant biological effects in preclinical cancer models, including GBM (17), medulloblastoma (18), pancreatic cancer, breast cancer, melanoma, and leukemia (19), with vorinostat (suberoylanilide hydroxamic acid) receiving approval by the U.S. Food and Drug Administration for the treatment of refractory cutaneous T-cell lymphoma (20). Moreover, it has been shown that HDACi equally sensitize cancer cells to the cytotoxic effects of other chemotherapeutic agents (21–24). To a
variable extent, HDACi induce growth arrest, differentiation, or apoptosis in vitro and in vivo (25, 26). Promising in vitro results have led to several brain tumor clinical trials using diverse HDACi, with a vorinostat phase II trial in adult recurrent GBM showing anticancer activity (27) and a vorinostat phase I trial in synergy with 13-cis-retinoic acid, recently commenced for pediatric medulloblastoma and CNS primitive neuroectodermal tumors (28). However, prior studies of HDACi in brain tumor cells did not investigate the mode of HDACi action or the temporal order of cellular events post HDACi exposure.

The mechanism(s) by which HDACi exert downstream effects that attenuate tumor growth is unknown. One attractive candidate for HDACi-mediated inhibition is the holoenzyme telomerase. In ~90% of malignant cells, unlimited replicative potential is conferred in considerable part by the maintenance of telomere length at chromosomal termini, above a critical minimum required to bypass senescence and apoptosis. This is achieved through the de novo addition of telomeric repeats by telomerase, which consists of an intrinsic RNA template (human telomerase RNA, hTR) and a catalytic component [human telomerase reverse transcriptase (hTERT)]. High telomerase activity is evident in ~90% of cancers tested, whereas in most somatic tissues the enzyme is undetectable or transiently active at low levels due to repression of hTERT transcription. Telomerase has been detected in an increasing number of brain tumors (29), including those arising in childhood as shown by our laboratory and others (30). With preclinical and clinical advances in telomerase therapeutics for cancer, there is a need to investigate whether such therapy is of importance to childhood brain tumors. Derepression of hTERT is directly linked to telomerase activation in tumor cells (31), and a number of transcription factors are known to regulate hTERT, including c-Myc (activator), Smad3, and Mad1 (repressors). Recently, exposure to the HDACi trichostatin A (TSA) was shown to reduce telomerase activity and hTERT gene expression in two brain cancer cell lines, including one childhood medulloblastoma line (17). This was concurrent with cellular growth arrest and apoptosis. Whether these antitelomerase effects are consequential or causal to the observed growth arrest remains unclear, as is the mechanism of TSA-mediated telomerase inhibition. HDACi exert antiproliferative effects rapidly in a telomere length–independent manner. This contrasts with classic antitelomerase approaches that require a lag period of telomere shortening upon telomerase inhibition (32). Concerns that HDACi may interfere with critical cellular functions seem unfounded as evidence thus far indicates that HDACi display selective toxicity against tumor cells compared with untransformed cells (33, 34).

Although there are more than 100 trials ongoing or recently concluded with HDACi as monotherapy or combination therapy, to date comprehensive preclinical testing of HDACi in diverse CNS brain tumors, particularly those of childhood, is lacking. Furthermore, the mechanism by which HDACi may be exerting an antiproliferative effect has been poorly studied (29, 35). The present study was therefore undertaken to assess the antitumor activities upon histone deacetylase inhibition in high-grade pediatric CNS PNET, medulloblastoma, ependymoma, and GBM tumor cells. We herein report effects on tumor cell proliferation, cell cycle progression, genomic instability, and apoptosis after exposure to the HDACi TSA, with cellular toxicity investigated using primary rat ependymal cells. Additionally, we assess HDACi as a mode of abrogating telomerase function in brain tumor cells, with reference to telomerase activity and telomere length data from primary pediatric brain tumors and cell lines. A hypothesis is presented pertaining to the mechanism of HDACi-mediated telomerase repression in this context.

Materials and Methods

Childhood primary brain tumors and cell lines

Brain tumor samples were obtained from the Children’s Cancer and Leukaemia Group and the Cooperative Human Tissue Network. A total of 18 snap-frozen CNS PNETs, all located in the cerebral hemispheres, 20 medulloblastomas, and 8 GBMs were obtained. Five CNS PNETs were recurrences, four with the paired primary. One CNS PNET and two medulloblastomas were recurrences. Constitutional blood samples were received for five CNS PNETs and three medulloblastomas. Clinical information, including gender, age at diagnosis, and time to recurrence, was obtained from Children’s Cancer and Leukaemia Group and Cooperative Human Tissue Network. Multiple Centre Research Ethics Committee approval was obtained for the study. Consent for use of tumor samples was taken in accordance with national tumor banking procedures and the Human Tissue Act. PFSK-1 (CNS PNET, isolated from the cerebral hemisphere) and DAOY (medulloblastoma, isolated from the cerebellum) cell lines were obtained from American Type Culture Collection and isolated from the cerebral hemisphere and cerebellum, respectively. The EPN-2 (ependymoma) cell line was derived at the Children’s Brain Tumor Research Centre, University of Nottingham, United Kingdom, and the GB-1 (GBM) cell line was derived at the University of Birmingham, United Kingdom. Each line has been passaged by standard monolayer culture for over 50 passages and fully characterized by high-resolution genotyping to establish their relationship to the tumor of origin, retention of brain tumor stem cells (BTSC), and degree of tumorigenicity in mouse xenografts.  

Cell culture and drug treatment

Cells were cultured in DMEM (DAOY, EPN-2, GB-1, C17.2, and HeLa) or RPMI 1640 (Sigma; PFSK-1), supplemented with 10% fetal bovine serum [or 10% fetal bovine serum/5% horse serum (C17.2); PAA Labs], 5 mmol/L sodium pyruvate, 5 mmol/L l-glutamine, and 5 mmol/L sodium pyruvate, and maintained in a humidified incubator at 37°C and 5% CO2. TSA (Sigma) stock solution (5 mmol/L in DMSO vehicle) was diluted in culture medium to obtain 0.5 to 3.0 μmol/L working concentrations. Etoposide was administered at 50 μmol/L for 48 hours.

Cell proliferation/viability assay

Etoposide was administered at 50 μmol/L to obtain 0.5 to 3.0 cycles of 94°C for 30 s, followed by 59°C for 30 s, repeated 30 times. Products were resolved on a 10% polyacrylamide gel, detected using SyBR Green 1, and visualized by Southern blotting. Gel electrophoresis was conducted using 200 ng cDNA template and amplified for 30 cycles: 94°C, 45 seconds; 60°C, 30 seconds; 72°C, 45 seconds. Primer sequences used were as follows: hTERT-W1, 5′-AGCCGACTACTCAGCTATG-3′; hTERT-W2, 5′-GTTCCTGCTTCTCAGGATGG-3′; GAPDH-F, 5′-CGGACCTAAGGTTGTCGTAT-3′; GAPDH-R, 5′-AGCCTTCTCCATGGTGTTAGAC-3′.

Telomere repeat amplification protocol assay

Telomerase activity was analyzed using the TRAPeze telomerase detection kit (Millipore). Briefly, 100 to 500 ng protein from homogenized tumor tissue or total cell lysate was used. Telomerase extension of a telomere oligonucleotide was conducted by heating samples at 30°C for 30 minutes. Amplification of the telomerase elongation product was subsequently carried out using a thermal cycle of 94°C for 30 s, followed by 59°C for 30 s, repeated 30 times. Products were resolved on a 10% polyacrylamide gel, detected using SyBR Green 1, and visualized with a Fujifilm FLA-2000 phosphorimager (Amersham Biosciences).

Telomere restriction fragment length assay

Mean telomere length was determined using the TeloTAGGG kit (Roche). Briefly, 3 μg genomic DNA was digested with a Hinf1/RsaI frequent cutter mix, separated on 0.8% agarose, and transferred to a nylon membrane by Southern blotting. Gel electrophoresis was conducted at 60 V for 16 hours using 1% agarose. Fragments were hybridized to a digoxigenin-labeled telomere probe, incubated with a digoxigenin-specific antibody, and visualized through chemiluminescent signal. The average telomere restriction fragment length was determined by comparing signals relative to a molecular weight standard, using ImageQuant version 5.1 software (GE Healthcare).

Reverse transcriptase-PCR

Total RNA was extracted using the PLG method for phenol/chloroform extraction (Eppendorf). First-strand cDNA synthesis was conducted using avian myeloblastosis virus reverse transcriptase (Roche), 2 μg RNA, and oligo(dT)15 in a total reaction volume of 20 μL (30°C, 8 minutes; 42°C, 1 hour; 95°C, 5 minutes). Each PCR was conducted using 200 ng cDNA template and amplified for 30 cycles: 94°C, 45 seconds; 60°C, 30 seconds; 72°C, 45 seconds. Primer sequences used were as follows: hTERT-W1, 5′-AGCCGACTACTCAGCTATG-3′; hTERT-W2, 5′-GTTCCTGCTTCTCAGGATGG-3′; GAPDH-F, 5′-CGGACCTAAGGTTGTCGTAT-3′; GAPDH-R, 5′-AGCCTTCTCCATGGTGTTAGAC-3′.

Ependymal cell culture and cilia function

Primary ependymal cells were grown as described in (37). Cerebellum from newborn Wistar rats (1–2 days old) was removed as were 3-mm edge regions of the frontal cortex and left and right cortical hemispheres. The remaining brain regions (containing ependymal cells and ventricles) were mechanically dissociated and grown in
2 mL culture medium [minimum essential medium (Gibco), penicillin (100 IU/mL), and streptomycin (100 μg/mL)]. Ciliated adherent ectodermal colonies were cultured with (1 and 10 μmol/L TSA) or without TSA. To determine cilia beat frequency and distance to cilia tip, cultured cells were placed in a humidified incubation chamber (37°C) and observed using an inverted microscope (Diphot, Nikon). Beating cilia were recorded using a digital high-speed video camera (Troubleshooter, Lake Image Systems) at a rate of 500 frames per second using a shutter speed of 1/2000. Each time point represents the measurement of four individual cilia from each well. Calculation of cilia beat frequency (Hz): 500 (no. of frames per second)/5 (frames elapsed for five cilary beat cycles) × 5 (conversion per beat cycle). The captured video sequences were played back at a slow rate, which allowed determination of the distance traveled by cilia tips within the power stroke of the beat cycle.

Statistics
SPSSv16 was used for statistical analyses. Independent-sample t tests with 95% confidence intervals were used to compare the mean patient age values between tumor groups, analyze percentage cell survival between HDACi treated and untreated cell lines, and explore differences in telomere length between tumor cohorts and specific tumor/blood groups, respectively. Correlation between results from the telomeric repeat amplification protocol and telomere restriction fragment length assays was determined by Pearson’s correlation test. P values less than 0.05 were deemed statistically significant, whereas effect sizes (through η² analysis) were calculated where appropriate.

Results
Telomerase is active in the majority of high-grade pediatric brain tumors
A comprehensive analysis of telomerase activity and telomere length in childhood brain tumors in situ is lacking as many studies rely on small quantities of tumor material, only allowing for quantitative reverse transcriptase-PCR, or rely on cell lines exclusively for telomerase and telomere length analysis (35). TRAP assay conducted using protein extracted from flash-frozen primary brain tumors revealed that all medulloblastomas (20 of 20) and GBMs (7 of 7) exhibited high levels of telomerase activity (Fig. 1A, left, and C left). In contrast, 33% of CNS PNET tumors (6 of 18) exhibited telomerase activity (Fig. 1B, left). WHO grade 1 dysmorphic/ependymal neuroepithelial tumors (2 of 2) and pilocytic astrocytomas (6 of 6) showed no detectable levels of telomerase activation (Fig. 1B, left and data not shown).

Contrasting telomere lengths in tumors of neuroectodermal and glial origin
Mean telomere length across each tumor population was measured using DNA extracted from flash-frozen primary brain tumors. Telomere length in 15 medulloblastomas ranged from 2.4 to 8.9 kb with a mean of 4.4 kb (Fig. 1A, right). Similarly, CNS PNET telomere length from 15 primary tumors ranged from 4.1 to 7.3 kb with a mean of 5.2 kb (Fig. 1B, right). [We note that there is no concordance between telomerase positive/negative CNS PNET tumors and telomere length (Pearson correlation coefficient: r = 0.151, P = 0.605).] For CNS PNET tumors, the mean telomere length was significantly shorter than the mean telomere length from constitutional blood (5.2 ± 0.28 kb versus 6.3 ± 0.09 kb; P = 0.002, large effect size; η² = 0.43), whereas medulloblastomas revealed a trend toward shorter mean telomere length compared with blood (4.4 ± 0.53 kb versus 6.9 ± 0.88 kb; P = 0.065). In contrast, telomere length from eight GBMs ranged from 7.2 to 8.9 kb, with a relatively longer mean telomere length of 8.3 kb (Fig. 1C, right). This is similar to our previous report of a mean primary ependymoma telomere length of 10.9 kb (30). To reinforce this finding, when neuroectodermal tumors (medulloblastoma and CNS PNET; 30 tumors) are grouped together, the mean telomere length across this combined population is 4.8 kb, which is significantly shorter than the 10.1 kb mean telomere length measured across a combined population of glial tumors (ependymoma and GBM; 29 tumors; 4.8 ± 0.31 kb versus 10.1 ± 0.54 kb; P < 0.0005, large effect size; η² = 0.55). Thus, the mean telomere length from tumors of neuroectodermal origin is significantly shorter compared with tumors of glial origin (Table 1). This marked difference cannot be attributed to patient age as no significant difference was observed with respect to age at diagnoses (6.3 ± 0.81 versus 5.5 years ± 0.92; P = 0.508). Telomere length was also measured in cell lines representing each tumor type to determine the accuracy of in vitro models to mimic primary brain tumor telomere length ranges. DA0Y (medulloblastoma) and PFSK-1 (CNS PNET) exhibited mean telomere lengths within the range observed in corresponding primary tumor types (5.9 and 3.8 kb, respectively). EPN-2 (ependymoma) and GB-1 (GBM) exhibited considerably shorter mean telomere length than the range observed in corresponding primary tumor types (3.7 and 4.5 kb, respectively; Fig. 1D). Thus, primary neuroectodermal and glial tumors showed significant differences in average telomere length, the majority of tumors using a telomerase-mediated telomere maintenance mechanism. Furthermore, the cell lines used in this study represent in vitro therapeutic models of brain tumors exhibiting relatively short telomere length and hence their amenability to telomerase inhibition mediated by HDACi.

TSA impairs proliferation, activates a DNA damage response, and induces apoptosis in brain tumor cells
All cell lines responded to TSA treatment, resulting in a significantly marked suppression of proliferation [PFSK-1, DA0Y, EPN-2: P < 0.0005, large effect sizes (η² = 0.95, 0.98, and 0.93, respectively); GB-1: P = 0.002, large effect size (η² = 0.56)]. Upon 48 hours of TSA exposure, the mean inhibition of cellular proliferation for PFSK-1,
Figure 1. Telomerase activity and telomere length in pediatric brain tumors. Telomerase activity in vitro was measured by the TRAP assay using 0.1 µg total protein lysate. A, left, all tumors analyzed from patients with medulloblastoma (20 of 20) showed telomerase activity, with the majority demonstrating high levels of enzyme processivity. B, left, in contrast, only 33% (6 of 18) of CNS PNETs showed telomerase activity, with the majority of tumors (12 of 18) and 2 of 2 dysembryoplastic neuroepithelial tumors (DNET) showing no detectable telomerase activity. C, left, high levels of telomerase activity were present in all GBMs (7 of 7). The mean telomere length across each tumor population was measured using the telomere restriction fragment assay using 2 to 3 µg genomic DNA. A, right, the mean telomere length of 15 medulloblastoma (MB) tumors was 4.4 ± 0.53 kb (range 2.4–8.9 kb), showing a trend toward a shorter mean telomere length from constitutional blood from three medulloblastoma patients (6.9 ± 0.88 kb; P = 0.065). B, right, the mean telomere length of 15 CNS PNETs was 5.2 ± 0.28 kb (range 4.1–7.3 kb), which is significantly shorter than the mean blood telomere length from five patients (6.3 ± 0.09 kb; P = 0.002). C, right, relative to telomere length from tumors of neuroectodermal origin, the mean telomere length from eight GBM tumors was longer (8.3 ± 0.24 kb; range 7.2–8.9 kb) and did not differ significantly from the mean blood telomere length from four patients (6.3 ± 0.29 kb; P = 0.521). D, the mean telomere lengths from the DAOY (6.8 ± 0.62 kb) and PFSK-1 (3.8 ± 0.43 kb) cell lines, derived from pediatric medulloblastoma and CNS PNET, respectively, were within the mean telomere length range observed in the primary tumors. The mean telomere lengths from the GB-1 (4.5 ± 0.58 kb) and EPN-2 (3.7 ± 1.1 kb) cell lines, derived from pediatric GBM and ependymoma, respectively, were considerably shorter than the mean telomere length range observed in the primary tumors (see Table 1 for ependymoma tumor telomere length data).
DAOY, and EPN-2 was 69%, 67%, and 55%, respectively. The concentration of TSA required for 50% growth inhibition (IC₅₀) was 0.40, 0.35, and 0.4 μmol/L for PFSK-1, DAOY, and EPN-2, respectively (Fig. 2A–C). GB-1 cells were the least responsive relatively, and the IC₅₀ value was not reached within the concentration range tested. The maximum growth inhibition for GB-1 was 31% (Fig. 2D). After an initial marked reduction in proliferation at the minimum dose utilized in this study (0.5 μmol/L), the percentage of viable and proliferating cells were not significantly reduced further upon increasing the concentrations of TSA (for 0.5 μmol/L versus 3.0 μmol/L TSA: PFSK-1, GB-1, DAOY, and EPN-2, respectively (Fig. 2A–C). GB-1 cells were the least responsive relatively, and the IC₅₀ value was not reached within the concentration range tested. The maximum growth inhibition for GB-1 was 31% (Fig. 2D). After an initial marked reduction in proliferation at the minimum dose utilized in this study (0.5 μmol/L), the percentage of viable and proliferating cells were not significantly reduced further upon increasing the concentrations of TSA (for 0.5 μmol/L versus 3.0 μmol/L TSA: PFSK-1, GB-1, DAOY, and EPN-2, respectively (Fig. 2A–C). GB-1 cells were the least responsive relatively, and the IC₅₀ value was not reached within the concentration range tested. The maximum growth inhibition for GB-1 was 31%

Flow cytometry was conducted to determine alterations in cell cycle distribution and to address whether apoptotic induction was evident on TSA (1.0 and 3.0 μmol/L) exposure. Following 16 hours of treatment, DAOY, EPN-2, and GB-1 showed a distinct increase of cells in the G₂-M phase with a concurrent decrease in S-phase cells. This G₂-M peak was evident in both concentrations of TSA tested. DAOY cells showed a similar subpopulation of cells, whereas a sub-G₀-G₁ population was not observed in EPN-2 and GB-1 cells. However, PFSK-1, DAOY, and EPN-2 cell cycle profiles indicate the presence of early-stage apoptotic cells independent of the sub-G₀-G₁ population (Fig. 3A). The prominent number of events to the right of the G₁ peak in PFSK-1 and DAOY TSA-treated cells was consistent with the high proportion of free-floating dead cells observed under a phase-contrast microscope (Supplementary Fig. S2). To more specifically characterize the nature of growth inhibition evident from the MTT cell proliferation assay, cell cycle analysis was conducted after 48 hours of TSA exposure (1.0 and 3.0 μmol/L). All cell lines showed a substantial dose-dependent increase in the sub-G₀-G₁ population (~50–80% of cells), whereas the sub-G₀-G₁ fraction was ~10% in untreated cells for all lines, indicative of a high level of apoptosis (Fig. 3B). Collectively, these results suggest that apoptosis is the key effector mechanism of TSA-mediated growth inhibition and genome instability in high-grade pediatric brain tumor cells.

Table 1. Telomere length is significantly shorter in neuroectodermal tumors compared with glial tumors

<table>
<thead>
<tr>
<th>No. tumors</th>
<th>Mean tumor telomere length (kb)</th>
<th>Median tumor telomere length (kb)</th>
<th>No. constitutional DNA</th>
<th>Mean constitutional DNA telomere length (kb)</th>
<th>Median constitutional DNA telomere length (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS primitive neuroectodermal tumor</td>
<td>15</td>
<td>5.2</td>
<td>4.8</td>
<td>5</td>
<td>6.3</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>15</td>
<td>4.4</td>
<td>3.8</td>
<td>3</td>
<td>6.9</td>
</tr>
<tr>
<td>Glioblastoma multiforme</td>
<td>8</td>
<td>8.3</td>
<td>8.6</td>
<td>4</td>
<td>8.5</td>
</tr>
<tr>
<td>Ependymoma* †</td>
<td>19</td>
<td>10.9</td>
<td>10.0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

NOTE: The mean telomere length in CNS PNETs was significantly shorter than the mean telomere length from constitutional blood (P = 0.02). The mean telomere length in medulloblastomas showed a trend toward significant shortening compared with constitutional blood (P = 0.07). The mean telomere length in GBM did not differ significantly from constitutional blood mean telomere length. When grouped together, the mean telomere length in tumors of neuroectodermal origin (medulloblastomas and CNS PNETs) were significantly shorter than those in tumors of glial origin (glioblastoma and ependymoma; P < 0.0005). No significant difference was observed with respect to age at diagnoses between tumors of neuroectodermal and glial origin (P = 0.508).

*Ependymoma: 6 = primary, 13 = recurrent.
†Ref. (30).
Telomerase activity is inhibited following TSA exposure in brain tumor cells

Following 48 hours of exposure to TSA, PFSK-1, DAOY, and EPN-2 cells showed a marked reduction in telomerase activity as measured by the TRAP assay. The minimal dose of TSA required for substantial telomerase inhibition was 0.5 μmol/L (DAOY and EPN-2) or 1.0 μmol/L (PFSK-1), whereas complete abrogation of telomerase activity was evident at high (3.0 μmol/L) TSA doses in PFSK-1 and EPN-2 cells. In contrast, TSA-treated GB-1 cells generally showed comparable telomerase processivity levels to untreated cells, with only a slight reduction of telomerase activity at the 3.0 μmol/L TSA dose (Fig. 4A). On a transcriptional level, TSA induced a marked reduction in hTERT mRNA transcripts in PFSK-1, GB-1 (from 0.5 μmol/L TSA), and DAOY (at 2.0–3.0 μmol/L TSA) cells. hTERT downregulation was only evident at the 3.0 μmol/L TSA dose in EPN-2 cells. Inhibition of hTERT transcription in PFSK-1 cells and at high TSA doses (3.0 μmol/L) in EPN-2 and DAOY cells was consistent with inhibition of telomerase activity in these respective lines. However, hTERT downregulation occurred at 2.0 to 3.0 μmol/L TSA in DAOY cells, not reflected by maximal telomerase inhibition at 0.5 μmol/L TSA. Similarly, hTERT downregulation in GB-1 does not reflect the degree of telomerase inhibition (Fig. 4B). To probe the specificity of HDACi-associated telomerase inhibition, cell lines were treated with a high
Figure 3. TSA-induced alterations in cell cycle dynamics in pediatric brain tumor cells. A, representative profiles of cell cycle distribution analyzed by flow cytometry in pediatric brain tumor cells treated for 16 h with TSA. EPN-2, GB-1, and DAOY cells showed a distinct reduction in S-phase cells with a concurrent increase in cells at the G2-M phase after drug treatment. No discernible cell cycle arrest was evident in PFSK-1 cells after 16 h TSA treatment. However, induction of apoptosis was evident in PFSK-1 (and DAOY) cells at both TSA concentrations, indicated by the emergence of a sub-G0-G1 population in these lines. Percentages represent the mean ± SE from three independent experiments. B, histogram showing the proportion of sub-G0-G1 cells, relative to the total cells in the population after 48 h treatment with TSA. Prolonged TSA exposure resulted in significantly high levels of apoptosis in all brain tumor cell lines. The proportion of untreated cells in sub-G0-G1 (~10%) was comparable for all cell lines. Results are expressed as the mean ± SE from three independent experiments.
Inhibition of telomerase activity and reduction in hTERT transcription in brain tumor cells after 48 h TSA treatment. A, left, TRAP assay using 0.1 μg total protein lysate was used to measure telomerase activity in vitro. The minimal dose required for a marked reduction in telomerase activity was 1.0 μmol/L TSA in PFSK-1 cells and 0.5 μmol/L TSA in DAOY cells. Telomerase inhibition persisted at higher TSA doses in PFSK-1 but enzyme activity was comparable with untreated cells at 3.0 μmol/L TSA in DAOY cells. B, left, semiquantitative reverse transcriptase-PCR was used to measure hTERT mRNA expression. The levels of hTERT transcript in PFSK-1 were markedly reduced in TSA-treated cells compared with vehicle-only untreated cells. A clear reduction in hTERT transcription was evident in DAOY cells at 2.0 to 3.0 μmol/L TSA. A right, telomerase inhibition in EPN-2 generally occurred in a dose-dependent manner, with almost no detectable telomerase activity at 3.0 μmol/L TSA. There was no obvious reduction in telomerase activity in GB-1 cells treated with TSA, with slight inhibition only occurring at the highest TSA dose. B, right, similarly, a marked reduction in hTERT transcript in EPN-2 cells was only observed at the 3.0 μmol/L dose of TSA. hTERT expression was reduced in all TSA-treated GB-1 cells, with lowest expression levels observed at the highest TSA dose. C, hTERT enzyme-linked immunosorbent assay was additionally used to detect hTERT protein levels. PFSK-1 and GB-1 showed a marked reduction in hTERT protein levels upon 0.5 μmol/L TSA exposure; DAOY and EPN-2 showed a moderate reduction in hTERT protein levels at 1.0 μmol/L TSA exposure. D, brain tumor lines treated with 50 μmol/L etoposide for 48 h showed high levels of telomerase activity, comparable with untreated TSA-controls described earlier (Fig. 2F). This is irrespective of apoptosis induction in these lines as inferred by caspase-3 activation.

Figure 4.

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Figure 5. *In vitro* cytotoxicity culture models and hypothetical model of HDACi-induced hTERT repression. A, no cytotoxicity was observed in normal rat ependymal cells exposed to 1 and 10 μmol/L TSA. Cytotoxicity was inferred as perturbation of ependymal cilia function with respect to cilia beat frequency (CBF, left) and cilia tip distance (right). NS, not significant. B, murine cerebellar progenitor cells (C17.2) transformed with v-Myc showed a high degree of sensitivity to TSA at all concentrations tested. C, top, hTERT transcriptional repressors such as Smad3 and Mad may be aberrantly silenced in neoplastic cells due to aberrant deacetylation of lysine tails in adjacent chromatin. This results in a failure to repress hTERT transcription initiation from c-Myc (i.e., derepression of the hTERT promoter). C, bottom, removal of histone deacetylases through the use of HDACi results in hyperacetylation of lysine tails and alleviation of Smad3/Mad silencing. Smad3 and Mad may then conduct the canonical roles of hTERT transcription repression through displacement of c-Myc in the case of Mad. This latter scenario may recapitulate the status of hTERT regulation in normal human somatic cells. Additionally, HDACi may result in upregulation of factors that mediate protein-protein inhibition of hTERT.
dose (50 μmol/L) of the cytotoxic chemotherapeutic, etoposide. Although caspase-3 activation was evident in all lines, telomerase activity levels remained comparable with untreated cells (Fig. 4C).

No cytotoxicity is observed with respect to normal brain tissue function

To address the level of cellular toxicity in normal brain tissue, normal rat ependymal cells were cultured and exposed to TSA. This model permits a series of functional analyses with respect to adverse effects on ependymal cilia beat frequency and cilia tip distance. Using both low TSA doses (1 μmol/L) and doses exceeding that used in previous experiments (10 μmol/L), no significant difference was observed with respect to cilia function between TSA-treated and untreated ependymal tissue (Fig. 5A). Thus, using one model of normal brain cellular function, no cytotoxicity was observed during treatment with the HDACI TSA.

In contrast, C17.2 cerebellar progenitors immortalized with v-Myc showed a high degree of sensitivity to TSA, comparable with PFSK-1, DAOY, and EPN-2 tumor cells, with maximal growth inhibition (~75%) achieved at 3.0 μmol/L TSA (Fig. 5B).

Discussion

The present study shows the potential utility of the HDACI TSA for the treatment of pediatric brain tumors. TSA caused a dramatic reduction in cell proliferation, activated a DNA damage response, altered cell cycle distribution, and induced caspase-3-dependent apoptosis in high-grade childhood CNS PNET, medulloblastoma, and ependymoma cell lines upon short-term (48 hours) drug exposure. The percentage of viable PFSK-1, DAOY, and EPN-2 cells from the MTT assay (Fig. 2A–D) and cell cycle analysis (Fig. 3B) is directly comparable (20%, 40%, and 50%, respectively). The growth-inhibitory effects in these lines were associated with marked telomerase inhibition and hTERT downregulation. These anticancer effects were achieved using nanomolar concentrations and 48 hours exposure time, suggesting that TSA may be a relatively potent chemotherapeutic. In contrast, childhood GBM cells showed relatively moderate antiproliferative and telomerase inhibitory responses upon TSA exposure. It is unclear why GB-1 GBM cells are intrinsically more resistant to TSA than the other tumor lines investigated. One suggestion is that TSA sensitivity is directly correlated to the expression of ABC transporters. Indeed, the efflux of HDACI by MDR1 and MRP1 transporters has been reported (38, 39). Importantly, TSA did not disrupt normal cellular function in a rodent ependymal primary cell culture model. As the cell lines used here are representative of brain tumors with relatively short mean telomere length and high telomerase activity (3.7–5.9 kb), the data indicate that HDACI may be an attractive approach for the treatment of like subsets of childhood brain tumors. However, the mean telomere length in primary tumors was significantly longer in glial tumors (8.3–10.9 kb) compared with neuroectodermal tumors (4.4–5.2 kb). This may reflect early neurogenesis in distinct brain regions with respect to cellular replicative histories, albeit the data do not exclude different tumor types exhibiting different telomere lengths regardless of anatomic location. It will therefore be important to test the efficacy of HDACI against brain tumor cell lines with relatively longer mean telomere lengths to establish whether HDACI-induced effects are telomere length independent. Additionally, it will be necessary to test HDACI efficacy against brain tumors that use the mechanism of alternative lengthening of telomeres for telomere maintenance. As a high proportion of CNS PNET tumors were telomerase negative in the present study, it is likely that alternative lengthening of telomeres is prevalent in these tumor subsets. Furthermore, no clinical parameter correlated to telomerase-negative CNS PNET tumors.

Typical HDACI studies have shown p21- or p53-mediated cell cycle arrest, proceeding to apoptosis. However, regardless of apoptotic induction in all cell lines, only DAOY cells showed accompanying marked upregulation of p21, whereas growth inhibition was p21 and p53 independent in PFSK-1, EPN-2, and GB-1 cells. Although unexpected, anticancer agents have previously induced apoptosis in a p21/p53-independent manner in cancer cells, including those of glioma (40, 41). Whereas an increase of cells in G2-M was evident in DAOY, EPN-2, and GB-1 lines after 16 hours TSA, no alterations in cell cycle distribution was evident in PFSK-1 cells. As a sub-G0-G1 population is prominent after only 16 hours TSA in PFSK-1 cells, induction of apoptosis must be abrupt with rapid cell cycle arrest at an earlier stage in this line. This notion is consistent with PFSK-1 showing the greatest degree of sensitivity to TSA in this study; inhibition of proliferation occurs in the highest proportion of PFSK-1 cells (80%) relative to the other lines, caspase-3 activation levels are highest, and the proportion of sub-G0-G1 cells are greatest. Additionally, mean telomere length in PFSK-1 (3.8 kb) is relatively short compared with the other lines, except EPN-2, suggesting that apoptotic pathways may be activated rapidly from critically short telomeres upon sufficient inhibition of telomerase activity. However, such pathways are not activated via ATM/ATR signaling from uncapped telomeres in this context.

Given the multitude of cellular effects triggered by HDACI, it is probable that several mechanisms contribute to HDACI-induced anticancer effects. The mode of action of a particular HDACI may also be dependent on the context of the cell type or tumor in question, and it is possible that more than one mechanism of action occurs during treatment of the same tumor (or cells). For example, PFSK-1 and DAOY cells show a marked reduction in cell viability at 0.5 μmol/L TSA, whereas caspase-3 activation occurs only at >2.0 μmol/L TSA. This paradox may at least be partially explained by the activation of
caspase-independent apoptosis, as reported by similar studies using HDACi including TSA (42–44). It is plausible that in PFSK-1 and DAOY cells, cell viability is reduced due to caspase-3-independent phenomena at low TSA doses, whereas at high TSA doses, this reduction in cell viability is reinforced by canonical caspase-3–dependent apoptosis. In addition, most studies have investigated the effect of HDACi on the regulation of gene transcription. However, recent reports document genomic instability as a consequential event to HDAC inhibition and may contribute to the cytotoxicity of these agents (45, 46). Our results are consistent with this phenomenon as TSA exposure leads to increased expression of the serine 139–phosphorylated histone H2A.X relative to vehicle-only controls, thereby eliciting a DNA damage response. Intriguingly, as no expression of ATM (typically phosphorylates H2A.X) was evident in any cell line, these results suggest a noncanonical means of H2A.X activation in this context. We cannot exclude the possibility that ATM-mediated H2A.X phosphorylation is a rapid upstream event not detected at the stage in which protein expression was assessed in this study. Although low levels of phosphorylated H2A.X are evident in all untreated cells, this is presumably above an activation threshold required to inhibit cellular proliferation.

Numerous groups have shown that hTERT gene expression and consequential abrogation of telomerase activity is an early upstream event following HDACi (17, 47, 48). Data presented here are consistent with such studies as PFSK-1, DAOY, and EPN-2 cells show substantial telomerase inhibition upon TSA treatment. Indeed, telomerase activity is completely abolished at the 3.0 μmol/L TSA dose for both the PFSK-1 and EPN-2 lines. This is consistent with the degree of TSA sensitivity apparent in PFSK-1 previously described. In contrast, GB-1 cells show low levels of telomerase inhibition only at the highest TSA dose. This suggests that HDACi-induced phenomena other than telomerase inhibition may account for the relatively moderate inhibition of cellular proliferation in this line. As caspase-3 activation occurs independently of telomerase inhibition in etoposide-treated cells, telomerase inhibition in brain tumor cells is a result of histone deacetylation inhibition rather than a consequence of apoptosis.

Telomerase activity is closely correlated with the repression and derepression of hTERT (49). These findings suggest that derepression of the hTERT promoter might be an important mechanism leading to the activation of hTERT and thereby of telomerase activity in cells during tumorigenesis. Several cancer studies have indicated that the c-Myc oncogene, deregulated in several tumors, can contribute to the transcriptional activation of hTERT (50–52). Sequence analysis has revealed that the hTERT promoter contains binding sites for a number of transcription factors, including the Myc/Max/Mad binding site (E-box; ref. 31), resulting in transcriptional activation (Myc/Max) or transcriptional repression (Mad/

Max; ref. 53). The identified cis-elements at the hTERT promoter also include several Sp1 binding sites at the proximal region, which have been shown to be important for hTERT transcriptional activation (50, 54, 55). TSA induces hyperacetylation of histones at the hTERT proximal promoter and directly transactivates the hTERT gene in normal telomerase-negative/low cells. Furthermore, recent manipulation of intracellular Smad3 gene expression has revealed that Smad3 interacts with a specific site on the hTERT promoter in response to transforming growth factor-β stimulation in vitro and in intact cells, leading to a significant inhibition of hTERT transcription. Smad3 may therefore constitute a negative regulatory system to balance c-Myc transcriptional activation of hTERT. Thus, HDAC-mediated repression could be the major transcriptional repression mechanism of hTERT in normal human somatic cells. Derepression of hTERT transcription is therefore likely to be involved in cellular immortalization as a result of telomerase activation in cancer cells. It is unclear from any study to date as to why HDACi induces hTERT transcription in normal somatic cells but suppresses hTERT transcription in cancer cells. It is plausible that the alleviation of hTERT and/or c-Myc repressor protein silencing overrides hyperacetylation at the hTERT promoter itself. In the current investigation, PFSK-1, DAOY, and EPN-2 cells show considerable transcriptional downregulation of hTERT on TSA exposure in a manner generally consistent with telomerase inhibition in these lines. The relative resistance to telomerase inhibition in GB-1 cells is not readily explained by hTERT expression levels as a marked reduction in transcript levels is evident at all concentrations of TSA. A compensatory mechanism with respect to the relative stability (half-life) of hTERT in GB-1 and/or increased hTERT mRNA translational efficiency may account for this discrepancy.

At present, the majority of HDACi in vitro and in vivo studies are proof-of-concept and largely descriptive. The temporal order of events upon HDACi exposure, from the alleviation of deacetylase-mediated gene repression (through hyperacetylation) to cellular growth arrest and/or apoptosis, is unclear. The precise nonhistone molecular targets for each tumor type shown to be amenable for HDACi anticancer strategies remain to be identified in most cases. True genomic substrates that are repressed by particular HDACs and from which transcription can reinitiate upon HDACi-mediated hyperacetylation must be distinguished from consequential gene expression changes and molecular alterations. Such knowledge will help refine development of novel, more potent HDACi that are selective for tumor cells. The difference in TSA sensitivity between GB-1 (relatively resistant) and all other brain tumor lines suggests that an HDACi that is efficacious in one tumor type may not be so against even a closely related type. Encouraging data from others have shown that HDACi display selective toxicity against tumor cells compared with nonmalignant cells (33, 34). This is supported by
the present study as no adverse effect on ependymal cilia function was evident. The sensitivity of tumor cells and relative resistance of normal cells to HDACi may reflect the multiple defects that render cancer cells less likely than normal cells to compensate for inhibition of one or more prosurvival factors or activation of pro-death pathways. In contrast, the sensitivity of the C17.2 neural progenitor line to TSA suggests that v-Myc-mediated immortalization is sufficient in this context to render the acetylation signature sufficiently “aberrant” to result in sensitivity to histone deacetylase inhibition. It is important to note that no tumors form in animals engrafed with C17.2, even following 2 years posttransplantation (36). It is likely that v-Myc (viral c-Myc homologue) contributes to derepression of hTERT in C17.2 cells, which is reversed considerably upon TSA exposure due to hyperacetylation at the promoters of hTERT repressor genes and/or inhibitors of Myc.

We propose a hypothetical model whereby HDACi in pediatric brain tumor cells proximally results in hyperacetylation of lysine tails on Mad or Smad3 (or alternatively an unidentified repressor specific to c-Myc), resulting in alleviation of Mad or Smad3 repression. The subsequent reactivation of hTERT repressor proteins would ultimately result in a shift of balance regarding acetylation status of hTERT modulators, leading to inhibition of hTERT gene expression, followed by cessation of proliferation and apoptotic induction as a result (Fig. 5C).

Further studies are required to refine our knowledge of HDACi targets such as hTERT. It is not known whether pan-HDACi that target several HDACs, or novel HDACi that are specific to one HDAC or one HDAC class, will emerge as the most potent HDACi for the next-generation brain cancer therapy trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

stabilization in papillary serous endometrial cancer cells. Mol Cancer Ther 2006;5:2767–76.
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