Histone deacetylase inhibition attenuates cell growth with associated telomerase inhibition in high grade childhood brain tumor cells.

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Abbreviations list: Central nervous system primitive neuroectodermal tumors (CNS PNET), Glioblastoma multiforme (GBM), Histone deacetylases (HDACs), Histone deacetylase inhibitors (HDACi), Trichostatin A (TSA)Human telomerase reverse transcriptase (hTERT), Telomere repeat amplification protocol (TRAP), Telomere restriction fragment length (TRF), Ataxia telangiectasia mutated (ATM), Ataxia telangiectasia mutated-related (ATR).
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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 anti-cancer effects of the HDACi Trichostatin A (TSA) in high grade childhood brain tumor cells. Acute exposure to TSA 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 hTERT downregulation in the majority of lines. In contrast, no cytotoxicity was observed in primary ependymal cells with respect to cilia function. Thus, inhibition of histone deacetylases leads to anti-proliferative and pro-apoptotic effects in childhood brain tumor cells, likely to involve altered chromatin regulation at the hTERT 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 origin (high risk medulloblastoma and central nervous system neuroectodermal tumors (CNS PNET)) 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 neurological disability. There is therefore the need to develop more effective and specific chemotherapeutic regimes that can successfully eradicate residual tumor cells, commonly spared by current therapies.

Histone deacetylases (HDACs) are known to regulate gene transcription and oncogenesis through remodelling of chromatin structure, canonically resulting in the repression of target genes (4, 5). Inhibition of HDACs increases acetylation of histone and non-histone proteins at the ε-amino 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 re-expression of proteins that repress tumor-propagating genes. As HDAC substrates include a vast array of non-histone 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 homoeostasis. As such, a number of histone deacetylase inhibitors (HDACi)
are under pre-clinical and clinical investigation as viable anticancer agents (9-16). Small molecule HDAC inhibitors have achieved significant biological effects in preclinical cancer models including glioblastoma multiforme (17), medulloblastoma (18), pancreatic cancer, breast cancer, melanoma and leukaemia (19), with Vorinostat (SAHA) receiving approval by the US-FDA 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 has lead to several brain tumor clinical trials using diverse HDACi, with a vorinostat phase II trial in adult recurrent glioblastoma multiforme showing anti-cancer 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 have neither investigated the mode of HDACi action nor the temporal order of cellular events post-HDACi exposure.

The mechanism(s) by which HDACi exerts 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 (hTR) and a catalytic component (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 pre-clinical and clinical advances in telomerase-therapeutics for cancer, there is a need to investigate whether such therapy is of importance to childhood brain tumors. De-repression 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 anti-telomerase effects are consequential or causal to the observed growth arrest remains unclear, as is the mechanism of TSA-mediated telomerase inhibition. HDACi exert anti-proliferative effects rapidly, in a telomere length independent manner. This contrasts with classical anti-telomerase approaches which require a lag period of telomere shortening upon telomerase inhibition (32). Concerns that HDACi may interfere with critical cellular functions seem unfounded as evidence so far indicates that HDACi display selective toxicity against tumor cells compared to untransformed cells (33, 34).

Although there are over 100 trials ongoing or recently concluded with HDACi as monotherapy or combination therapy, to date comprehensive pre-clinical testing of HDACi in diverse CNS brain tumors, particularly those of childhood, is lacking. Furthermore the mechanism by which HDACi may be exerting an anti-proliferative 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 (CCLG) and the Cooperative Human Tissue Network (CHTN). A total of 18 snap-frozen CNS PNETs, all located in the cerebral hemispheres, 20 medulloblastomas and 8 GBM 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 CCLG and CHTN. 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 ATCC and isolated from cerebral hemisphere and cerebellum respectively. The EPN-2 (ependymoma)
and cell line was derived at the Children’s Brain Tumor Research Centre, University of Nottingham, UK and the GB-1 (GBM) line derived at the University of Birmingham, UK. Each line has been passaged by standard monolayer culture for over 50 passages and fully characterised by high resolution genotyping to establish their relationship to the tumor of origin, their retention of brain tumor stem cells (BTSCs) and degree of tumorigenicity in mouse xenografts (Hussein D manuscript accepted). C17.2 neural progenitor cells isolated from neonatal mouse cerebellar cortex and immortalized with v-Myc have been previously described (36).

**Cell culture and drug treatment:**

Cells were cultured in DMEM (DAOY, EPN-2, GB-1, C17.2 and HeLa) or RPMI-1640 (Sigma, UK; PFSK-1), supplemented with 10% fetal bovine serum (or 10% FBS/5% horse serum (C17.2)) (PAA Labs, UK), 5mM sodium pyruvate, 5mM L-Glutamine and 5mM sodium pyruvate and maintained in a humidified incubator at 37°C and 5% CO2. Trichostatin A (TSA) (Sigma, UK) stock solution (5mM in DMSO vehicle) was diluted in culture media to obtain 0.5µM-3.0µM working concentrations. Etoposide was administered at 50µM for 48h.

**Cell proliferation/viability assay:**

1 x 10⁴ cells were plated in 96-well flat-bottomed plates 24h prior to TSA treatment. After 48h drug exposure, 10µl MTT (Sigma, UK) solution in PBS (100 µl) was added to each well for 3 hours at 37°C. PBS/MTT was replaced with 100µl MTT Solvent (0.1 N HCL in anhydrous isopropanol) prior to 1h incubation at room temperature with gentle shaking to dissolve formazan crystals. Sample absorbance was determined at 540nm and background at 670nm. Triplicate wells were assayed during three independent experiments to calculate standard error of the mean.
Cell cycle analysis:
TSA-treated cells were fixed with cold ethanol, washed with cold PBS (w/0.1BSA, 0.1% tween) and stained with 20µg propidium iodide for 20mins in the dark and at room temperature. Cell cycle analysis was conducted using a Coulter FC500 flow cytometer and analyzed using WinMDI2.8 and Cylchred software.

Western blot:
Total protein (30µg) was separated with 10% SDS-polyacrylamide, transferred to nitrocellulose membrane (overnight at 8°C), blocked with TBST-milk (10 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.5% Tween 20, 5% non-fat dry milk) and incubated with primary antibody (overnight at 4°C) at the indicated dilutions: [anti-caspase-3, anti-bcl2, anti-phospho-p53, anti-ATR, (1:1000; Cell Signalling, UK), anti-p21, anti-ATM (1:1000 and 1:500 respectively; Upstate, UK); anti-actin (1:7000 ; Calbiochem. UK)]. Membranes were washed twice in TBST and incubated with anti-mouse or anti-rabbit secondary antibody coupled to horseradish peroxidise at a dilution of 1:2000 (Cell Signalling, UK) (1 hour at room temperature). Membranes were washed four times in TBST and immunoreactivity detected using chemiluminescence (ECL+ reagent, Roche, UK).

Immunofluorescence:
Cells were fixed in 0.4% paraformaldehyde and blocked in 5% normal goat serum/0.1% Triton-X for 1 hour at room temperature. For immunolabelling, cells were incubated with anti-phospho-histone H2AX (ser139) (Cell Signalling) using a 1:25 dilution overnight at 4°C in a humidified chamber. Cells were washed and incubated with the anti-rabbit Alexa-fluro 555 (Dako) using a 1:300 dilution in the dark at room temperature. Nuclei were visualized using
DAPI and fluorescence signals were recorded using a Leica DMRB upright fluorescent microscope.

**Telomere repeat amplification protocol (TRAP) assay:**

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

**Telomere restriction fragment (TRF) length assay:**

Mean telomere length was determined using the TeloTAGGG kit (Roche, Burgess Hill, UK). Briefly, 3 μg genomic DNA was digested with a *Hinf1/Rsa1* 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 h using 1% agarose. Fragments were hybridized to a digoxigenin (DIG)-labelled telomere probe, incubated with a DIG-specific antibody, and visualized via chemiluminescent signal. The average TRF length was determined by comparing signals relative to a molecular weight standard, using ImageQuant version 5.1 software (GE Healthcare, Buckinghamshire, UK).

**Reverse-transcriptase (RT)- PCR:**

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

Ependymal cell culture and cilia function:

Primary ependymal cells were grown as described in (Hirst RA 2000). Cerebellum from newborn Wistar rats (1-2d old) were removed as were 3mm 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 2ml culture media (minimum essential media (Gibco, UK), penicillin (100IU/ml) and streptomycin (100µg/ml)). Ciliated adherent ependymal colonies were cultured with or without TSA (1µM and 10Mµ TSA). To determine cilia beat frequency (CBF) and distance to cilia tip, cultured cells were placed in a humidified incubation chamber (37°C) and observed using an inverted microscope (Diphot, Nikon, UK). Beating cilia were recorded using a digital high-speed video camera (Troubleshooter, Lake Image Systems, UK) at a rate of 400 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 CBF (Hz): 500 (no. frames per second) / 5 (frames elapsed for 5 ciliary beat cycles) x 5 (conversion per beat cycle). The captured video sequences were played back at a slow rate which allowed determination of the distance travelled by cilia tips within the power stroke of the beat cycle.
Statistics:
SPSSv16 was used for performing the statistical analyses. Independent-sample t-tests with 95% confidence intervals were used to compare the mean patient age values between tumor groups, analyse 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 TRAP and TRF length assays was determined by Pearson’s correlation test. P values less than 0.05 were deemed statistically significant, while effect sizes (via eta squared 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 RT-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, reveals that all medulloblastomas (20/20) and GBMs (7/7) exhibit high levels of telomerase activity (Figure 1a left and 1c left). In contrast, 33% of CNS PNET tumors (6/18) exhibited telomerase activity (Figure 1b left). WHO Grade 1 dysembryoplastic neuroepithelial tumors (DNET) (2/2) and pilocytic astrocytomas (6/6) showed no detectable levels of telomerase activation (Figure 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 2.4-8.9kb with a mean of 4.4kb (Figure 1A right). Similarly, CNS PNET telomere length from 15 primary tumors ranged from 4.1-7.3kb with a mean 5.2kb (Figure 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, mean telomere length was significantly shorter than mean telomere length from constitutional blood (5.2kb±0.28 vs 6.3kb±0.09; \( p=0.002 \), large effect size (eta squared = 0.43)), while medulloblastomas revealed a trend towards shorter mean telomere length compared to blood (4.4kb±0.53 vs 6.9kb±0.88; \( p=0.065 \)). In contrast, telomere length from 8 GBMs ranged from 7.2-8.9kb with a relatively longer mean telomere length of 8.3kb (Figure 1C right). This is similar to our previous report of mean primary ependymoma telomere length of 10.9kb (30). To reinforce this finding, when neuroectodermal tumors (medulloblastoma and CNS PNET; 30 tumors) are grouped together, mean telomere length across this combined population is 4.8kb, significantly shorter than the 10.1kb mean telomere length measured across a combined population of glial tumors (ependymoma and GBM; 29 tumors) (4.8kb±0.31 vs 10.1kb±0.54; \( p<0.0005 \), large effect size (eta squared = 0.55)). Thus, mean telomere length from tumors of neuroectodermal origin is significantly shorter compared to tumors of glial origin (Table1). This marked difference cannot be attributable to patient age as no significant difference was observed with respect to age at diagnoses (6.3yrs±0.81 vs 5.5yrs±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. DAOY (medulloblastoma) and PFSK-1 (CNS PNET) exhibit mean telomere lengths within the range observed in corresponding primary tumor types (5.9kb and 3.8kb respectively). EPN-2 (ependymoma) and GB-1 (GBM) exhibit considerably shorter mean telomere length than the range observed in corresponding primary tumor types (3.7kb and 4.5kb respectively) (Figure 1D). Thus primary neuroectodermal and glial tumors show significant differences in average telomere length, the majority of tumors utilising a telomerase-mediated telomere maintenance mechanism. Furthermore, the cell lines deployed 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.

**Trichostatin A 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, DAOY, EPN-2: \( p<0.0005 \), large effect sizes (eta squared = 0.95, 0.98, 0.93 respectively), GB-1: \( p=0.002 \), large effect size (eta squared = 0.56)). Upon 48h TSA exposure, mean inhibition of cellular proliferation for PFSK-1, DAOY and EPN-2 was 69%, 67% and 55% respectively. The concentration of TSA required for 50% growth inhibition (IC\(_{50}\)) was 0.40\(\mu\)M, 0.35\(\mu\)M and 0.4\(\mu\)M for PFSK-1, DAOY and EPN-2 respectively (Figure 2A-C). GB-1 cells were the least responsive relatively and an IC\(_{50}\) value was not reached within the concentration range tested. Maximum growth inhibition for GB-1 was 31% (Figure 2D). After an initial marked reduction in proliferation at the minimum dose utilised in this study
(0.5µM), the percentage of viable and proliferating cells were not significantly reduced further upon increasing concentrations of TSA (for 0.5 µM vs 3.0 TSA: PFSK-1: p=0.106; DAOY: p=0.916; EPN-2: p=0.159; GB-1: p=0.371). All lines show increased activation of the pro-apoptotic cleaved caspase-3 protein, which is dose-dependent in PFSK-1 and DAOY cells. Consistent with this, Bcl-2 anti-apoptotic protein levels are downregulated in a dose-dependent manner in PFSK-1 and DOAY. Caspase-3-dependent apoptosis is p53- and p21-independent in PFSK-1, EPN-2 and GB-1 cells. In contrast, p21 is upregulated in DAOY cells following high doses of TSA. No role for ATM and/or ATR signalling was evident from these studies (Figure 2E-F). Densitometric analysis was used to quantitate protein expression and reveals a 2-2.5 fold increase in cleaved caspase-3 for all four lines after 3.0µM TSA exposure and a 1.5 fold increase of p21 in DAOY cells after 2.0µM TSA exposure (Supplementary Figure 1). Immunolabelling revealed a marked upregulation of the genome instability marker, phosphorylated-histone H2A.X in PFSK-1, DAOY, EPN-2 and BT-4 cells following 1.0µM TSA for 24h, relative to vehicle-only controls (Figure 2G-H). Flow cytometry was conducted to determine alterations in cell cycle distribution and to address whether apoptotic induction was evident upon TSA exposure (1.0µM and 3.0µM). Following 16h treatment, DAOY, EPN-2 and GB-1 show a distinct increase of cells in the G2/M phase with a concurrent decrease in S-phase cells. This G2/M peak is most prominent at the 1.0µM TSA dose for EPN-2 and GB-1, but 3.0µM for DAOY cells. No alteration in cell cycle profile was evident in proliferating PFSK-1 cells after 16h TSA treatment; however a population of cells in the sub G0/1 phase was evident at both concentrations of TSA tested. DAOY cells showed a similar subpopulation of cells, whereas a sub G0/1 population was not observed in EPN-2 and GB-1 cells. However, PFSK-1, DAOY and EPN-2 cell cycle profiles do indicate the
presence of early-stage apoptotic cells outwith the sub G0/1 population (Figure 3A). The prominent number of events to the left of the G1 peak in PFSK-1 and DAOY TSA-treated cells is consistent with the high proportion of free-floating dead cells observed under a phase-contrast microscope (Supplementary Figure S2). To more specifically characterize the nature of growth inhibition evident from the MTT cell proliferation assay, cell cycle analysis was conducted after 48h TSA exposure (1.0µM and 3.0µM). All cell lines showed a substantial dose-dependent increase in the sub G0/1 population (~50-80% of cells), whereas the sub G0/1 fraction was ~10% in untreated cells for all lines, indicative of a high level of apoptosis (Figure 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.

**Telomerase activity is inhibited following TSA exposure in brain tumor cells**

Following 48h exposure to TSA, PFSK-1, DAOY and EPN-2 cells show a marked reduction in telomerase activity as measured by the TRAP assay. The minimal dose of TSA required for substantial telomerase inhibition is 0.5µM (DAOY and EPN-2) or 1.0µM (PFSK-1), while complete abrogation of telomerase activity is evident at high (3.0µM) TSA doses in PFSK-1 and EPN-2 cells. In contrast, TSA-treated GB-1 cells generally show comparable telomerase processivity levels to untreated cells, with only slight reduction of telomerase activity at the 3.0µM TSA dose (Figure 4A). On a transcriptional level, TSA induces a marked reduction in hTERT mRNA transcripts in PFSK-1, GB-1 (From 0.5µM TSA) and DAOY (at 2.0-3.0µM TSA) cells. hTERT downregulation is only evident at the 3.0µM TSA dose in EPN-2 cells. Inhibition of hTERT transcription in PFSK-1 cells and at high TSA doses (3.0µM) in EPN-2 and DAOY cells.
is consistent with inhibition of telomerase activity in these respective lines. However, hTERT downregulation occurs at 2.0-3.0µM TSA in DAOY cells, not reflected by maximal telomerase inhibition at 0.5µM TSA. Similarly, hTERT downregulation in GB-1 does not reflect the degree of telomerase inhibition (Figure 4B). To probe the specificity of HDACi associated telomerase inhibition, cell lines were treated with a high dose (50µM) of the cytotoxic chemotherapeutic, etoposide. Although caspase-3 activation was evident in all lines, telomerase activity levels remained comparable to untreated cells (Figure 4C).

**No cytotoxicity 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 (1µM) and doses exceeding that utilized in previous experiments (10µM), no significant difference was observed with respect to cilia function between TSA-treated and untreated ependymal tissue (Figure 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 show a high degree of sensitivity to TSA, comparable to PFSK-1, DAOY and EPN-2 tumor cells, with maximal growth inhibition (~75%) achieved at 3.0µM TSA (Figure 5B).

**Discussion**

The present study demonstrates 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 (48h) drug exposure. The percentage of viable PFSK-1, DAOY and EPN-2 cells from the MTT assay (Figures 2A-D) and cell cycle analysis (Figure 3B) is directly comparable (20%, 40% and 50% respectively). Growth inhibitory effects in these lines were associated with marked telomerase inhibition and hTERT downregulation. These anti-cancer effects were achieved using nanomolar concentrations and 48h exposure time, suggesting TSA may be a relatively potent chemotherapeutic. By contrast, childhood GBM cells showed relatively moderate anti-proliferative 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 (37, 38). 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 (3.7-5.9kb), the data indicates that HDACi may be an attractive approach for the treatment of like subsets of childhood brain tumors. However mean telomere length in primary tumors was significantly longer in glial tumors (8.3-10.9kb) compared to neuroectodermal tumors (4.4-5.2kb). This may reflect early neurogenesis in distinct brain regions with respect to cellular replicative histories, albeit the data does not exclude different tumor types exhibiting different telomere lengths regardless of anatomical 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 alternative lengthening of telomeres (ALT) mechanism for telomere maintenance. As a high proportion of CNS PNET tumors were telomerase negative in the present study, it is likely that ALT is prevalent in these tumor subsets. Furthermore, no clinical parameter correlated to telomerase negative CNS PNET tumors.

Typical HDACi studies have demonstrated 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, anti-cancer agents have previously induced apoptosis in a p21/p53-independent manner in cancer cells including those of glioma (39, 40). Whereas an increase of cells in G2/M was evident in DAOY, EPN-2 and GB-1 lines after 16h TSA, no alterations in cell cycle distribution was evident in PFSK-1 cells. As a sub G0/1 population is prominent after only 16h 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 G0/1 cells are greatest. Additionally mean telomere length in PFSK-1 (3.8kb) is relatively short compared to 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 signalling 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 µM TSA, while caspase-3 activation occurs only at >2.0µM 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 (41-43). 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 (44, 45). Our results are consistent with this phenomenon as TSA exposure leads to increased expression of the serine139-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 non-canonical 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 protein expression was assessed in this study. Although low levels
of phosphorylated H2A.X are evident in all untreated cells, this is presumably below 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, 46, 47). Data presented here is 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 3.0µM TSA dose for both 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 deacetylase inhibition, rather than a consequence of apoptosis.

Telomerase activity is closely correlated with the repression and de-repression of hTERT (48). These findings suggest that de-repression of the hTERT promoter might be one 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, de-regulated in several tumors, can contribute to the transcriptional activation of hTERT (49-51). 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).
(31), resulting in transcriptional activation (Myc/Max) or transcriptional repression (Mad/Max) (52). 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 (49, 53, 54). 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 TGF-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. De-repression 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, whereas 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 upon 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 (via hyperacetylation) to cellular growth arrest and/or apoptosis is unclear. The precise non-histone molecular targets for each tumor type shown to be amenable for HDACi anticancer strategies remain to be identified in most cases. True genomic substrates which are repressed by particular HDACs and from which transcription can re-initiate 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 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 to non-malignant 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 pro-survival 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 engrafted with C17.2, even following two years post-transplantation(36). It is likely that v-Myc (viral c-Myc homolog) contributes to
de-repression 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
(Figure 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 next
generation brain cancer therapy trials.

Acknowledgements

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Stuart Smith for a critical review of the manuscript.
Table 1. Telomere length is significantly shorter in neuroectodermal tumors compared to glial tumors. Mean telomere length in CNS PNETs was significantly shorter than mean telomere length from constitutional blood ($p=0.02$). Mean telomere length in medulloblastomas showed a trend towards significant shortening compared to constitutional blood ($p=0.07$). Mean telomere length in GBM did not differ significantly from constitutional blood mean telomere length. When grouped together, mean telomere length in tumors of neuroectodermal origin (medulloblastomas and CNS PNETs) were significantly shorter than those tumors of glial origin (glioblastoma and ependymoma) ($p<0.0005$). No significant difference was observed with respect to age at diagnoses between tumors of

<table>
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<th>Median Tumor Telomere Length (kb)</th>
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neuroectodermal and glial origin ($p=0.508$). $^a$Ependymoma 6=primary, 13=recurrent; $^b$ Ridley L, Rahman R et al 2008 (30).

**Figure 1: Telomerase activity and telomere length in pediatric brain tumors.** Telomerase activity *in vitro* was measured by the TRAP assay using 0.1µg total protein lysate. (A, left) All tumors analyzed from patients with medulloblastoma (20/20) showed telomerase activity, with the majority demonstrating high levels of enzyme processivity. (B, left) In contrast, only 33% (6/18) of CNS PNETs showed telomerase activity, with the majority of tumors (12/18) and 2/2 dysembryoplastic neuroepithelial tumors (DNET) showing no detectable telomerase activity. (C, left) High levels of telomerase activity was present in all GBM (7/7). Mean telomere length across each tumor population was measured using the TRF assay using 2-3µg genomic DNA. (A, right) Mean telomere length of 15 medulloblastoma (MB) tumors was 4.4kb±0.53 (range 2.4-8.9kb), shows a trend towards shorter mean telomere length from constitutional blood from three medulloblastoma patients (6.9kb±0.88; $p=0.065$). (B, right) Mean telomere length of 15 CNS PNETs was 5.2kb±0.28 (range 4.1-7.3kb), significantly shorter than mean blood telomere length from five patients (6.3kb±0.09; $p=0.002$). (C, right) Relative to telomere length from tumors of neuroectodermal origin, mean telomere length from 8 GBM tumors was longer (8.3kb±0.24; range 7.2-8.9kb) and did not differ significantly to mean blood telomere length from four patients (8.5kb±0.29; $p=0.521$). (D) Mean telomere length from the DAOY (5.9kb±0.62) and PFSK-1 (3.8kb±0.43) cell lines, derived from pediatric medulloblastoma and CNS PNET respectively, is within the mean telomere
length range observed in the primary tumors. Mean telomere length from the GB-1 (4.5kb±0.58) and EPN-2 (3.7kb±1.1) cell lines, derived from pediatric GBM and ependymoma respectively, is considerably shorter than the mean telomere length range observed in the primary tumors (see Table 1 for ependymoma tumor telomere length data).

**Figure 2: Anti-proliferative and pro-apoptotic effects induced by TSA in high grade pediatric brain cancer cells are associated with genomic instability.** Cells were treated with 0.5-3.0µM TSA for 48 h and cell growth determined using the MTT assay. (A-D) All cell lines responded to treatment and showed a similar proliferation profile. (A) Mean inhibition of tumor cell proliferation in TSA-treated cells was 69% within the PFSK-1 line, (B) 67% within DAOY cells, (C) 55% within EPN-2 cells and (D) 26% within GB-1 cells. IC₅₀ values for PFSK-1, DAOY and EPN-2 were extrapolated as 0.40µM, 0.35µM and 0.40µM TSA respectively. GB-1 did exhibit 50% growth inhibition within the experimental set-up; rather, maximum inhibition (31%) was evident at the highest TSA dose. Results are expressed as the percentage of cells surviving treatment, with mean +/- S.E. of three independent experiments. 0.06% DMSO vehicle in each set-up was used to monitor any non-specific toxicity. (E) Both tumors of neuroectodermal origin show induction of apoptosis in a dose-dependent manner, evidenced by increased activation of cleaved (cl) caspase-3, concomitant with a reduction in full-length (fl) caspase-3 levels. The anti-apoptotic protein Bcl-2 in contrast, is downregulated in a dose-dependent manner. Caspase-3-dependent apoptosis is p53- and p21-independent in PFSK-1 cells and p53-independent in DAOY cells. Upregulation of p21 is evident at 2-3.0µM TSA in DAOY cells. Neither line shows evidence of induced ATM or ATR signalling. (F) Both tumors of glial origin show evidence of apoptotic
induction as indicated by activation of cleaved caspase-3. No difference was observed in expression levels of Bcl-2 in untreated compared to TSA-treated cells. In both cases, caspase-3-dependent apoptosis is p53- and p21- independent. Neither line shows evidence of induced ATM or ATR signalling. HeLa cells were used as positive controls for caspase-3, ATM and ATR. (G) TSA treatment (1µM) for 24h results in a rapid induction of DNA damage as observed by phosphorylated-histoneH2A.X activation. HeLa cells treated with 50µM etoposide were used as positive controls. Images are representative of three independent experiments and at x40 magnification. (H) Enlarged images of EPN-2 and BT-4 cells showing intense H2A.X nuclear foci. Scale bar in G and H represents 100µM.

**Figure 3: TSA-induced alterations in cell cycle dynamics in pediatric brain tumour 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 show 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 is evident in PFSK-1 cells after 16 h TSA treatment. However, induction of apoptosis is evident in the PFSK-1 (and DAOY) cells at both TSA concentrations, indicated by the emergence of a sub G0/1 population in these lines. Percentages represent the mean +/- S.E. from three independent experiments. (B) Histogram showing the proportion of cells that are sub G0/1, relative to the total cells in the population after 48 h treatment with TSA. Prolonged TSA exposure results in significantly high levels of apoptosis in all brain tumor cell lines. The proportion of untreated cells in sub G0/1 (~10%) is comparable for all cell lines. Results are expressed as the mean +/- S.E. from three independent experiments.
Figure 4: Inhibition of telomerase activity and reduction in hTERT transcription in brain tumor cells after 48 h TSA treatment. (A, left) TRAP assay using 0.1µg total protein lysate was used to measure telomerase activity in vitro. The minimal dose required for a marked reduction in telomerase activity was 1.0µM TSA in PFSK-1 cells and 0.5µM TSA in DAOY cells. Telomerase inhibition persists at higher TSA doses in PFSK-1 but enzyme activity is comparable to untreated cells at 3.0µM TSA in DAOY cells. (B, left) Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was used to measure hTERT mRNA expression. Levels of hTERT transcript in PFSK-1 are markedly reduced in TSA-treated cells compared to vehicle-only untreated cells. A clear reduction in hTERT transcription is evident in DAOY cells at 2.0-3.0µM TSA. (A, right) Telomerase inhibition in EPN-2 generally follows in a dose-dependent manner, with almost no detectable telomerase activity at 3.0µM TSA. There is 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 is only observed at the 3.0µM dose of TSA. hTERT expression is reduced in all TSA-treated GB-1 cells with lowest expression levels observed at the highest TSA dose. (C) hTERT ELISA was additionally used to detect hTERT protein levels. PFSK-1 and GB-1 show a marked reduction in hTERT protein levels upon 0.5µM TSA exposure; DAOY and EPN-2 show a moderate reduction in hTERT protein levels at 1.0 µM TSA exposure. (D) Brain tumor lines treated with 50µM Etoposide for 48h, show high levels of telomerase activity, comparable to untreated TSA-controls described earlier. (Figure 2F) This is irrespective of apoptosis induction in these lines as inferred by caspase-3 activation.
Figure 5: In vitro cytotoxicity culture models and hypothetical model of HDACi-induced hTERT repression.

(A) No cytotoxicity is observed in normal rat ependymal cells exposed to 1µM and 10µM TSA. Cytotoxicity was inferred as perturbation of ependymal cilia function with respect to cilia beat frequency (CBF, left) and cilia tip distance (right). (B) Murine cerebellar progenitor cells (C17.2) transformed with v-Myc show 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. de-repression 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, via 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.
Figure 4 - Ruman Rahman

A

B

C

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Histone deacetylase inhibition attenuates cell growth with associated telomerase inhibition in high grade childhood brain tumor cells


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