Valproic acid induces growth arrest, apoptosis, and senescence in medulloblastomas by increasing histone hyperacetylation and regulating expression of p21Cip1, CDK4, and CMYC

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Abstract

Valproic acid is a well-tolerated anticonvulsant that has been identified recently as a histone deacetylase inhibitor. To evaluate the antitumor efficacy and mechanisms of action of valproic acid in medulloblastoma and supratentorial primitive neuroectodermal tumor (sPNET), which are among the most common malignant brain tumors in children with poor prognosis, two medulloblastoma (DAOY and D283-MED) and one sPNET (PFSK) cell lines were treated with valproic acid and evaluated with a panel of in vitro and in vivo assays. Our results showed that valproic acid, at clinically safe concentrations (0.6 and 1 mmol/L), induced potent growth inhibition, cell cycle arrest, apoptosis, senescence, and differentiation and suppressed colony-forming efficiency and tumorigenicity in a time- and dose-dependent manner. The medulloblastoma cell lines were more responsive than the sPNET cell line and can be induced to irreversible suppression of proliferation and significantly reduced tumorigenicity by 0.6 and 1 mmol/L valproic acid. Daily i.p. injection of valproic acid (400 mg/kg) for 28 days significantly inhibited the in vivo growth of DAOY and D283-MED s.c. xenografts in severe combined immunodeficient mice. With Western hybridization and real-time reverse transcription-PCR, we further showed that the antitumor activities of valproic acid correlated with induction of histone (H3 and H4) hyperacetylation, activation of p21, and suppression of TP53, CDK4, and CMYC expression. In conclusion, valproic acid possesses potent in vitro and in vivo antimedulloblastoma activities that correlated with induction of histone hyperacetylation and regulation of pathways critical for maintaining growth inhibition and cell cycle arrest. Therefore, valproic acid may represent a novel therapeutic option in medulloblastoma treatment.

Introduction

Embryonal tumors of childhood, including medulloblastoma and supratentorial primitive neuroectodermal tumor (sPNET), are among the most common malignant brain tumors of childhood. Although medulloblastomas and sPNETs are histologically similar, patients with medulloblastoma tend to have a better therapeutic response than sPNET when treated with same therapy. Furthermore, infants and young children with these tumors have a poor overall survival rate. Even among survivors, many will have long-term neurocognitive and neuroendocrine sequelae resulting from craniospinal radiation. Thus, effective new therapies and treatment paradigms are needed for these diseases.

Histone deacetylase (HDAC) inhibitors represent a novel class of therapeutic agents that may provide an alternative approach for the treatment of these tumors. Recent studies have shown that HDACs play an important role in the regulation of gene transcription and oncogenesis through remodeling of chromatin structure and dynamic changes in nucleosomal packaging of DNA (1–3). Inhibition of HDAC increases histone acetylation and maintains chromatin structure in a more open conformation. This conformational change may lead to restoration of transcriptionally silenced pathways or suppression of aberrantly expressed genes through recruitment of repressor proteins (2), resulting in cell cycle arrest, apoptosis, and cellular differentiation in human cancers. Several structurally diverse HDAC inhibitors have shown preclinical activities in a variety of adult and pediatric tumor models (1), some of them, including suberoylanilide hydroxamic acid, depsipeptide, and MS-275, have recently entered clinical trials (4, 5). For malignant pediatric brain tumors, such as medulloblastoma and sPNET, however, there is still a lack of HDAC inhibitors that are ready for clinical trials.

Valproic acid, a well-tolerated anticonvulsant with an extensively characterized toxicity profile, has been identified recently as a HDAC inhibitor. It inhibits both class I and II HDACs (excluding HDAC6 and HDAC10) with
resultant hyperacetylation of histone H3 and H4 (3, 6–8). Altered expression of multiple genes, including the cyclin-dependent kinase inhibitor p21Cip1, glycogen synthase kinase-3β, and peroxisome proliferator-activated receptors, have been reported in cells exposed to valproic acid treatment (8–10). Valproic acid has displayed potent in vitro and in vivo antitumor activities against neuroblastoma (11, 12), glioma (13, 14), leukemia (15, 16), breast cancer (17) and prostate cancer (18), but effect of valproic acid in medulloblastomas and sPNET tumors remains unknown.

Valproic acid possesses several established and yet special properties that make it an attractive drug for treating brain tumors in children, especially in patients with medulloblastomas and sPNETs. Valproic acid can pass the blood-brain barrier and has a long half-life of 9 to 20 hours in human being; its concentration in cerebrospinal fluid is nearly the same as the free valproic acid concentration in plasma. Therefore, effective drug delivery to brain tumors is feasible. In a pediatric patient with relapsed sPNET, valproic acid was reported recently to have induced histologically confirmed signs of tumor cell differentiation (19). Furthermore, valproic acid is already a commercially available drug with very well defined pharmacokinetic properties; it has greater potential of being quickly translated into clinical trials once its antitumor activities are established in preclinical models of medulloblastomas and sPNETs.

The present study was therefore undertaken to assess the antitumor activities of valproic acid in medulloblastoma and sPNET by using two medulloblastoma and one sPNET cell lines that are available from American Type Culture Collection (Manassas, VA). In this report, we describe the in vitro effects of valproic acid on cell proliferation, cell cycle regulation, apoptosis, differentiation, cellular senescence, colony-forming efficiency (CCE), and tumorigenicity in severe combined immunodeficient (SCID) mice as well as the in vivo growth inhibition of medulloblastoma xenografts. In addition, we studied the changes in histone (H3 and H4) and TP53 acetylation and the alterations of p21, TP53, p16, CDK4, and CMYC gene expression during in vitro valproic acid treatment to investigate the molecular mechanisms of the antitumor effects of valproic acid. Our findings formed the basis of a recently approved phase I clinical study of valproic acid in pediatric patients by the Children’s Oncology Group.

Materials and Methods

Valproic Acid

Valproic acid (2-propyl-pentanoic acid) was purchased from Sigma (St. Louis, MO) and dissolved in serum-free medium to make a stock solution of 360 mmol/L. The stock solution was further diluted with cell culture medium to yield final valproic acid concentrations that have been established in patients with epilepsy ranging from 0.2 mmol/L (the typical cerebrospinal fluid concentration), 0.6 mmol/L (the typical therapeutic serum concentration), 1 mmol/L (upper limit of antiepileptic range), 2.7 mmol/L (associated with limited toxicity, such as lethargy with benign outcome), and 3.6 and 5.1 mmol/L (associated with life-threatening toxicities, such as coma).

Cell Lines

Human medulloblastoma cell lines (D283-MED and DAOY) and a sPNET cell line (PESK) were obtained from American Type Culture Collection (20–22) and maintained in DMEM supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA).

Cell Proliferation Assay

Cells were seeded into 96-well plates at 2,000 to 3,000 live cells per well and treated with valproic acid (0.2–5.1 mmol/L) for up to 45 days. Culture medium was replaced every 3 to 4 days at which time the antiproliferative effect of valproic acid was assessed using Cell Count Kit-8 (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). Washout experiments to assess the reversibility of the valproic acid–associated antiproliferative effect were done at various time points by replacing drug-containing medium with drug-free medium.

Cell Cycle Analysis with Flow Cytometry

Cells treated with or without valproic acid (1 and 2.7 mmol/L) were harvested for flow cytometry analysis on days 1, 2, 3, and 7 and weekly thereafter until day 42. Cells were fixed and stained with 0.1 mg/mL propidium iodide for DNA analysis with Becton Dickinson FACScan (Franklin Lakes, NJ) as described previously (23).

Detection of Apoptosis

Apoptosis was evaluated with flow cytometry and on cell smears using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (In situ Cell Death Detection kit, AP; Boehringer Mannheim GmbH, Mannheim, Germany). Samples were incubated with 50 μL of reaction mixture in a humidified chamber at 37°C for 90 minutes as described previously (23). The percentage of apoptotic cells was determined by counting at least 1,000 cells from 10 to 20 high-power fields (×400) under both phase-contrast and fluorescent microscopy.

Cell Senescence Assay

Histochemical detection of senescence-associated expression of β-galactosidase activity (24) was done with a Senescence Detection kit (BioVision, Mountain View, CA) on fixed cells treated with or without valproic acid (0.6 and 1 mmol/L). The development of cytoplasmic blue was detected and photographed using a Nikon (Nikon Instruments, Inc., Lewisville, TX) inverted microscope equipped with a color CCD camera.

Immunofluorescent Staining

Protein expression of glial marker glial fibrillary acidic protein (GFAP) and neuronal marker synaptophysin was evaluated during valproic acid treatment. Monoclonal antibodies against human GFAP (DAKO, Glostrup, Denmark) and synaptophysin (Boehringer Mannheim) were used as primary antibody. FITC-conjugated secondary antibodies (Molecular Probes, Eugene, OR) were subsequently applied. The staining intensity was scored as negative (−), marginal (+), low (+), medium (++), or high (+++).
Tumor cells were resuspended in DMEM with 0.3% agar and plated in 24-well plates at 2,000 per well on top of a 0.5 mL precast semisolid 1% agar underlayer following treatment with valproic acid (1 or 2.7 mmol/L) for 1 or 3 weeks as described previously (23). The CFE was defined as the percentage of plated cells that formed colonies relative to an untreated control.

**Tu morigen eticity an d In vivo Treatment of DAOY and D283-MED Xenografts in SCID Mice**

All animal experiments were conducted according to an Institutional Animal Care and Use Committee–approved protocol. RAG2 SCID mice, ages 8 to 12 weeks, were bred and maintained in a specific pathogen-free animal facility. Heterotransplantation was done by s.c. injection of $10^7$ live cells as described previously (23). Xenograft growth was measured weekly with a sliding caliper. Tumor size ($M$) was calculated using the formula: $M = \frac{a^2b}{2}$, where $a$ is the minimum width and $b$ is the maximum length. For tumorigenicity assay, cells were pretreated with valproic acid (0.6 and 1 mmol/L) for 4 weeks before s.c. injection, and tumor take and xenograft growth were compared with untreated cells. For efficacy of in vivo valproic acid treatment, tumors were allowed to reach ~0.5 cm in diameter before the initiation of daily i.p. administration of valproic acid (400 mg/kg), which lasted up to 28 days. Tumor size was measured weekly, and at the end of treatment, all mice were sacrificed and remnant tumors were examined histologically.

**Western Hybridization**

For analysis of histone acetylation, histones were prepared by acid extraction. For analysis of the remaining selected genes, protein pellets were collected with Trizol reagent (Invitrogen, Inc., Carlsbad, CA) and dissolved in 8 mol/L urea. Protein or histones (40 μg) were separated with 4% to 20% SDS-polyacrylamide gels, which were either stained with Coomassie blue or transferred to polyvinylidene difluoride membranes for blotting with primary antibodies against acetylated histone H3 (AcH3) and H4 (AcH4) and acetylated p53 (Lys373 and Lys382; Upstate Biotechnology, Inc., Waltham, MA) and p21 (sc-397), TP53 (sc-6243), p16 (sc-9968), CDK4 (sc-260), and CMYC (N-262; Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were visualized with horseradish peroxidase–conjugated secondary antibody and Chemiluminescence Plus kit (Amersham, Piscataway, NJ). Because recent reports have shown that levels of housekeeping proteins can be affected by HDAC inhibitors (25), and glyceraldehyde-3-phosphate dehydrogenase may not be the optimal protein as internal control for analyzing the effect of HDAC inhibitors by Western blot (26, 27), we therefore measured the protein concentration in each sample and also estimated the amount of proteins applied onto each lane by using a Coomassie blue–stained duplicate SDS-PAGE gel as protein loading control.

**Table 1. Summary of GFAP and synaptophysin expression in cells treated with valproic acid**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Valproic acid (mmol/L)</th>
<th>GFAP 2 wk</th>
<th>GFAP 4 wk</th>
<th>Synaptophysin 2 wk</th>
<th>Synaptophysin 4 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>D283-MED</td>
<td>0</td>
<td>+</td>
<td>±</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D283-MED</td>
<td>1</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>D283-MED</td>
<td>2.7</td>
<td>+++</td>
<td>ND</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>DAOY</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DAOY</td>
<td>1</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>DAOY</td>
<td>2.7</td>
<td>+++</td>
<td>ND</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>PFSK</td>
<td>0</td>
<td>–</td>
<td>–</td>
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<tr>
<td>PFSK</td>
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<td>+</td>
<td>–</td>
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<tr>
<td>PFSK</td>
<td>2.7</td>
<td>±</td>
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</tr>
</tbody>
</table>

Abbreviation: ND, not done due to very few viable cells.

**Figure 1.** Time- and dose-dependent antiproliferative effects of valproic acid. D283-MED cells were the only ones that responded to 0.2 mmol/L valproic acid (A). Minimum required dose for inhibiting cell proliferation in DAOY and PFSK was 0.6 mmol/L, and the exposure time required in PFSK cells is much longer than in DAOY cells (B and C). Dotted lines, growth curves of washout (W/O) experiments. Experiments were repeated at least twice.
Quantitative Real-time PCR
Quantitative real-time PCR was done with SYBR Green Master Mix and ABI 7000 DNA Detection System (ABI, Columbia, MD) as described previously (23). Five normal brain tissue cDNAs were employed as references, which include two adult cerebellum and one fetal brain tissues purchased from Clontech (Paulo Alto, CA) and ILSbio (Bethesda, MD) and two normal cerebellar tissues collected from patients (ages 8 and 14 years) undergoing resection of benign tumors at Texas Children’s Hospital in accordance to institutional review board–approved protocols. Gene-specific primers were designed to flank more than one exon to ensure that all the expected PCR products were generated from mRNA (Table 1, Supplementary Material). Gene expression levels were determined with standard \( \Delta \Delta Ct \) method (23) and normalized to the internal standard glyceraldehyde-3-phosphate dehydrogenase. All reactions were done in duplicate on two occasions. Reaction specificity was confirmed with dissociation curves.

Statistical Analysis
The effects of valproic acid on cell proliferation, CFE, cell cycle arrest, apoptosis, and xenograft growth in SCID mice were analyzed with two-way ANOVA and presented as the mean ± SD.

Results
Valproic Acid Suppresses Cell Proliferation
Valproic acid suppressed tumor cell proliferation in a time- and dose-dependent manner (\( P < 0.05 \)) in all three central nervous system embryonal tumor cell lines (Fig. 1). In D283 cells, treatment with 0.6 mmol/L valproic acid for 10 days or with 0.2 mmol/L valproic acid for 14 days resulted in >50% suppression of cell proliferation (Fig. 1A), whereas in DAOY cells it required 10 days of exposure to 1 mmol/L valproic acid or 21 days to 0.6 mmol/L valproic acid (Fig. 1B). PFSK cells were the least responsive. Cell growth was initially suppressed on day 10 after exposure to 1 mmol/L valproic acid; however, the cell number continued to increase, albeit at a slower rate than untreated cells. PFSK cell growth was not completely suppressed following 4 weeks of exposure to valproic acid concentrations as high as 3.6 mmol/L (Fig. 1C). It should be pointed out that the growth curves of the untreated cells in all three cell lines reached plateau in ~21 days mainly due to the limited
growth areas that were available in 96-well plates that were used in the cell proliferation assay. This phenomenon, however, was not observed in any of the treated cells, suggesting that the differences of cell numbers between treated and control groups could have been more significant had the untreated cells been given additional space.

To evaluate whether cell lines regained proliferative capacity following cessation of valproic acid exposure, washout experiments in which valproic acid–containing medium was replaced with valproic acid–free medium revealed that following continuous exposure to 0.6 mmol/L valproic acid for at least 4 weeks suppression of cell proliferation became irreversible in the medulloblastoma cell lines (Fig. 1A and B).

Valproic Acid Induces Cell Cycle Arrest

To investigate the effects of valproic acid on cell cycle distribution, cells were treated with concentrations that are either clinically safe (1 mmol/L) or with mild toxicities (2.7 mmol/L). In D283-MED and DAOY cells, shift of cell population to G1-G0 phases started after 3 days of valproic acid (1 or 2.7 mmol/L) treatment. More significant cell cycle arrest, however, was detected on day 7, when cells in G1-G0 phases increased and cells in G2-M phases decreased concurrently. Higher concentration (2.7 mmol/L in D283-MED treated for 1 week) or longer exposure (up to 5 weeks in DAOY and D283-MED cells) also resulted in a remarkable increase of subdiploid apoptotic cells. In PFSK cells, however, valproic acid did not produce significant cell cycle arrest (Fig. 2A).

Valproic Acid Augments Apoptosis

In addition to flow cytometry analysis, terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay was also used to examine the induced apoptosis in cells treated with valproic acid (1 and 2.7 mmol/L; Fig. 2B). The increased apoptosis was time and dose dependent. In D283-MED cells, following an initial induction of apoptosis in the first 24 hours, apoptotic cells increased ~20-fold (10%) over untreated cells (0.5%) on day 7. Due to excessive accumulation of dying D283-MED cells and debris as a result of valproic acid treatment, exact quantification of apoptotic assay beyond 7 days was unreliable. In DAOY cells, longer exposure time (up to 5 weeks) was required to elicit significant increase of apoptosis. In PFSK cells, only minimal increase (2- to 3-fold) in apoptosis was detected after 2 to 6 weeks of treatment.

Valproic Acid Induces Cellular Senescence

Cellular senescence has been identified as one of the mechanisms mediating the anticancer effects of chemotherapies (24). One of the morphologic changes that was frequently observed in our valproic acid–treated cells is the flattening of cells with increased granularity, which is a typical morphologic change associated with cellular senescence (24). By examining senescence-associated expression of β-galactosidase activity (24), we confirmed that cellular senescence was indeed induced in those flattened D283-MED and DAOY cells by valproic acid (0.6 and 1 mmol/L) in a time- and dose-dependent manner (Fig. 3). More interestingly, in D283-MED cells treated

![Figure 3. Cellular senescence induced by valproic acid (0.6 and 1.0 mmol/L). Arrowhead, changes found in multicell spheroids in D283-MED cells; arrows, senescent cells with the typical flattened appearance. Magnification, ×100.](https://example.com/figure3.png)
with valproic acid (0.6 mmol/L), we observed blue staining in the gradually dissociating cell spheroids as well. The induced senescence started from day 3 and peaked on day 7 when the whole spheroids were densely stained. A higher valproic acid concentration (1 mmol/L) led to more dramatic increase of cellular senescence as evidenced by further depletion of spheroids and increased β-galactosidase staining of attached D283-MED cells. The PFSK cells showed only minimal increase of positive β-galactosidase staining after valproic acid treatment.

Valproic Acid Induces Glial and Neuronal Marker Expression in Medulloblastoma Cells

To evaluate the differentiation-inducing ability of valproic acid, the upper limit of clinically achievable concentration (1 mmol/L) and concentration with low toxicity (2.7 mmol/L) were included. For glial marker GFAP, treatment with 1 mmol/L valproic acid for 2 weeks dramatically increased its expression from negative (−) to high level (+++) in DAOY and D283-MED cells (Table 1, Supplementary Fig. S1A-D).3 Such elevation of GFAP expression in PFSK cells, however, was not observed until after 4 weeks of treatment at 2.7 mmol/L (Supplementary Fig. S1E and F).3 For neuronal marker synaptophysin, elevated expression was observed in DAOY cells after 2 weeks of exposure to 1 mmol/L valproic acid (Supplementary Fig. SIG and H.),3 whereas longer exposure for up to 4 weeks was required with D283-MED cells (Table 1), suggesting that the predominantly apoptotic and growth arrest effects of valproic acid on D283-MED cells delayed the few surviving cells from being induced to differentiate. Synaptophysin protein was not induced in PFSK cells even after 4 weeks of valproic acid (2.7 mmol/L) treatment (Table 1). Altogether, these results showed that the medulloblastoma cell lines (DAOY and D283-MED) are more responsive to valproic acid than the sPNET cell line PFSK and can be induced to more differentiated phenotype with concentration (1 mmol/L) that is clinically achievable.

Valproic Acid Suppresses CFE

To examine the suppressive effects of valproic acid on CFE, D283-MED and PFSK cells were treated with valproic acid (1 and 2.7 mmol/L) for 1 or 3 weeks. DAOY cells do not form colonies in soft agar and were not tested in this assay. Our results showed that valproic acid exerted time- and dose-dependant suppression of CFE in both D283-MED and PFSK cells (P < 0.01; Fig. 4). Treatment with 1 mmol/L valproic acid for 1 week resulted in 75% inhibition of colony formation in D283-MED cells and >85% inhibition in PFSK cells. Higher concentration (2.7 mmol/L) and/or longer treatment time (3 weeks) produced more significant suppressive effects in both cell lines (Fig. 4).

Valproic Acid Inhibits Tumorigenicity of Medulloblastoma Cells

To examine the inhibitory effects of valproic acid on tumorigenicity, cells were pretreated with valproic acid (0.6 and 1 mmol/L) for 4 weeks before heterotransplanted s.c. into SCID mice. As shown in Fig. 5A, valproic acid exerted dose-dependent suppression of tumorigenicity in the two medulloblastoma cell lines (D283-MED and DAOY). In D283-MED cells, pretreatment with 0.6 mmol/L valproic acid significantly reduced the tumor growth, and with 1 mmol/L valproic acid, the tumor formation was completely suppressed (P < 0.01). In DAOY cells, both tumor take and growth were inhibited (P < 0.01). In the sPNET cell line PFSK, however, no suppression of tumorigenicity was observed (data not shown).

Valproic Acid Suppresses the Growth of D283-MED and DAOY S.c. Xenografts in SCID Mice

To further assess the in vivo antimeculloblastoma effects of valproic acid in s.c. xenografts, valproic acid was given through daily i.p injection of 400 mg/kg, which is the most commonly used route and dose in mouse (28), for up to 28 days. Such treatment resulted in significant suppression of xenograft growth from both cell lines (P < 0.01; Fig. 5B). Although no significant histologic changes were observed, intense GFAP positivity was detected in the majority of DAOY cells in the treated tumors. The expression of synaptophysin, which was undetectable in the untreated tumors, was increased in a fraction of cells in xenografts treated with valproic acid (data not shown).

Valproic Acid Induces Hyperacetylation of Histone H3 and H4 Both In vitro and in Animal Models

To evaluate the temporal changes of histone H3 and H4 acetylation status, cells treated with valproic acid (1 mmol/L) for 0, 3, 7, 14, and 28 days in vitro were analyzed

Figure 4. Time- and dose-dependent suppression of CFE of D283-MED (A) and PFSK (B) following valproic acid treatment (P < 0.01). DAOY cells do not form colonies in soft agar and were not tested in this assay.

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by Western hybridization. As shown in Fig. 6A, increased AcH3 and AcH4 levels were detected as early as day 3 in all three cell lines. With extended treatment, the medulloblastoma cell lines (D283-MED and DAOY) displayed progressive accumulation of AcH3 and AcH4 until day 28. In the sPNET cell line, however, no additional increases in AcH3 or AcH4 levels were observed beyond day 7 of valproic acid treatment. To further examine the \textit{in vivo} effects of valproic acid, remnant s.c. xenografts of D283-MED and DAOY cells were analyzed. Treatment with valproic acid (400 mg/kg/d i.p.) significantly increased AcH3 and AcH4 levels in both D283-MED and DAOY xenografts (Fig. 6B).

\textbf{Valproic Acid Activates p21\textsuperscript{Cip1} (p21) and Inhibits TP53 Expression}

To perform detailed temporal analysis of gene expression alteration, both mRNA and protein levels before and after valproic acid (1 mmol/L) treatment were examined. Using cDNAs from normal human brains, including two from age-matched cerebella, as references, we found that the intrinsic expression levels of p21 and TP53 were significantly different among the three cell lines and were associated with some differences of their response modes toward valproic acid treatment. In agreement with previously published results (29), our data showed that valproic acid activated \textit{p21} gene by increasing its mRNA transcription and/or protein translation (30–33). In D283-MED cells, which showed near-normal level of pretreatment \textit{p21} mRNA, the increased p21 protein level was observed on day 3 before the induction of a marginal increase (<50%) in \textit{p21} mRNA transcripts on day 7. In DAOY and PFSK cells, both of which showed lower than normal levels of pretreatment \textit{p21} mRNA, their increases in \textit{p21} protein translation paralleled the increases in mRNA transcription (Fig. 7).

The tumor suppressor TP53 induces cell cycle arrest or apoptosis in response to a variety of stress signals (34). Because \textit{p21} is believed to be the main target for TP53-induced cell cycle arrest (35), we examined if valproic acid also induces TP53 expression. As shown in Fig. 7, the expression of TP53 mRNA and protein were induced only in DAOY cells, which is known to harbor a mutated TP53 gene (36), whereas in D283-MED cells the expression of TP53 was steadily down-regulated by valproic acid (1 mmol/L). In PFSK, the TP53 protein expression was not affected by 1 mmol/L valproic acid, although its near-normal expression of TP53 mRNA was slightly (<50%) decreased. Altogether, our data showed that effects of valproic acid on apoptosis, cell cycle arrest, and activation of \textit{p21} gene in medulloblastomas does not correlate with the increased expression of tumor suppressor gene TP53.

Post-translational modifications of TP53, such as ubiquitination, phosphorylation, and acetylation, have profound effects on TP53 function (37). Acetylation of TP53, in

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Effects of valproic acid on tumorigenicity and \textit{in vivo} growth of xenografts in SCID mice. To examine the suppressive activities of valproic acid on tumorigenicity, tumor cells were pretreated with valproic acid (0.6 and 1.0 mmol/L) for 4 wks before heterotransplanted s.c. into SCID mice; both tumor take and growth rate were presented. For D283-MED cells, two mice bearing tumors that were too big in the untreated group were euthanized on week 6 (arrow: A). To examine the \textit{in vivo} growth inhibitory effects of valproic acid, cells from the medulloblastoma cell lines (D283-MED and DAOY) were heterotransplanted s.c. into SCID mice and allowed to grow for 4 wks to reach approximate size of 0.5 $\times$ 0.5 cm\textsuperscript{2} when daily i.p. injections of valproic acid (400 mg/kg) were started and lasted 21 d for D283-MED and 28 d for DAOY xenografts ($P < 0.01$; B).}
\end{figure}
particular, can dramatically stimulate its sequence-specific DNA-binding activity in vitro, and treatment with trichostatin A, a HDAC inhibitor, has been shown to increase the levels of acetylated TP53. To further investigate the effect of valproic acid on TP53 acetylation, Western hybridization was done using a monoclonal antibody against specific acetylated lysine residue (Lys\textsuperscript{373} and Lys\textsuperscript{382}) in TP53 protein in the three cell lines treated with 1 mmol/L valproic acid for up to 28 days. In all the cell lines examined, valproic acid failed to induce TP53 acetylation. Instead, it reduced the levels of acetylated TP53 in both DAOY (with mutated TP53) and PFSK (with wild-type TP53) cells starting from day 7 of treatment while maintaining undetectable levels of acetylated TP53 in D283-MED cells until the end of valproic acid treatment on day 28 (Fig. 7B), indicating that valproic acid did not activate p21 via increasing TP53 acetylation.

Effects of Valproic Acid on p16INK4a and CDK4 Expression

The tumor suppressor p16INK4a arrests cells at G\textsubscript{1} phase and mediates cellular senescence by inhibiting kinase activities of CDK4 and phosphorylation of RB tumor suppressor protein (24). Our results showed that, in the medulloblastoma cell lines, p16 mRNA levels were much lower compared with normal references and its protein expression was completely absent. Valproic acid treatment had no effects on p16 expression in D283-MED cells. Although p16 mRNA expression in DAOY was induced 6-fold by valproic acid on day 28, the mRNA transcript level was still <30% of the levels in age-matched cerebellum, and no p16 protein was detected. Contrary to medulloblastoma cells, PFSK expressed normal level of p16 mRNA, which was increased 2-fold with valproic acid treatment, but p16 protein levels remained unchanged. These results suggest that p16 did not play a major role in valproic acid induced cellular senescence.

The expression of CDK4, however, was significantly altered by valproic acid. Suppression of CDK4 mRNA expression was most prominent in D283-MED cells (Fig. 7A), and a corresponding decline in protein levels, although less dramatic, was also observed (Fig. 7B). In DAOY cells, although the inhibition of mRNA transcript levels was not major, a dramatic depletion of CDK4 protein was observed (Fig. 7B), suggesting that valproic acid treatment may also affect CDK4 protein levels in a translational or post-translational manner. Taken together, our data suggest that altered CDK4 transcription and/or translation may mediate valproic acid–induced senescence in the two medulloblastoma cell lines. Because PFSK cells exhibited minimal growth inhibition and cellular senescence despite significant decline in CDK4 mRNA and protein levels, we hypothesize that this sPNET cell line may possess redundant pathways and/or compensatory mechanisms to escape the antitumor effects of valproic acid.

Valproic Acid Down-Regulates the Expression of Oncogene CMYC

A series of studies have documented that CMYC regulates a wide range of genes involved in processes, such as proliferation, differentiation, and apoptosis.
including p21 and CDK4 (38, 39). In our study, all three cell lines expressed high levels (>6-fold of normal) of CMYC mRNA. Treatment with valproic acid (1 mmol/L) dramatically reduced CMYC mRNA transcription levels in D283-MED (>50% on day 28), DAOY (down to normal levels after day 14), and PFSK (45% on day 14) cells. Significant reductions in CMYC protein levels were also observed, especially in D283-MED and DAOY cell lines, suggesting that suppression of CMYC correlated with valproic acid responsiveness of medulloblastoma cells.

**Discussion**

In the current study, we showed that the potent antimedulloblastoma activities of valproic acid in medulloblastomas are correlated with induction of histone (H3 and H4) hyperacetylation, activation of p21, restoration of p16/CDK4 pathway, and suppression of CMYC oncogene. With absent cell cycle arrest, minimal apoptosis, and cellular senescence, the sPNET cell line PFSK seems less sensitive to valproic acid than medulloblastoma cell lines. However, because of significant differences in gene expression patterns among the three cell lines before they were exposed to valproic acid treatment, some of the differential responses observed in these cell lines may be related to their genetic backgrounds, including the genes and pathways that were not evaluated in the current study.

Our study showed that the *in vitro* antimedulloblastoma effects of valproic acid were time and concentration dependent, and irreversible inhibition of cell growth could be achieved with extended treatment. This finding is in agreement with our previous results with another HDAC inhibitor phenylbutyrate (23). More importantly, we further showed that valproic acid possesses strong inhibitory activities on tumorigenicity of medulloblastoma cells. Pretreatment with 1 mmol/L valproic acid for 4 weeks before heterotransplantation into SCID mice resulted in complete abrogation of tumorigenicity in D283-MED cells and significantly reduced tumor take (down to 50%) and growth rate in DAOY cells. Even pretreatment with 0.6 mmol/L valproic acid was able to significantly decrease the growth of xenografts from both medulloblastoma cell lines. These results provided strong evidence to support the notion that irreversible epigenetic reprogramming has taken place and are responsible for the reduced tumorigenicity. Because long-term valproic acid administration in children is well tolerated (40, 41), these results suggest that chronic treatment with valproic acid should be maintained in children with medulloblastomas after radiation and chemotherapy, which may possibly decrease recurrence and improve survival.

The antimedulloblastoma effect of valproic acid was confirmed *in vivo* using s.c. heterotransplanted D283-MED and DAOY xenografts in SCID mice. Because the half-life of valproic acid in mice is only 0.8 hour compared with 9 to 18 hours in humans (42), we expected that with once daily injection of 400 mg/kg the xenografts were exposed to therapeutic valproic acid concentrations for only 4 hours daily. Nonetheless, we observed significant growth inhibition of the treated xenografts. It is therefore reasonable to infer that more prominent tumor suppression would have been observed had steady-state therapeutic valproic acid concentrations been maintained.

In agreement with previous reports (7, 8), our data showed valproic acid–induced histone (H3 and H4) hyperacetylation both *in vitro* and *in vivo*. We also found that the levels of accumulated AcH3 and AcH4 correlated with the degree of *in vitro* growth suppression in the valproic acid–sensitive medulloblastoma cell lines, suggesting that the antimedulloblastoma effects of valproic acid were at least partly mediated through histone H3 and H4 hyperacetylation. Recent cDNA microarray profiles of human medulloblastomas documented their overexpressions of HDAC1 and HDAC2 (43), lending further support for using HDAC inhibitors as novel agents for treating these tumors.

The expression of cyclin-dependent kinase inhibitor p21 has been implicated in HDAC inhibitor–induced cell cycle arrest in numerous human cancers (29). In this study, we confirmed that valproic acid is capable of activating p21 gene in medulloblastoma cells. Our results also showed that the p21 activation does not correlate with increased p53 expression or with increased acetylation of TP53. In fact, the mRNA expression of TP53 gene was inhibited in D283 and PFSK cell lines, both of which have functional wild-type TP53 gene. The p16/CDK4/RB pathway, now believed to be the molecular link between cellular senescence and tumor suppression, also seemed to mediate HDAC inhibitor–induced senescence in human cells (44–46). Our results showed that, in medulloblastoma cell lines lacking intrinsic p16INK4a expression, suppression of CDK4 expression seemed to have compensated for losses of p16 and restored significant cellular senescence in both cell lines. In PFSK cells, although valproic acid induced significant suppression of CDK4 mRNA and protein levels, induced cellular senescence was not observed, suggesting that there may be redundant pathways or compensatory mechanisms allowing these cells to be resistant to the antitumor effects of valproic acid.

Overexpression of CMYC had been frequently detected in medulloblastomas and is associated with shorter survival and tumor anaplasia (47–49). CMYC has also been reported to promote cell cycle reentry and proliferation (39) through repression of p21 expression and activation of CDK4 mRNA transcription (50). Therefore, the suppression of CMYC expression by valproic acid may render substantial therapeutic benefits in medulloblastoma patients by inhibiting the driving activities of CMYC in cell proliferation and cell cycle progression.

In summary, we showed that valproic acid possesses potent *in vitro* and *in vivo* antimedulloblastoma activities by suppressing cell proliferation, promoting apoptosis, inducing cell cycle arrest and cellular senescence, enhancing cell differentiation, and inhibiting tumorigenicity at
concentrations within the established therapeutic ranges of valproic acid for epilepsy. These results may lay the groundwork for further studies using specific genetically engineered models to establish the causal relationship between valproic acid antitumor activity and specific genetic pathways and to identify molecular markers that will predict drug responsiveness and guide the development of future clinical therapies.

References


Valproic acid induces growth arrest, apoptosis, and senescence in medulloblastomas by increasing histone hyperacetylation and regulating expression of p21Cip1, CDK4, and CMYC

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