Phenybutyrate and Phenylacetate Induce Differentiation and Inhibit Proliferation of Human Medulloblastoma Cells

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ABSTRACT

Purpose: Phenylbutyrate (PB) and phenylacetate (PA) have antiproliferative and differentiation-inducing effects in malignant tumors, and had been evaluated in Phase I/II clinical trials. This study was undertaken to evaluate their antitumor activities in medulloblastomas.

Experimental Design: The biological effects of PB and PA, ranging from 0.1 mM to 3 mM, on two medulloblastoma cell lines (DAOY and D283-MED) were examined using various long-term in vitro and in vivo assays for morphology, proliferation, differentiation, anchorage-independent growth, apoptosis, and tumorigenicity.

Results: PB and PA can both induce morphological changes and suppress proliferation in a time- and dose-dependent manner. These effects were more pronounced with PB and became irreversible in D283-MED cells after continuous exposure to 3 mM PB for 28 days. Both PB and PA were able to increase expression of glial marker glial fibrillary acidic protein and neuronal marker synaptophysin in two cell lines. For anchorage-independent growth, PB showed a more significant suppression than PA in D283-MED cells. PB caused more pronounced cell cycle arrest and remarkably reduced tumorigenicity in D283-MED cells than in DAOY cells. Apoptosis was readily induced in D283-MED cells with either low dose of PB or short-term treatment. In contrast, much higher concentrations of PB or longer treatment were required to achieve similar effect with DAOY cells. PB induced increased histones H3 acetylation in both cell lines, but histone H4 acetylation was only observed in D283-MED cells.

Conclusions: PB, through induction of hyperacetylation of histone H3 and H4, is a much more potent antitumor agent than PA. 283-MED cells are more responsive to PB than DAOY cells, which may be dependent on their original state of differentiation as well as the changes of histone H4 acetylation status.

INTRODUCTION

Neoplasms of the central nervous system rank second only to leukemia in incidence among children. Among those <15 years of age, medulloblastoma is one of the most common types of malignant brain tumors. In addition to frequent leptomenigeal spread through the cerebrospinal fluid compartment or to the spinal cord, medulloblastoma also has the greatest propensity among primary malignant central nervous system tumors for disseminating to extraneural tissues such as bone, bone marrow, lymph nodes, liver, and lung (1), displaying a highly malignant and very aggressive phenotype. The tumor cells are very primitive and express various markers of cell differentiation, most commonly neuronal and glial lineage, and to a lesser extent cartilage, muscle, fat, epithelial, mesenchymal (2), and retinal markers (1). Despite the use of sophisticated multimodality therapy, many of the children with advanced medulloblastoma eventually die of progressive disease. Even among the survivors, there is still the problem of long-term neuropsychological and cognitive impairment caused by the therapies on the developing brain (3). Thus, there is an urgent need to develop new and innovative therapies that can improve survival and reduce toxicity.

A novel approach that has shown dramatic results both in vitro and in animal models involves the use of agents that induce differentiation in tumor cells (4). Recent advances in molecular oncology studies have demonstrated that the genes required for normal growth regulation and differentiation are not necessarily lost during malignant transformation. The regulating mechanisms may still remain intact, but the regulatory signals governing cell proliferation, differentiation, and apoptosis are defective. Unlike the use of cytotoxic agents to kill tumor cells, differentiation therapy aims to restore the regulating machinery by overriding the defective signals or activating alternative pathways that will cause malignant tumor cells to mature and lose their malignant phenotype (5, 6). Such induced differentiation could also cause tumor cells to become more responsive to normal growth regulatory signals or more sensitive to chemotherapy (7–10) and other differentiation agents (11). The remarkable success of all-trans-retinoic acid in producing complete remission in patients with acute promyelocytic leukemia has sparked interest in differentiation induction as an alternative form of cancer chemotherapy (12). In two previously reported medulloblastoma cases, neuronal maturation associated with extensive therapy was observed (13), suggesting that these tumor cells can be induced to differentiate.
Phenylbutyrate (PB) is one of the histone deacetylase inhibitors that was reported to have antiproliferative, differentiation-inducing, and apoptosis-enhancing effects by affecting the dynamics of chromatin folding during gene transcription (10, 14–19). It has also been demonstrated that PB enhanced the antigungrowth effect of chemotherapeutic agents in colon carcinoma cells (7, 18); induced cytostasis and maturation in multidrug-resistant breast, ovarian, and colon carcinoma cells (20); and increased the radiosensitivity of colon, breast, brain, and prostate cancer cells (8). The detailed mechanisms by which PB or phenylacetate (PA) exert their effects remain unclear. One of the unique features of PB and its metabolite PA is that they are both relatively nontoxic (21). PA is used in the therapy of hyperammonemia associated with urea cycle defects and has a track record of safety with doses giving rise to plasma levels of 3–6 mM (22). Both PA and PB have high free fractions in human plasma, so more unbound molecules are available for interactions with tumor cells (23). This provides PB and PA a greater potential for clinical application over other differentiation-inducing agents such as hexamethylene bisacetamide (24), and dimethylformamide (25). Both PA and PB have been studied in Phase I/II clinical trials (14, 19, 26, 27).

The other advantage that PA and PB have toward future treatment of central nervous system tumors is that both are small aromatic fatty acids, enabling them to pass through the blood brain barrier more easily. The lipophilicity of PB is even higher than PA, which will aid the delivery of PB to brain tumors (28, 29). PA has been reported to suppress proliferation of medulloblastoma cells causing them to accumulate in G0/G1 phase of the cell cycle and increase the expression of neurofilament proteins (30). PB, on the other hand, has been found to be a more potent differentiation-inducing agent than PA in prostate cancer, myeloid leukemia cells, and erythroleukemia cells (16, 17, 31). It is also capable of enhancing the effectiveness of other agents such as retinoids, β-interferon, suramin, 5-aza-2′-deoxy-cytidine, hydroxyurea, 5-fluorodeoxyuridine in colorectal cancer, cisplatin and lovastatin in ovarian cancer, and multiple chemotherapeutic agents in malignant B cells (7, 9, 18, 28). Its effect on medulloblastoma cells, however, has not been investigated yet. Therefore, the present study was undertaken to evaluate the antiproliferative, differentiation-inducing, and pro-apoptotic effects of PB and PA, on two medulloblastoma cell lines, in preparation to further studies of the mechanisms of their action through detailed gene expression profiling.

MATERIALS AND METHODS

Cell Cultures. Human medulloblastoma cell lines (DAOY and D283-MED) were obtained from American Type Culture Collection (Manassas, VA). DAOY cell line was established from a desmoplastic medulloblastoma of a 4-year-old male, and grows as attached polygonal cells with a population doubling time of 70% confluent.

D283-MED cells growing in suspension tend to aggregate into multicellular spheroids. These cell lines were routinely maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA). Cells were plated in T-75 tissue culture flasks, and adherent cells were subcultured approximately every 3 days when the cells were 70% confluent.

PA and PB Treatment of Cell Lines. PA and PB were supplied by the National Cancer Institute and were dissolved in DMEM to make a 100 mM stock solution. Final concentrations ranging from 0.1 to 30 mM were prepared by serial dilutions using DMEM supplemented with 10% fetal bovine serum.

Cell Proliferation Assay. DAOY cells in log-phase of growth were harvested by trypsinization, resuspended at a concentration of 1000 cells/ml, and replated into 24-well plates at 1 ml/well. After 24 h, the medium was replaced with media containing various concentrations of PA and PB ranging from 0.3 to 100 mM. D283-MED cells were centrifuged before replacing medium to also retain the population of cells in suspension. Cells were counted in triplicate at intervals of 3 days using a Coulter Z1 counter (Beckman Coulter, Inc., Miami, FL). To study the reversibility of the antiproliferation effects of PB, washout experiments were performed in which the media containing PB were removed, cells washed and the media replaced with PB-free DMEM on days 7, 15, and 28, and cells maintained in drug-free medium for the remaining duration of the experiment. Cell viability was determined by trypan blue exclusion. The differences in cell proliferation between the treated and untreated cells were statistically analyzed with two-way ANOVA.

Immunohistochemical Detection of Glial and Neuronal Differentiation Markers. Cells were cytospun on to Super-frost slides, which were subsequently fixed with acetone and blocked with normal goat serum (1:20). For detection of glial marker glial fibrillary acidic protein (GFAP), rabbit anticoic GFAP polyclonal antibody (Dako A/S, Glostrup, Denmark; 1:1000), which has strong cross-reaction with human GFAP, was used as primary antibody. For neuronal marker synaptophysin (SYN), mouse monoclonal anti-SYN antibody (Boehringer Mannheim GmbH, Mannheim, Germany; 1:20) was used. Primary antibodies were applied overnight at 4°C. Appropriate biotinylated secondary antirabbit (for GFAP) and antimouse (for SYN) antibodies were applied for 30 min at room temperature followed by avidin-biotin complex for 45 min at room temperature. 3,3′-Diaminobenzidine/hydrogen peroxide was used as the chromogenic substrate. The intensity of the staining was rated as 0 (none), + (≤25% of cells stained), + + (25–50% of cells stained), + + + (>50% of cells stained).

Western Blot Analysis. D283-MED and DAOY cells were treated with PB (3 mM) for 0, 3, 7, 14, and 28 days. Protein pellets collected with TRizol reagent (Invitrogen Inc.) were dissolved in 8 M urea. Protein concentration was determined with Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA). Forty μg of proteins were separated with 4–20% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. To detect the expression of GFAP and SYN proteins, the same antibodies used in immunohistochemical staining as described above were used. To determine the changes of histone H3 and H4 acetylation status induced by PB, polyclonal antibodies directed against acetylated histone H3 (AcH3; 1:1000) and H4 (1:330; AcH4; Upstate Biotechnology, Wallath, MA) were applied. β-Tubulin was used as loading control.
Induced Differentiation of Medulloblastoma Cells

Both layers of agar contained identical concentrations of PA or semisolid 1% agar underlayer as described previously (5, 6). 24-well plates at 2000 cells/well on top of a 0.5-ml precast were resuspended in DMEM with 0.3% agar and plated into days, respectively. At the end of each treatment, tumor cells changed every 3 days while maintaining the drug concentration. Concentrations as low as 1 mM of PB, however, decreased spheroid formation in D283-MED cells as early as day 4. Cells exposed to 3 mM PB spread out as shown in Figure 1.

Quantitative Reverse Transcription-PCR Analysis of GFAP and SYN mRNA Expression. To check the expression of GFAP and SYN mRNA during the treatment of PB (3 mM), real-time reverse transcription-PCRs were performed with SYBR green master mix in ABI 7000 DNA detection system (ABI, Columbia, MD). D283-MED and DAOY cells were harvested after they have been treated with 3 mM PB for 0, 3, 7, 14, and 28 days. Total RNAs were extracted with TRIzol reagent, and dissolved in diethyl pyrocarbonate water. cDNA was synthesized with murine leukemia virus reverse transcriptase and random hexamers (Perkin-Elmer, Foster City, CA) in a total volume of 20 μl from 1 μg of total RNA. The primers were designed to flank more than one exon of GFAP (accession No.: NM_002055, Forward 5'-TGGAACCGAAGAACAACCT-3', Backward 5'-CCTCCAGCGACTCAATCTTC-3') and SYN (accession No.: NM_003179.1, Forward 5'-TGACCTCGG-GACCTCAACAC, Backward 5'-AGGCTCTCCCTAAGACGCAA). Their expression levels were normalized to the value of the internal standard glyceraldehyde-3-phosphate dehydrogenase (accession No.: J04038, Forward 5'-AAGTG-GAAAGTCCGGAGTCA, Backward 5'-AATGAAGGGTG-CATTGATGG) using standard ΔΔCt method. All of the reactions were performed in duplicate and repeated twice, the specificity of reactions was checked with dissociation curve generated from each reaction and periodic running on 2% agarose gel as well.

Anchorage-Independent Growth in Soft Agar. DAOY and D283-MED cells were pretreated with PA and PB at doses of 0 mM (control), 0.3 mM, 1 mM, and 3 mM for 3, 7, 14, and 28 days, respectively. At the end of each treatment, tumor cells were resuspended in DMEM with 0.3% agar and plated into 24-well plates at 2000 cells/well on top of a 0.5-ml precast semisolid 1% agar underlayer as described previously (5, 6). Both layers of agar contained identical concentrations of PA or PB that were used for the pretreatment. Colonies of >50 cells were scored after 2 weeks of growth. The difference between treated and untreated cells was statistically analyzed with one-way ANOVA.

Flow Cytometry (FCM) Analysis. DAOY and D283-MED cells were treated with PB ranging from 0.1 mM to 3 mM for 3, 7, 14, and 28 days, during which time the medium was changed every 3 days while maintaining the drug concentration. Cells grown in PB-free medium were used as control at each time point. At the end of the treatment, cells were either trypsinized (DAOY) or scraped (D283-MED) from culture flasks and harvested by centrifugation. Methanol-fixed whole cells for DNA analysis were stained with staining solution (0.1 mg/ml of propidium iodide, 0.001 mg/ml of Triton X-100, and 0.037 mg/ml of EDTA in PBS) and analyzed with a Becton Dickinson FasScan (Franklin Lakes, NJ) according to the manufacturer’s protocols.

Tumorigrenicity of PB-treated DAOY and D283-MED cells was studied in Rag-2 SCID mice. To evaluate the dose effects, DAOY and D283-MED cells were first treated with 3 mM PB in vitro for 2 or 4 weeks. At the end of treatment, cells were harvested and counted with trypan blue staining. Ten million viable cells from each treatment group were suspended in 0.15 ml of complete growth medium and injected s.c. at two sites per mouse for a total of four sites. Untreated cells were injected as control. The growth of xenografts was measured weekly with a sliding caliper for 10 weeks. The tumor size was calculated by the formula

\[ M = a^2b/2 \]

where M refers to the tumor size, a is the minimum width, and b is the maximum length. The growth of the xenografts were plotted with GraphPad Prism software package (GraphPad, San Diego, CA) and statistically analyzed through two-way ANOVA.

Detection of Apoptosis. In addition to estimating the fraction of subdiploid population of cells analyzed with FCM, terminal deoxynucleotidyl transferase-mediated nick end labeling assay was used to detect apoptosis on cell smears with the In Situ Cell Death Detection kit, AP (Boehringer Mannheim GmbH) using PB-treated cells that had been prepared for FCM analysis. Cell smears on the Superfrost/Plus slides were fixed, washed with PBS, and incubated with permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice. For terminal deoxynucleotidyl transferase-mediated nick end labeling reaction, slides were incubated with 50 μl of reaction mixture in a humidified chamber at 37°C for 90 min. For positive control, DNA strand breaks were induced by DNase I before adding the reaction mixture. In the negative control, 50 μl of label solution without terminal transferase was used. After the incubation, slides were rinsed three times with PBS and analyzed by both phase contrast and fluorescence microscopy from the same field.

RESULTS

Morphology. Neither DAOY nor D283-MED cells showed a significant change in morphology after PA treatment. Concentrations as low as 1 mM of PB, however, decreased spheroid formation in D283-MED cells by day 4, and increased the fraction of attached cells as compared with untreated cells. Attached cells appeared to be spread out and larger in size. In some attached cells, outgrowth of neurite could be seen after 2 weeks of exposure to 3 mM PB. DAOY cells showed decreased cell density without cytotoxicity after PB treatment at 1 mM and 3 mM as early as day 4. Cells exposed to 3 mM PB spread out as monolayer and appeared much larger in size. They did not, however, show any evidence of neurite formation (Fig. 1). Concentrations of 10 mM and above were toxic to both cell lines and were therefore not used in subsequent experiments.

Cell Proliferation. There was no significant effect of PA on cell proliferation. PB, however, caused a significant suppression of cell proliferation in a dose-dependent manner, with maximal effect seen at 3 mM (Fig. 2). After an initial suppression of proliferation during the first 3 days of treatment, cells treated with lower PB concentrations (<1 mM) began to proliferate at similar rates as the untreated cells, whereas cells ex-
posed to higher concentrations (3 mM) maintained a decreased rate of proliferation.

With washout experiments, the antiproliferative effect of PB on DAOY cells was found to be reversible even after exposure for 4 weeks. D283-MED cells, however, showed an irreversible suppression of proliferation after 28 days of exposure to 3 mM PB. There was no pronounced effect of PB on cell viability in the first 2 weeks in either DAOY or D283-MED cells. About 80% or more of the cells were viable at the end of 2 weeks of exposure. With continuous exposure, however, the cell viability decreased significantly, such that at the end of 4 weeks of exposure to PB, only ~60% of the cells were viable.

**Protein Expression of Glial Marker GFAP and Neurological Marker SYN.** With immunohistochemical staining, increased expression of SYN induced by both PA and PB became apparent after 2 weeks of exposure. The expression of GFAP, however, was induced only by PB (Fig. 3). In DAOY cells, PB (3 mM) induced the expression of GFAP and SYN beginning as early as 3 days of exposure (data not shown). Both the total number of positive cells and staining intensity increased remarkably after 2 weeks of treatment with 3 mM PB (Fig. 3). PA caused only a marginal increase in GFAP and SYN expression after 2 weeks of exposure at low dose (0.3 mM), but showed similar induction effects as PB at higher dose (3 mM; Table 1).

Western hybridization confirmed the pattern of increased expression of GFAP and SYN proteins in cells treated with PB (3 mM) for up to 28 days (Fig. 4A). GFAP protein was induced in both cell lines, but the response of DAOY cells was much slower than that of D283-MED. The changes of SYN protein level in both cell lines, however, were not as much as GFAP.

**Quantitative RT-PCR Analysis of GFAP and SYN mRNA Expression.** Treatment of PB (3 mM) resulted in a remarkably elevated expression of GFAP and SYN mRNA in both D283-MED and DAOY cells. Compared with ~4-fold increase in DAOY cells after 3 days of exposure, D283-MED cells were more responsive, and the GFAP mRNA elevated >30-fold starting from day 3 and lasted until day 28 (Fig. 5). The overall level of SYN mRNA expression induced by 3 mM PB was lower than that of GFAP (2-fold in DAOY and 8-fold in D283-MED cells) and required longer treatment time.

**Anchorage-Independent Growth.** Anchorage-independent growth has been shown to be a useful in vitro correlate of in vivo tumorigenicity. Both PA and PB caused a significant decrease in the colony-forming efficiency of D283-MED cells in soft agar as compared with the untreated control ($P < 0.01$; Fig. 6). These effects became evident as early as after 6 days of exposure and persisted with continuous exposure. But these effects remained reversible even up to 15 days of treatment. DAOY cells failed to show colony formation in double agar assay.

**Flow Cytometry Analysis.** The increase of cell population in $G_0/G_1$ phase with concomitant decrease of cells in $S$ and $G_2/M$ phases was observed with DAOY cells only after exposure to 3 mM PB for 28 days. Lower concentrations of PB failed to produce significant changes of cell cycle distribution. With D283-MED cells, however, PB produced a more prominent accumulation of cells in $G_0/G_1$ phase with reduced cell population in $S$ and $G_2/M$ phases in a dose- and time-dependent manner. The maximum effect was observed in cells treated with 3 mM PB for 28 days (Fig. 7).

**Heterotransplantation into SCID Mice.** For DAOY cells, 2 or 4 weeks of treatment with 3 mM PB did not alter their tumorigenicity in SCID mice. Tumors were produced in all four of the injected sites (100%). In addition, PB treatment of DAOY cells did not change the latency period, but size of the tumors generated from the treated cells was decreased ($P < 0.001$). Interestingly, DAOY cells treated with PB for 4 weeks grew faster than those treated for only 2 weeks (Fig. 8).

With D283-MED cells, however, both the tumor take and growth of xenografts were significantly decreased, whereas the latency period was increased after treatment with 3 mM PB. The tumor take decreased from 75% (3 of 4) in untreated cells to 25% in cells treated with 3 mM PB for 4 weeks, and the latency period increased from 5 weeks to 10 weeks. The size of tumors generated from cells treated with PB for 4 weeks was remarkably suppressed ($P < 0.01$; Fig. 8).
Detection of Apoptosis. Flow cytometry analysis failed to detect any increase in subdiploid apoptotic cells in DAOY cells treated with lower doses (0.1–1 mM) of PB for up to 14 days (Table 2). Prolonged exposure to 3 mM PB up to 28 days resulted in a dramatic increase of apoptotic cells (16.4%), whereas 0.1 mM PB also caused a slight elevation of apoptotic cells after 28 days of treatment (0.5%). D283-MED was once again found to be more sensitive to PB. After 14 days of treatment, a dose-dependent increase of apoptotic cells was observed. With 28 days of exposure to PB, concentrations as low as 0.1 mM were able to produce significantly more apoptotic cells (22.8%). The results obtained with FCM were additionally confirmed by terminal deoxynucleotidyl transferase-mediated nick end labeling assay, which detected a similar dose-effect relationship that was consistent with the FCM findings after 28 days of treatment (Table 2).

Determination of Histone H3 and H4 Acetylation Status. Accumulation of AcH3 was observed in both D283-MED and DAOY cells. Their response time to 3 mM PB treatment, however, was different. D283-MED cells reacted much faster. The remarkably elevated AcH3 levels could be detected as early as day 3 and maintained until day 28. A similar pattern was observed for AcH4 in D283-MED cells. The accumulation of AcH3 in DAOY cells, however, occurred gradually, and did not reach its peak until day 28. AcH4 levels did not increase in treated DAOY cells (Fig. 4, B and C).

DISCUSSION

There have been ongoing attempts to identify effective differentiation-inducing agents for the treatment of medulloblastomas. None of the agents evaluated thus far, including retinoic acid (35, 36), PA (30), and cyclic AMP (36) was found to induce irreversible differentiation in medulloblastoma cells. In the current study, we have shown the antigrowth and differentiation-induction effects of PB in medulloblastoma cells, similar to those described in many other human malignant tumors (7, 8, 10, 11, 14–18, 20, 28). We also demonstrated for the first time that irreversible antiproliferation and differentiation-inducing effects could be achieved with some medulloblastoma cells.

Reversibility of the effects of differentiation-inducing agents has been one of the obstacles that hinder their clinical applications. As the effects of most differentiation-inducing agents, including PB and PA, on malignant tumor cells are time- and dose-dependent, it has been suggested that higher doses and/or prolonged exposure may lead to irreversible arrest of cell proliferation and/or terminal differentiation (37, 38). For PB, however, most of the published works were based on short-term exposure, ranging from 72 h (39, 40) up to 12 days (11, 16, 18, 19, 31). To address the efficacy of the long-term exposure of medulloblastoma cells to PB, we performed the extensive washout experiments in vitro with cell proliferation assay. By removing PB after the cells had been treated for various periods of time and then monitoring the proliferation of cells in PB-free medium for as long as 60 days, we were able to demonstrate that a minimum of 28 days of continuous treatment with 3 mM PB was required to cause irreversible suppression of cell proliferation in D283-MED cells. This result not only confirmed the feasibility of achieving permanent growth arrest, but also determined the optimal duration of drug exposure. In D283-MED cells, significantly elevated expression of glial (GFAP) and neuronal (SYN) markers both at protein and mRNA levels, remarkable suppression of colony forming efficiency in soft agar, and substantial cell cycle arrest in $G_0/G_1$ phase all suggested that a more differentiated phenotype had been induced. The tumorigenicity assay in SCID mice demonstrated that treatment with 3 mM PB for 28 days resulted in a significantly reduced tumor take and growth rate, as well as the doubling of the latency period. All of these data indicate that the D283-MED
cells have reached a more differentiated phenotype with significantly reduced malignancy.

In addition to inducing cytostasis and differentiation, PB has been found to promote apoptosis in prostate cancer cells (10, 31), neoplastic myeloid cells (17), and malignant B cells (9). Our data show that the antiproliferative effect of PB in D283-MED cells was in parallel with its apoptotic effect, whereas DAOY cells displayed resistance to both the antiproliferative and apoptotic effects. The apoptotic effect of PB on D283-MED cells after 28 days of treatment, however, did not seem to be dose-dependent. In fact, the maximal effect was observed with 0.1 mM PB. One explanation for this phenomenon is that the results obtained at the end of 28-day treatment were the cumulative effects from all 28 days of treatment. Many more cells that were sensitive to higher concentrations such as 3 mM of PB might have died before they were harvested for FCM and

**Table 1**  Expression of GFAP and SYN in D283-MED and DAOY cells treated with PA or PB for 2 weeks

<table>
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<th>Cells</th>
<th>Markers</th>
<th>0 mM</th>
<th>0.3 mM</th>
<th>1 mM</th>
<th>3 mM</th>
<th>0.3 mM</th>
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<tr>
<td>D283-MED</td>
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<td>DAOY</td>
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*GFAP, glial fibrillary acidic protein; SYN, synaptophysin; PA, phenylacetate; PB, phenylbutyrate.*
terminal deoxynucleotidyl transferase-mediated nick end labeling assay, as confirmed by the cell viability decreasing from 80% with 14-day treatment to 60% at the end of 28-day treatment. Our results suggest that apoptosis did play an important role in suppressing the growth of D283-MED cells. Similar results were observed in peripheral primitive neuroectodermal tumors treated with lovastatin in which treated cells underwent apoptosis shortly after the appearance of the differentiated phenotype (41). This suggests that PB may have an additional therapeutic advantage in that if the differentiation program of tumor cells is defective and they cannot proceed further into differentiation, they may be directed to apoptosis.

PB and PA are two closely related aromatic fatty acids. PB is used as a prodrug of PA in the treatment of urea cycle disorders. Once administered, PB is converted into PA, which conjugates with glutamine \textit{in vivo} to form a water-soluble compound, phenylacetylglutamine, that is excreted in urine (21, 22). Mixed results have been reported in comparing the potency of PB and PA in malignant tumors; equal or similar effects were found in various tumors, including malignant B cells, melanoma, and cancer cells from prostate, colon, and breast (8, 9, 20, 28). Our data, however, showed that PB is much more potent than PA in terms of their effects on cell morphology, cell proliferation, and expression of GFAP and SYN in medulloblastoma, which is consistent with many other previous reports (16, 17, 31). In their studies of the effects of PA in brain tumor cell lines, Stockhammer \textit{et al.} (30) did not observe significant morphological changes in DAOY and D283-MED cells when they were treated with PA. Our results support the notion that in medulloblastoma, PA and PB may have distinct pharmacological properties (21) and PB may induce an effect at the molecular level before it is metabolized to PA (31).

DAOY and D283-MED cells have been shown to express different markers of cell differentiation, and they posses different biological properties \textit{in vitro}. D283-MED cells are believed to be more differentiated than DAOY cells (32, 42, 43). They were selected in our study to evaluate whether the responsiveness of medulloblastoma cells to PB is related to their initial degree of differentiation. Our data showed that DAOY cells are more resistant to PB. When compared with D283-MED cells, DAOY cells showed no irreversible arrest of cell proliferation by PB, or G_0/G_1 arrest, and the suppression of tumorigenicity was less significant. In a previous report, the effect of PB was found to vary among different glioma cell lines (29). Our results are in agreement with the findings in melanoma cells and colon carcinoma cells in which the ability of PB to induce cytostasis and terminal differentiation correlated with the degree of maturation before treatment (18, 28). These observations warrant future search for more effective agents and/or modified treatment strategies for those very immature tumor cells that are resistant to current treatment of PB.

The mechanisms by which PA and PB inhibit proliferation and induce differentiation remain unknown. It was postulated that the depletion of glutamine could play a role in inhibiting the growth of tumor cells (23), but the results obtained in mouse erythroleukemia cells and human leukemic cells did not support terminal deoxynucleotidyl transferase-mediated nick end labeling assay, as confirmed by the cell viability decreasing from 80% with 14-day treatment to ~60% at the end of 28-day treatment. Our results suggest that apoptosis did play an important role in suppressing the growth of D283-MED cells. Similar results were observed in peripheral primitive neuroectodermal tumors treated with lovastatin in which treated cells underwent apoptosis shortly after the appearance of the differentiated phenotype (41). This suggests that PB may have an additional therapeutic advantage in that if the differentiation program of tumor cells is defective and they cannot proceed further into differentiation, they may be directed to apoptosis.

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Fig. 4 Western hybridization on cells treated with phenylbutyrate (PB; 3 mm) from 0 to 28 days. \(A\), elevated protein expression of GFAP was more potent than SYN in both cell lines. D283-MED cells were more responsive than DAOY cells. \(B\), PB induced AcH3 in both cell lines. Increased AcH4, however, was observed only in PB-responsive D283-MED cells. \(C\), expression of β-tubulin was included as loading control.

Fig. 5 Expression of GFAP (top) and SYN (bottom) mRNA as detected by real-time reverse transcription-PCR in D283-MED and DAOY cells during the treatment of phenylbutyrate (3 mm). In D283-MED cells, both GFAP and SYN mRNA levels were remarkably elevated \((P < 0.01)\). In DAOY cells, significantly increased expression was only observed in GFAP mRNA; bars, ±SD.
this hypothesis (44). Recent findings suggest that PB may act by inhibiting histone deacetylase so as to impact histone acetylation, which, in turn, alters the structure of chromatin, modulating binding activity of transcriptional regulators (19, 44, 45). Our data showed that PB induced increased histone H3 acetylation in both cell lines, but histone H4 acetylation only in D283-MED and not in DAOY cells. This combined with the differential responsiveness of the two cell lines, i.e., D283-MED being more sensitive and DAOY being resistant, toward PB treatment lead us to postulate that it is the hyperacetylation of histone H4 that played a more important role in determining drug responsiveness. Numerous candidate genes and pathways affected by PA and PB have been identified. PB regulates the G1 phase growth arrest through p21\textsuperscript{Waf1/Cip1/Shi1} pathway (40) and Rb hypophosphorylation (16). In colon cancer cells, PB treatment also resulted in an increase in the expression of p130 and p107, which was associated with increased binding and subsequent inactivation of E2F, a transactivator of genes regulating cell cycle transition (39, 46). PB treatment was also found to induce peroxisome proliferator-activated receptor activation

Fig. 6  Anchorage-independent growth of D283-MED cells in soft agar. Cells were pretreated with either phenylacetate (PA) or phenylbutyrate (PB; 0–3 mM) for 3, 7, 14, and 28 days, and plated in double-layer agar that contained identical doses of PA or PB in both layers. Both PA and PB resulted in a significant suppression of colony formation in a time- and dose-dependent manner (P < 0.01); bars, ±SD.

Fig. 7  Effects of 4 weeks of phenylbutyrate treatment on cell cycle distribution in DAOY and D283-MED cells. A more consistent shift of the cell population toward quiescence was observed in D283-MED cells as demonstrated by an increased fraction of cells in G0/G1 phase and a concomitant decrease of cells in S and G2/M phases; bars, ±SD.

Fig. 8  Growth of DAOY and D283-MED cells in SCID mice following treatment with 3 mM phenylbutyrate (PB) for 4 weeks. For each group, tumor cells were inoculated s.c. into a total of four sites (2 mice). The number of tumors in parentheses represents tumor take based on four injected sites. Significant suppression of tumorigenicity was produced by PB in D283-MED cells in terms of tumor take, latency period, and size of tumors (P < 0.01); bars, ±SD.
Table 2 Detection of apoptotic cells by TUNEL-assay and FCM in D283-MED and DAOY cells treated with PA or PB for 2–4 weeks

<table>
<thead>
<tr>
<th>Cells</th>
<th>FCM (%)</th>
<th>TUNEL assay (%)</th>
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<tbody>
<tr>
<td></td>
<td>2 weeks</td>
<td>4 weeks</td>
</tr>
<tr>
<td>D283-MED</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D283-MED</td>
<td>0.1</td>
<td>0</td>
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<tr>
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</tr>
<tr>
<td>D283-MED</td>
<td>1.0</td>
<td>9.9 ± 1.2</td>
</tr>
<tr>
<td>D283-MED</td>
<td>3.0</td>
<td>11.9 ± 2.3</td>
</tr>
<tr>
<td>DAOY</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DAOY</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>DAOY</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
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<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>DAOY</td>
<td>3.0</td>
<td>0</td>
</tr>
</tbody>
</table>

*TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PB, phenylbutyrate; PA, phenylacetate; FCM, flow cytometry analysis.

(47); the activated peroxisome proliferator-activated receptor-γ results in decreased E2F-DNA binding that is independent of pRb phosphorylation (48). PB-induced apoptosis in prostate cancer cells was associated with the repression of Bcl-2 and up-regulation of Bax and Fas proteins, and was independent of the p53 status (49). With the recently developed high-density DNA microarray, we have performed the gene expression profiling of D283-MED and DAOY cells treated with PB (3 mM) for 0, 3, 7, 14, and 28 days to additionally demonstrate the molecular mechanisms that underly the different responsiveness of the two cell lines. A total of 815 differentially expressed genes were identified in D283-MED cells, as compared with 207 genes in DAOY cells. Gene associated with more differentiated phenotype, such as GFAP, SYN, gama-aminobutyric acid receptor, and dopamine receptors were identified in PB-responsive D283-MED cells. These data suggest that D283-MED and DAOY cells could be used as a functional model system to additionally elucidate the molecular mechanisms of action of PB.

REFERENCES


Phenylbutyrate and Phenylacetate Induce Differentiation and Inhibit Proliferation of Human Medulloblastoma Cells

Xiao-Nan Li, Suhag Parikh, Qin Shu, et al.


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