Advances in Brief

Selective Activity of Phenylacetate against Malignant Gliomas: Resemblance to Fetal Brain Damage in Phenylketonuria

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Abstract

Phenylacetate, a deaminated metabolite of phenylalanine, has been implicated in damage to immature brain in phenylketonuria. Because primary brain tumors are highly reminiscent of the immature central nervous system, these neoplasms should be equally vulnerable. We show here that sodium phenylacetate can induce cytostasis and reversal of malignant properties of cultured human glioblastoma cells, when used at pharmacological concentrations that are well tolerated by children and adults. Tumor cells exhibited biochemical alterations similar to those observed in phenylketonuria-like conditions, including selective decline in de novo cholesterol synthesis from mevalonate. Because gliomas, but not mature normal brain cells, are highly dependent on mevalonate for production of steroids and isoprenoids vital for cell growth, sodium phenylacetate would be expected to affect tumor growth in vivo while sparing normal tissues. Systemic treatment of rats bearing intracranial gliomas resulted in significant tumor suppression with no apparent toxicity to the host. The data indicate that phenylacetate, acting through inhibition of protein prenylation and other mechanisms, may offer a safe and effective novel approach to treatment of malignant gliomas and perhaps other neoplasms as well.

Introduction

Malignant glioma, the most common primary brain cancer, affects patients of all ages. The median survival of adults with high-grade gliomas is <1 year, despite aggressive treatments that include surgical resection, radiotherapy, and cytotoxic chemotherapy (1). We describe here a novel nontoxic treatment approach based on the discovery of phenylacetate as an antitumor agent with selective activity against undifferentiated glial cells (2-4).

Phenylacetate, a common metabolite of phenylalanine, controls growth and differentiation in diverse organisms throughout phylogeny (5-7). In human plasma, phenylacetate is normally found in micromolar concentrations. We have recently shown that, when used at higher (millimolar) levels, NaPA, can induce selective cytostasis and reversal of malignancy of cultured leukemic cells and hormone-refractory prostate carcinoma (2, 8, 9). Clinical experience, obtained during phenylacetate treatment of children with urea cycle disorders, indicates that millimolar levels can be achieved without significant adverse effects (10, 11). The lack of neurotoxicity in these patients is, however, in marked contrast to the severe brain damage documented in PKU, an inborn error of phenylalanine metabolism associated with excessive production of phenylacetate, microcephaly, and mental retardation (12). The differences in clinical outcome can be explained by the fact that, although phenylacetate readily crosses the blood-brain barrier in both prenatal and postnatal life, neurotoxicity is limited to the immature brain (3, 4, 12-14). Compelling evidence for a developmentally restricted window of susceptibility is provided by the phenomenon of “maternal PKU syndrome”; female patients with PKU who are diagnosed early and maintained on a phenylalanine-restricted diet develop normally and subsequently tolerate a regular diet. These women often give birth to genetically normal but mentally retarded infants due to the untreated maternal PKU; the elevated levels of circulating phenylacetate, while sparing the mature tissues of the mother, are detrimental to the fetal brain (13, 14). The primary pathological changes in PKU involve rapidly developing glial cells and are characterized by alterations in lipid metabolism and myelination, with subsequent neuronal dysfunction. The vulnerable fetal glial tissues resemble neoplastic glial cells in numerous molecular and biochemical aspects, including unique dependence on MVA metabolism for synthesis of steroids and isoprenoids critical to cell replication (15-17) and on circulating glutamate as the nitrogen donor for DNA, RNA, and protein synthesis (18, 19). The hypothesis underlying our studies was that phenylacetate, which is known to conjugate and deplete serum glutamine in humans (11) and to inhibit the MVA pathway in circulating glutamate, while sparing the mature tissues of the mother, are detrimental to the fetal brain (13, 14). The primary pathological changes in PKU involve rapidly developing glial cells and are characterized by alterations in lipid metabolism and myelination, with subsequent neuronal dysfunction. The vulnerable fetal glial tissues resemble neoplastic glial cells in numerous molecular and biochemical aspects, including unique dependence on MVA metabolism for synthesis of steroids and isoprenoids critical to cell replication (15-17) and on circulating glutamate as the nitrogen donor for DNA, RNA, and protein synthesis (18, 19). The hypothesis underlying our studies was that phenylacetate, which is known to conjugate and deplete serum glutamine in humans (11) and to inhibit the MVA pathway in immature brain (20-22), might attack these critical control points in malignant gliomas. The efficacy of phenylacetate was demonstrated using both in vitro and in vivo tumor models.

Materials and Methods

Cell Cultures and Reagents. Human glioblastoma cell lines were purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, antibiotics, and 2 mM L-glutamine, unless otherwise specified. Human umbilical vein endothelial cells, isolated from freshly obtained cords, were provided by D. Grant and H. Kleinman (NIH, Bethesda, MD). Sodium salts of phenylacetic acid and of phenylbutyric acid were provided by Elan Pharmaceutical Corporation (Gainesville, GA). Phenylacetylglutamine was a gift from S. Brusilow (Johns Hopkins University, Baltimore, MD).

Evaluation of Cell Replication and Viability. Growth rates were determined by an enzymatic assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) (23), by cell enumeration with a hemocytometer following detachment with trypsin/EDTA, and by measurement of thymidine incorporation into DNA (see below). The different assays produced essentially the same results. Cell viability was assessed by trypan blue exclusion.

Colonies were formed in monolayer cultures in RPMI 1640 medium supplemented with 10% FCS, and assessed in parallel by trypan blue exclusion.

Invasion through Matrigel. Tumor cells were treated with trypsin/EDTA, resuspended in growth medium containing 0.36% agar, and placed onto a base layer of solid agar (0.9%) in the presence or absence of drugs. Colonies composed of 30 or more cells were scored after 3 weeks. The capacity of cells to invade a reconstructed basement membrane was determined using a Biocoat Matrigel invasion chamber (Becton Dickinson Labware, Bedford MA).

Immunocytochemistry. Cells were immunostained with antivimentin monoclonal antibodies using Dako peroxidase-antiperoxidase kit K537 (Dako Corporation, Carpenteria, CA).

Received 11/11/93; accepted 1/5/94.

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3 The abbreviations used are: NaPA, sodium phenylacetate; NaPB, sodium phenylbutyrate; MVA, mevalonate; PKU, phenylketonuria; IC50, inhibitory concentration causing 50% reduction in cell proliferation.
medium and were labeled with (RS)-[2\textsuperscript{J4C}]mevalonate (16 \textmu Ci/ml; specific activity, 15 \mu Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) during the final 15 h of treatment. Total cell proteins were extracted, resolved on 10% sodium dodecyl sulfate-polyacrylamide gels, and stained with Coomassie Brilliant Blue. Gels were then dried and exposed to Kodak X-Omat film for 4 days.

Animal Studies. To determine the effect of phenylacetate on the tumorigenic phenotype of human glioblastoma cells, cultures were pretreated for 1 week, harvested, resuspended in medium containing 30% Matrigel (Collaborative Biomedical Products, Bedford, MA), and transplanted s.c. (2.5 \times 10^6 cells/site) into 5-week-old female athymic mice (Division of Cancer Treatment, National Cancer Institute Animal Program, Frederick Cancer Research Facility). To further evaluate drug efficacy in vivo, Fischer 344 rats received a stereotaxic inoculation of syngeneic 9L gliosarcoma cells (4 \times 10^5) into the deep white matter of the right cerebral hemisphere, as described previously (24, 25). The animals were then subjected to 2 weeks of continuous treatment with sodium phenylacetate (550 mg/kg/day s.c.), using osmotic minipumps transplanted s.c. In control rats the minipumps were filled with saline. Statistical analysis of data was by Fisher’s exact test.

Results and Discussion

Induction of Cytostasis and Phenotypic Reversion in Cultured Human Glioblastoma Cells. Treatment of glioblastoma cells with NaPA resulted in time- and dose-dependent growth arrest (Fig. 1A), accompanied by similarly diminished DNA synthesis. After 4–6 days of continuous treatment with 4 mM NaPA, there was approximately 50% inhibition of growth in U87, A172, U373, U343, and HS683 cultures (IC\textsubscript{50}  4.4 \pm 0.6 mM). Reflecting the heterogeneous nature of tumor cell responses, glioblastoma U251 and U138 cells were less sensitive to NaPA, exhibiting 50% inhibition of growth at concentrations of 6 and 10 mM, respectively. At these concentrations, NaPA was also toxic to the normal human umbilical vein endothelial cells (HUVC).

Measurement of Cholesterol, Protein, and DNA Synthesis. For studies of steroid synthesis, cells were labeled for 24 h with 5 \times 10^6 dpm of [\textsuperscript{3H}]mevalonate (35 Ci/mmol; New England Nuclear, Boston, MA) in growth medium containing 3 \mu M lovastatin and 0.5 mM unlabeled mevalonate. In the presence or absence of 6 mM phenylacetate or 2.5 mM phenylbutyrate, cellular sterols were extracted with hexane and separated by silica thin layer chromatography. Reduction in cell number paralleled changes in \textit{de novo} DNA synthesis (data not shown). B, selective cytostasis induced by NaPA (5 mM) combined with glutamine starvation (0.2 mM glutamine, \textit{i.e.}, 2–4-fold below the normal plasma levels). The results indicate increased vulnerability of glioblastoma A172, compared to actively replicating normal human umbilical vein endothelial cells (HUVC). Cell viability was >95% in all cases.

Analysis of Protein Isoprenylation. Cell cultures were incubated for 24 h with 10 mM phenylacetate or 2.5 mM phenylbutyrate in complete growth medium and were labeled with (RS)-[\textsuperscript{14C}]mevalonate (16 \mu Ci/ml; specific activity, 15 \mu Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) during the final 15 h of treatment. Total cell proteins were extracted, resolved on 10% sodium dodecyl sulfate-polyacrylamide gels, and stained with Coomassie Brilliant Blue. Gels were then dried and exposed to Kodak X-Omat film for 4 days.

Fig. 1. Growth arrest of human glioblastoma cells. A, dose-dependent inhibition by NaPA of human glioblastoma cell proliferation. Growth rates were determined, after 4–5 days of continuous treatment, by an enzymatic assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and were confirmed by cell enumeration with a hemocytometer. Reduction in cell number paralleled changes in \textit{de novo} DNA synthesis (data not shown). B, selective cytostasis induced by NaPA (5 mM) combined with glutamine starvation (0.2 mM glutamine, \textit{i.e.}, 2–4-fold below the normal plasma levels). The results indicate increased vulnerability of glioblastoma A172, compared to actively replicating normal human umbilical vein endothelial cells (HUVC). Cell viability was >95% in all cases.

Fig. 2. Immunocytochemical analysis of vimentin in phenylacetate-treated human glioblastoma U87 cells. A, light microscopy (\times 200) of untreated U87 cells subjected to immunostaining with anti-vimentin monoclonal antibodies. Note formation of multilayered “tumoroids” composed of highly refractile spindle cells with poor cytoskeletal organization. B, U87 cells exposed to 5 mM phenylacetate for 1 week. Treated cells stained for vimentin display altered morphology and cytoskeletal filament patterns. These changes, confirmed by immunolabeling for glial fibrillary acidic protein (data not shown), are consistent with cell maturation and correlate with reduced proliferative capacity and regained contact inhibition of growth.
sensitive, with IC\textsubscript{50} values of 8–10 mM. Further studies, mimicking pharmacological conditions that are expected in patients, involved exposure of cells to phenylacetate in glutamine-depleted medium; these conditions completely blocked glioblastoma cell growth but had little effect on the replication of normal endothelial cells (Fig. 1B). Phenylbutyrate, an intermediate metabolite of phenylacetate formed in the brain by fatty acid elongation (26), also inhibited tumor cell replication (IC\textsubscript{50} 2.2 ± 0.2 mM in A172, U87, and U373 cells), while the end metabolite, phenylacetylglutamine, was inactive. In addition to inducing selective tumor cytostasis, both NaPA and NaPB promoted cell maturation and reversion to a nonmalignant phenotype, as manifested by an altered pattern of cytoskeletal intermediate filaments (Fig. 2), loss of anchorage independence and invasive capacity, and reduced tumorigenicity in athymic mice (Table 1). These profound changes in tumor behavior were accompanied by alterations in the expression of genes implicated in growth control, angiogenesis, and immunosuppression (e.g., basic fibroblast growth factor and transforming growth factor β2).4

**Phenylacetate Inhibition of the Mevalonate Pathway and Protein Isoprenylation.** The most consistent biochemical change observed in glial cells exposed to phenylacetate involved alterations in lipid metabolism and inhibition of the MVA pathway (see diagram in Fig. 3A). Active de novo synthesis of cholesterol and isoprenoids from precursors such as acetyl-CoA and MVA is an important feature of the developing brain (but not the mature brain), coinciding with myelination (17). It is also a hallmark of malignant gliomas (16, 27, 28). Cholesterol production and protein isoprenylation diminished within 24 h of glioblastoma treatment with either NaPA or NaPB (Fig. 3), preceding changes in DNA and total protein synthesis, which were detectable after 48 h. The reduction in isoprenylation was paralleled by a decrease in MVA decarboxylation (to <50% of control), an effect previously observed in embryonic brain in PKU-like conditions (20, 21). MVA-5-pyrophosphate decarboxylase, a key enzyme regulating cholesterol synthesis in brain, is inhibited by phenylacetate under conditions in which MVA kinase and MVA-5-phosphate kinase are only minimally affected (22). Phenylacetate and its metabolite phenylacetyl-CoA might also interfere with MVA synthesis from acetyl-CoA (16, 18). Glioblastoma cells could not, however, be rescued by exogenous MVA (0.3–3 mM), suggesting that MVA utilization, rather than its synthesis, is the prime target.

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**Table 1 Reversal of malignancy of human glioblastoma cells**

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Clonogenicity in soft agar (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Invasion through Matrigel (% of control)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tumor incidence (positive/infected sites)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.1</td>
<td>100</td>
<td>9/10</td>
</tr>
<tr>
<td>NaPA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 mM</td>
<td>0.5</td>
<td>51.8</td>
<td>ND</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>&lt;0.01</td>
<td>18.9</td>
<td>ND</td>
</tr>
<tr>
<td>NaPB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.75 mM</td>
<td>ND</td>
<td>44.6</td>
<td>ND</td>
</tr>
<tr>
<td>1.25 mM</td>
<td>0.15</td>
<td>17.5</td>
<td>ND</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>&lt;0.01</td>
<td>ND</td>
<td>1/10</td>
</tr>
</tbody>
</table>

<sup>a</sup> U87 cells were detached with trypsin/EDTA, resuspended in growth medium containing 0.36% agar, and placed onto a base layer of solid agar (0.9%) in the presence or absence of drugs. Colonies composed of 30 or more cells were scored after 3 weeks.

<sup>b</sup> A172 cells that had been pretreated for 3 days were detached and assayed for their ability to invade a reconstituted basement membrane, using a Matrigel Invasion Chamber, according to the manufacturer’s instructions. Under the experimental conditions used, 2–3% of the untreated A172 cells invaded the Matrigel within 24 h.

<sup>c</sup> U87 cells that had been pretreated in culture for 1 week were harvested, resuspended in medium containing 30% Matrigel, and transplanted s.c. into 5-week-old female athymic mice (2.5 × 10⁶ cells/mouse). Data were recorded 5 weeks after cell inoculation.

<sup>d</sup> ND, not determined.

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Mevalonate is a precursor of several isopentenyl moieties required for progression through the cell cycle, such as sterols, dolichol, the side chains of ubiquinone and isopentenyladenine, and prenyl groups that modify a small set of critical proteins (29–31). The latter include plasma membrane G and G-like proteins (e.g., ras) involved in mitogenic signal transduction (M, 20,000–26,000), the myelination-
related enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase, and nuclear envelope lamins that play a key role in mitosis (M, 44,000-74,000). Inhibition of sterol and isoprenoid synthesis during rapid development of the brain could lead to the microcephaly and impaired myelination seen in untreated PKU. Targeting MVA in dedifferentiated malignant gliomas, on the other hand, would be expected to inhibit tumor growth in vivo without damaging the surrounding normal tissues, because the MVA pathway is significantly less active in mature brain (17).

Activity of Phenylacetate in Experimental Gliomas in Rats. To evaluate the in vivo antitumor effect of NaPA, Fisher rats were inoculated with stereotaxic intracerebral injection of syngeneic 9L gliosarcoma cells. This tumor model is known for its aggressive growth pattern, which results in nearly 100% mortality of rats within 3–4 weeks (24, 25). NaPA was continuously administered by implanted s.c. osmotic minipumps that delivered a clinically achievable dose of 550 mg/kg/day. Systemic treatment for 2 weeks of rats bearing intracranial glioma cells markedly suppressed tumor growth (P < 0.05) (Table 2; Fig. 4), with no detectable adverse effects. Further studies in experimental animals indicate that phenylacetate (plasma and cerebrospinal fluid levels of 2–3 μM) induces tumor cell maturation in vivo and significantly prolongs survival.5

Summary and Perspective. Phenylacetate has long been implicated in damage to the developing fetal brain. Because primary central nervous system tumors are highly reminiscent of immature fetal brain, malignant gliomas should be equally vulnerable. Moreover, viewing maternal PKU syndrome as a natural human model, phenylacetate would be expected to suppress the growth of brain neoplasms without harming normal tissues. Our experimental data support this hypothesis. Phenylacetate induced selective cytostasis and promoted maturation of glioma cells in vitro and in vivo; premature growth arrest and differentiation could also underlie the damage to fetal brain in PKU. Multiple mechanisms of action might be involved, including inhibition of protein isoprenylation and depletion of plasma glutamine in humans. The demonstrable antitumor activity, lack of toxicity, and ease of administration (p.o. or i.v.) suggest potential clinical efficacy of phenylacetate in the management of malignant gliomas. Ongoing phase I clinical studies with phenylacetate in the treatment of adults with cancer indicate that therapeutic levels can be achieved in the plasma and cerebrospinal fluid with no significant toxicities and result in some clinical improvement in some patients with high-grade gliomas.6

Acknowledgments

We thank V. Sykes for outstanding technical assistance, S. Brusilow for phenylacylglutamine, and D. Grant and H. Kleinman for providing endothelial cell cultures.

This paper is dedicated to the memory of Beni Amdurski.

References


Phenylacetate activity in experimental brain cancer

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