

# Growth Inhibition, Tumor Maturation, and Extended Survival in Experimental Brain Tumors in Rats Treated with Phenylacetate<sup>1</sup>

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## ABSTRACT

Phenylacetate is a naturally occurring plasma component that suppresses the growth of tumor cells and induces differentiation *in vitro*. To evaluate the *in vivo* potential and preventive and therapeutic antitumor efficacy of sodium phenylacetate against malignant brain tumors, Fischer 344 rats ( $n = 50$ ) bearing cerebral 9L gliosarcomas received phenylacetate by continuous s.c. release starting on the day of tumor inoculation ( $n = 10$ ) using s.c. osmotic minipumps (550 mg/kg/day for 28 days). Rats with established brain tumors ( $n = 12$ ) received continuous s.c. phenylacetate supplemented with additional daily i.p. dose (300 mg/kg). Control rats ( $n = 25$ ) were treated in a similar way with saline. Rats were sacrificed during treatment for electron microscopic studies of their tumors, *in vivo* proliferation assays, and measurement of phenylacetate levels in the serum and cerebrospinal fluid.

Treatment with phenylacetate extended survival when started on the day of tumor inoculation ( $P < 0.01$ ) or 7 days after inoculation ( $P < 0.03$ ) without any associated adverse effects. In the latter group, phenylacetate levels in pooled serum and cerebrospinal fluid samples after 7 days of treatment were in the therapeutic range as determined *in vitro* (2.45 mM in serum and 3.1 mM in cerebrospinal fluid). Electron microscopy of treated tumors demonstrated marked hypertrophy and organization of the rough endoplasmic reticulum, indicating cell differentiation, in contrast to the scant and randomly distributed endoplasmic reticulum in tumors from untreated animals. In addition, *in vitro* studies demonstrated dose-dependent inhibition of the rate of tumor proliferation and restoration of anchorage dependency, a marker of phenotypic reversion.

Phenylacetate, used at clinically achievable concentrations, prolongs survival of rats with malignant brain tumors through induction of tumor differentiation. Its role in the treatment of brain tumors and other cancers should be explored further.

## INTRODUCTION

Phenylacetate is a common metabolite of phenylalanine that is present in human plasma in micromolar concentrations. High concentrations (millimolar) of the sodium salt of phenylacetate, NaPA,<sup>3</sup> induce cytostasis and reversal of malignant phenotypes in a variety of cultured human cancer cells, including malignant glioma (1-4). Clinical experience from patients treated with NaPA for inborn errors of urea synthesis and hyperammonemia (5, 6) indicate that millimolar concentrations are easily attained and well tolerated by children and adults.

The exact mechanism of action of phenylacetate is unclear but may stem from its ability to deplete plasma glutamine, which is essential for the growth of malignant but not normal cells (1, 4). Another possible mechanism is inhibition of the mevalonate pathway in glioma cells (4). Neoplastic cells depend upon mevalonate metabolism to

synthesize sterols and isoprenoids that are critical to cell replication (7, 8). Phenylacetate also constitutes a component of some complex mixtures used to treat cancer patients (9, 10).

We evaluated the *in vitro* and *in vivo* antitumor efficacy of NaPA in a rat model of malignant gliosarcoma. The results indicate that phenylacetate induces tumor maturation, prevents tumor growth, and has a significant antitumor effect in established malignant 9L gliosarcoma in rats.

## MATERIALS AND METHODS

### *In Vitro* Studies

**Cell Proliferation.** The effect of NaPA on cell proliferation was evaluated using [<sup>3</sup>H]thymidine incorporation assay on cultured 9L gliosarcoma cells and cell enumeration using a hemocytometer following detachment with trypsin-EDTA. 9L is a syngeneic malignant glial tumor derived from Fischer 344 rats and is associated with 100% mortality within 3- to 4-weeks after intracerebral inoculation (11). Tumor cells were plated at  $5 \times 10^4$  tumor cells/well in 24-well plates (Costar, Cambridge, MA) in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 2 mM L-glutamine (GIBCO BRL, Gaithersburg, MD), 50 units/ml penicillin (GIBCO), 50  $\mu$ g/ml streptomycin (GIBCO), and 2.5  $\mu$ g/ml Fungizone (ICN Biomedicals, Inc., Costa Mesa, CA). After 24 h, the medium was changed, and NaPA (Elan Pharmaceutical Research Corp., Gainesville, GA) was added to the medium at 0, 2.5, 5, and 10 mM for 5 days. Six h before harvest, 0.5  $\mu$ Ci [<sup>3</sup>H]thymidine (ICN Radiochemicals, Irvine, CA) was added to each well. Thymidine incorporation was determined by scintillation counting in triplicates.

**Colony Formation in Semisolid Agar.** Anchorage-independent growth (the ability of cells to form colonies in semisolid agar) is characteristic of malignant glial cells. 9L cells were harvested with trypsin-EDTA and resuspended at  $1.0 \times 10^4$  cells/ml in growth medium containing 0.36% agar (Difco). Two ml of the cell suspension were added to 60-mm plates (Costar) which were precoated with 4 ml of solid agar (0.9%). Phenylacetate was added to the agar at different concentrations (0, 1.25, 2.5, and 5 mM). In a second experiment, 9L cells were grown for 7 days in tissue culture containing 5 mM NaPA. The cells were then transferred, as described, to agar plates without NaPA. Colonies composed of 30 or more cells were counted after 3 weeks.

### 9L Brain Tumor Inoculation and Phenylacetate Administration

Fischer 344 rats ( $n = 50$ ) weighing 230-350 g were anesthetized using i.p. ketamine (90 mg/kg; Fort Dodge Laboratories, Inc., Fort Dodge, IA) and Xylazine (10 mg/kg; Mobay Corp., Shawnee, KS) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Syngeneic 9L gliosarcoma cells ( $4 \times 10^4$ ) in 5  $\mu$ l (Hanks') balanced salt solution were injected into the deep white matter (depth of inoculation, 3.5 mm) of the right cerebral hemisphere using a 10- $\mu$ l Hamilton syringe connected to the manipulating arm of the stereotaxic apparatus. In 10 rats (group 1), phenylacetate was administered by continuous s.c. release of the drug using two 2ML2 osmotic pumps (release rate of 5  $\mu$ l/h for 14 days; Alza Corp., Palo Alto, CA). On the day of tumor inoculation, the pumps were implanted in the s.c. tissue of both flanks. The concentration of the drug in the pumps was 650 mg/ml (total of 2600 mg for both pumps) for a daily dose of 550 mg/kg per rat. The minipumps were replaced after 14 days for a total treatment of 28 days. Fifteen additional rats (group 2) received NaPA as described starting 7 days after intracerebral inoculation of the tumor. Since no more than two pumps could be implanted

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<sup>3</sup> The abbreviations used are: NaPA, sodium phenylacetate; BrdUrd, bromodeoxyuridine; CSF, cerebrospinal fluid.

in the s.c. tissues, to increase the dose of NaPA we empirically chose to add a single daily injection of NaPA (300 mg/kg, i.p.) for 28 days; this dose proved to be nontoxic after i.p. administration to rats (data not shown). Control rats ( $n = 25$ ) received continuous saline from two s.c. 2ML2 osmotic pumps. Perioperative penicillin (100,000 units/kg, i.m.) was given to all rats before implantation of the minipumps. Survival was recorded in each group. Three rats treated for established tumors (group 2) and two control rats were sacrificed 7 days after initiation of NaPA (14 days after tumor inoculation). These were used for electron microscopic studies of treated tumors, *in vivo* proliferation assays, and measurement of NaPA levels in the serum and CSF (see below). Peripheral organs (heart, lung, spleen, liver, kidney, bowel, adrenal, and gonads) were harvested for routine histological examination. Brain specimens were sectioned and stained with hematoxylin and eosin and myelin stains (Luxol-fast blue) to assess drug-related toxicity.

### Electron Microscopy

Animals (two NaPA-treated; group 2, one saline-treated) were sacrificed by intracardiac perfusion with 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. Two h later, the fixed brains were washed in buffer and sliced into 1-mm thick coronal sections. The areas containing tumors were further dissected into 1-mm<sup>3</sup> cubes, postfixed with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h, washed in buffer, mordanted *en bloc* with 1% uranyl acetate at pH 5 overnight, and then washed, dehydrated, and embedded in Epon. Thin sections were cut at several levels into each block to ensure greater sampling. Electron micrographs of tumor cells were taken at random for morphology.

### In Vivo Proliferation Assay

One NaPA-treated (group 2) and one saline-treated rat received an i.p. injection of 9 mg/3 ml of BrdUrd (Amersham, IL) 14 days after tumor inoculation and 7 days after initiation of treatment. Two h later, the rats were sacrificed, and the brains were removed and sectioned. Mouse anti-BrdUrd monoclonal antibodies were used for immunostaining of the tissues, which were then counterstained with hematoxylin. Tumor cells in 10 high-power fields were enumerated in each specimen, and the percentage of positively staining cells (indicating incorporation of BrdUrd during active cell division) was recorded.

### Measurement of NaPA Levels in Serum and CSF

Three NaPA-treated and two saline-treated rats were sacrificed after 7 days of combined s.c. and i.p. NaPA or saline administration. Animals were sacrificed 18 h after the last i.p. supplement injection of NaPA. Blood was drawn from the heart, and CSF was aspirated from the cisterna magna. Due to volume limitations of CSF, pooled serum, and CSF samples were assessed. A standard curve was generated by adding known amounts of NaPA to a commercial preparation of pooled serum. Standard values spanned a range of serum and CSF concentrations (0–1500  $\mu\text{g/ml}$ ). Protein extraction of a 200- $\mu\text{l}$  aliquot of fluid was carried out with 100  $\mu\text{l}$  of a 10% perchloric acid solution. Supernatant (150  $\mu\text{l}$ ) was neutralized with 25  $\mu\text{l}$  of 20% potassium bicarbonate and centrifuged. Supernatant (125  $\mu\text{l}$ ) was then pipeted into sampling tubes. Chromatography was performed on a Gilson 715 high-performance liquid chromatography system using a 30-cm Waters C18 column (internal diameter, 3.9 mm) at 60°C. A 75- $\mu\text{l}$  injectate was eluted with an acetonitrile/water gradient ranging from 5 to 30% over 20 min and flowing at 1 ml/min. UV monitoring was performed at a wavelength of 208 nm. Elution time for phenylacetate was 14.8 min. The lower limit of detection for NaPA in our assay was 5  $\mu\text{g/ml}$ , and the interassay coefficient of variation (for the tested range of concentrations) was less than 6%.

### Statistical Analysis

The Mantel-Haenszel test (12) was used to compare survival between NaPA-treated and saline-treated rats in the survival experiments.

The animal protocol was reviewed and approved by the NIH Animal Care and Use Committee. The procedures were performed in accordance with the NIH guidelines for the care of laboratory animals.

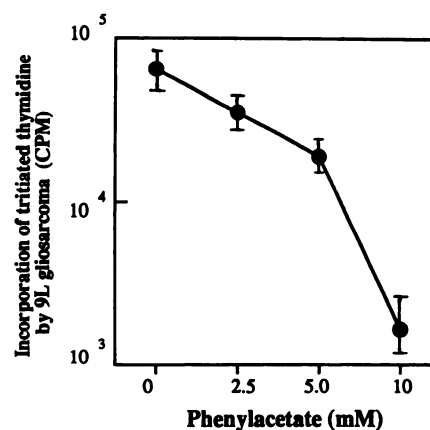


Fig. 1. Effects of phenylacetate on DNA synthesis after *in vitro* exposure of 9L tumor cells to various concentrations of phenylacetate for 5 days. A significant concentration-related decline in DNA synthesis occurred. Points, cpm; bars, SD.

Table 1 Effect of phenylacetate on anchorage-independent growth of 9L gliosarcoma cells

Treatment in culture	Phenylacetate in agar (mM)	Colony Formation	
		No. of colonies	% inhibition
none	0	628 $\pm$ 50	—
none	1.25	326 $\pm$ 20	48.0
	2.5	111 $\pm$ 13	82.4
	5	8 $\pm$ 4	98.7
Phenylacetate <sup>a</sup>	0	375 $\pm$ 25	40.3

<sup>a</sup> 9L cells were treated with 5 mM phenylacetate in culture for 7 days before being plated on soft agar.

## RESULTS

### In Vitro Studies

**In Vitro Effect of NaPA on Cell Proliferation and Anchorage Dependency.** Treatment of 9L cells with NaPA for 5 days resulted in a dose-dependent decrease in [<sup>3</sup>H]thymidine incorporation (Fig. 1). In addition, phenylacetate induced a dose-dependent decrease in anchorage-independent growth. Almost complete loss of the ability to form colonies in soft agar was observed at NaPA concentrations that produced only a partial (<50%) decrease in DNA synthesis (Fig. 1). These data are consistent with *in vitro* reversion of the malignant phenotype (Table 1). 9L cells that were exposed to NaPA for 7 days before plating in agar that did not contain NaPA still showed >40% inhibition in colony formation (Table 1).

### In Vivo Studies

**In Vivo Proliferation Assay and Electron Microscopy Findings.** Treatment of the established brain tumor with NaPA decreased the rate of proliferation. In the tumor from an animal treated with NaPA, 285 of 1283 tumor cells stained for BrdUrd, compared to 429 of 1347 tumor cells in a saline-treated animal (mitotic index of 0.22 in NaPA-treated versus 0.33 in saline-treated tumors).

Electron microscopy of these tumors (Fig. 2, a and b) showed a striking abundance of well-organized rough endoplasmic reticulum in the cells of the NaPA-treated tumor (Fig. 2, a and b), indicating a higher degree of cell differentiation. (13) By contrast, the untreated tumor generally had scant rough endoplasmic reticulum and numerous polyribosomes (Fig. 2, c and d), which are characteristics of highly malignant cells. In addition, mitotic cells were more frequently found in untreated tumors.

**Simultaneous Tumor Inoculation and Administration of NaPA.** Seven of 10 NaPA-treated rats (550  $\mu\text{g/kg/day}$ ) survived for >12

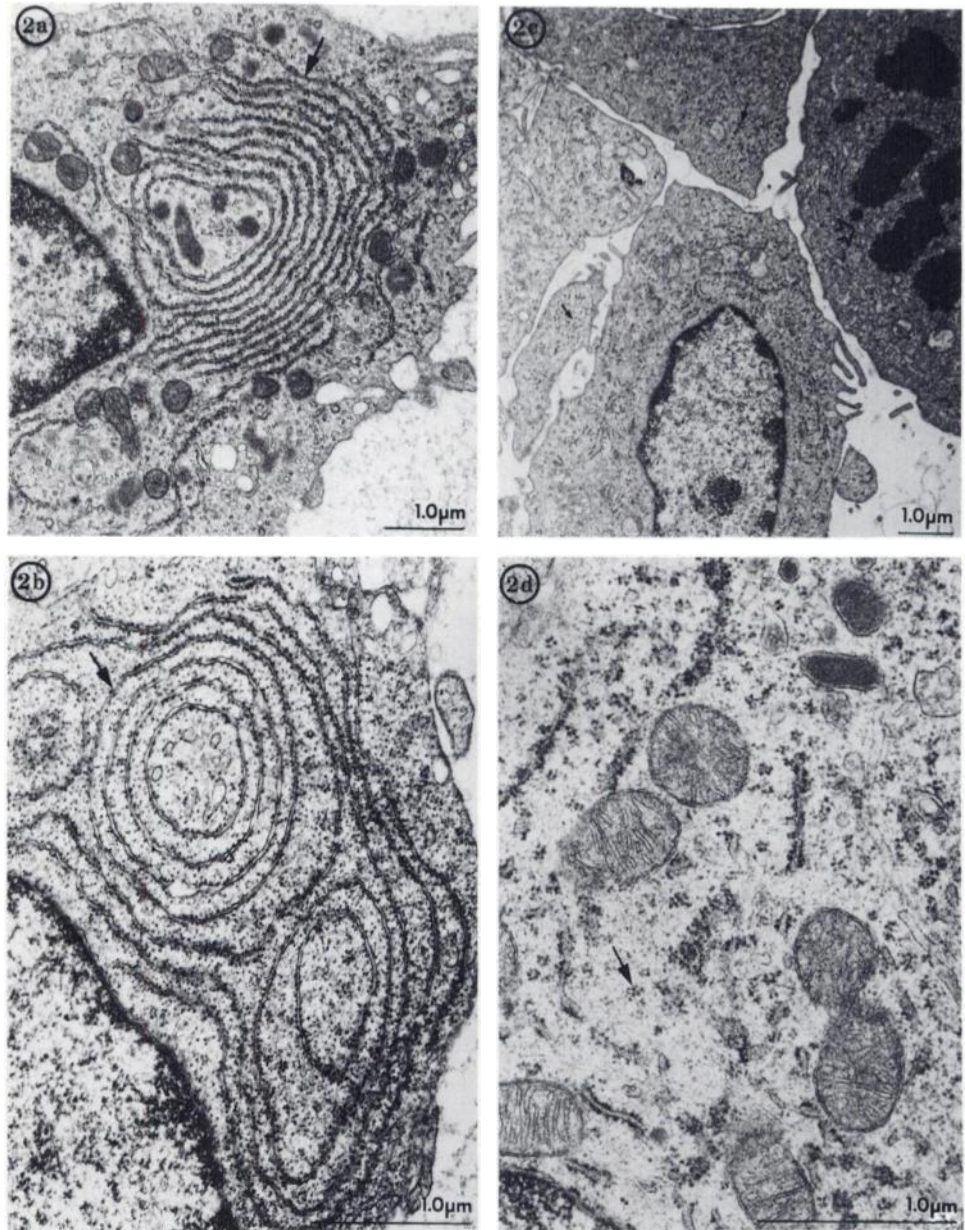


Fig. 2. Electron micrographs of intracerebral 9L tumors from animals treated with NaPA (a and b) and saline (c and d). a, phenylacetate-treated tumor has hyperplastic, well-organized, rough endoplasmic reticulum (arrow).  $\times 15,000$ . b, high-power magnification of treated 9L tumor cell showing the hyperplastic endoplasmic reticulum (arrow).  $\times 30,000$ . c, untreated 9L tumor. Note the scattered cytoplasmic polyribosomes (arrow) and the cell undergoing mitosis (open arrow).  $\times 10,000$ . d, higher magnification of untreated tumor cell showing scattered polyribosomes (arrow).  $\times 37,500$ .

months after tumor inoculation when NaPA was administered for 4 weeks starting on the day of tumor inoculation. Nine of 10 control rats died within 34 days after intracerebral tumor inoculation ( $P < 0.01$ , Mantel-Haenszel test; Fig. 3). The surviving rats are still being followed for long-term observation, and all appear to be disease free.

**Treatment of Established Tumors with NaPA.** Five of 12 rats treated with s.c. and i.p. NaPA (total of 850  $\mu\text{g}/\text{kg}/\text{day}$ ) for 4 weeks (starting 7 days after tumor inoculation) are still alive >12 months after tumor inoculation, whereas 12 of 13 saline-treated rats died by day 36 ( $P < 0.03$ , Mantel-Haenszel test; Fig. 4). All five surviving rats are still being observed, and all appear to be free of disease.

**Serum and CSF Levels of NaPA.** Assays of pooled serum and CSF, obtained after 7 days of combined s.c. and i.p. NaPA (total daily dose of 850 mg/kg) or saline administration, revealed a mean phenylacetate level of 2.45 mM in the serum and 3.1 mM in the CSF. No phenylacetate was detected in the serum or CSF samples from saline-treated rats. Although no formal pharmacokinetic studies were performed, these data indicate that mM concentrations of NaPA can be detected in both the serum and CSF of the treated rats.

**Toxicity.** No adverse effects of NaPA treatment were detected in any treated rats. Histological evaluation of the major peripheral organs and nontumoral brain showed no abnormalities.

## DISCUSSION

Phenylacetate induced a potent cytostatic and antitumor effect *in vitro* and in the *in vivo* brain tumor model used in these studies. This effect extended beyond the duration of drug administration, indicated by the long-term survival (>12 months) and apparent cure of rats which received NaPA, either simultaneously with tumor inoculation or after tumors were established. This extended effect of NaPA suggests that the malignant phenotype of treated tumor cells reverted, perhaps irreversibly in some animals, to one that was more benign and differentiated. Phenylacetate-induced tumor maturation has previously been documented in cultured leukemic and solid tumor cells (1-3). Phenylacetate induced dose-dependent restoration of anchorage dependency, indicating reversion of the glioma cells to a nonmalignant phenotype. More than 80% inhibition of colony formation was

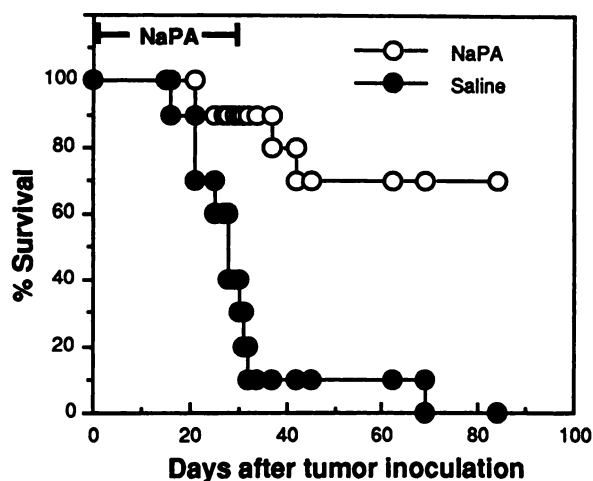


Fig. 3. Treatment with phenylacetate from the day of intracerebral tumor inoculation extended survival compared with saline treatment ( $P < 0.01$ ; Mantel-Haenszel test).

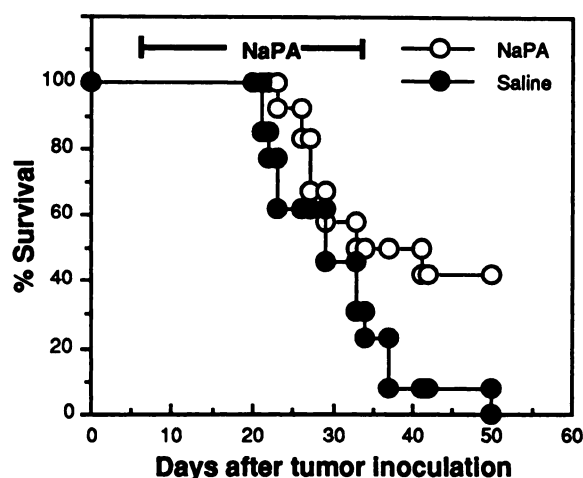


Fig. 4. Treatment of established tumors with phenylacetate extended survival compared to saline treatment ( $P < 0.03$ ; Mantel-Haenszel test).

achieved at a concentration of NaPA in the agar plate of 2.5 mM, which was similar to the serum and CSF levels measured in treated rats. Furthermore, after 1 week of exposure to NaPA, more than 40% of tumor cells maintained a benign growth pattern despite the absence of NaPA in the agar plates (Table 1). Phenylacetate itself, or one of its intermediate metabolites such as phenylacetyl coenzyme A, are believed to mediate this differentiating effect. The conjugated end-metabolites of phenylacetate, such as phenylacetyl glutamine, were shown to have no *in vitro* biological effect on tumor growth (1–3).

In our study of the subcellular organelles of treated brain tumor cells, an indication of cell differentiation was observed. The disorganized cytoplasmic polyribosomes in the saline-treated tumor cells were transformed by NaPA to a hyperplastic, well organized, rough endoplasmic reticulum. The endoplasmic reticulum is a highly specialized structure that performs many distinct functions; a well-developed endoplasmic reticulum represents cell differentiation and functional activity (13, 14). An inverse relationship has been noted between the amount of rough endoplasmic reticulum and the growth rate and degree of malignancy of tumors (15, 16). The numerous polyribosomes in the untreated tumor cells, which correlated well with the number of mitoses seen by light microscopy, have been described in other tumor systems previously. (13) Although determined in two

representative animals only, the effect of phenylacetate on the rate of replication of tumor cells *in vivo* was supported in our study using the BrdUrd proliferation assay. These changes underscore the differentiating effect of NaPA on the malignant glial cells and correlate with the *in vivo* decrease in cell proliferation and extended survival that occurred in treated animals with brain tumors. Therapeutic blood and CSF phenylacetate levels were reached in the treated rats. These measurements do not represent a pharmacokinetic profile of the drug but indicate good penetration of NaPA into the central nervous system, and presumably, into the developing tumor.

The mechanism(s) by which phenylacetate exerts its therapeutic effects are not completely understood. Its ability to deplete plasma glutamine, which is essential for the growth of malignant but not normal glial cells (4) does not appear to play a major role in the rat brain tumor model. In humans, the conjugation of glutamine by phenylacetate to phenylacetylglutamine is the process by which glutamine levels are reduced, hence depriving malignant glial cells of an essential source of this amino acid (4). However, in rodents a different metabolic pathway, involving the conjugation of glycine rather than glutamine, detoxifies phenylacetate (17). Accordingly, the antitumor effect of NaPA may be more pronounced in the treatment of human cancer. Other possible mechanisms, such as inhibition of the mevalonate pathway in glioma cells by inhibition of synthesis of sterols and isoprenoids, also may effect growth arrest (7, 8).

Based on its glutamine-depleting capacity in humans, phenylacetate was first proposed as an antitumor drug in 1971, (18) although the earlier studies in animal models showed no antitumor activity. More recently, phenylacetate was reported to be a component of some complex mixtures used to treat cancer patients (9). The use of these mixtures has been criticized for lacking experimental or clinical evidence of antitumor efficacy (10). In view of our findings, it is reasonable to assume that the phenylacetate component of these mixtures could account, at least in part, for the reported antitumor effect (9).

Our data indicate that phenylacetate, given to rats at a nontoxic dose, has a profound effect on regulation of tumor growth and cell maturation. Its potential use and safety in the treatment and prevention of cancer should be explored further.

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