Advances in Brief

Phenylacetate: A Novel Nontoxic Inducer of Tumor Cell Differentiation[1]

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Abstract

Sodium phenylacetate was found to affect the growth and differentiation of tumor cells *in vitro* at concentrations that have been achieved in humans with no significant adverse effects. Treatment of promyelocytic leukemia HL-60 cells resulted in the rapid decline of *myc* oncogene expression followed by growth arrest and granulocyte differentiation. Phenylacetate also induced highly efficient adipocyte conversion in immortalized mesenchymal C3H 10T½ cultures; yet, unlike the differentiating chemotherapeutic drug 5-aza-2'-deoxycytidine, phenylacetate did not cause neoplastic transformation in these susceptible cells. The results indicate that phenylacetate is both effective in inducing tumor cell maturation and free of cytotoxic and carcinogenic effects, a combination that warrants attention to its potential use in cancer intervention.

Introduction

Phenylacetate is a naturally occurring plasma component capable of conjugating glutamine to yield PAG,[3] which is subsequently excreted in the urine (1). The latter, leading to waste nitrogen excretion, has been the basis for using sodium phenylacetate (NaPA) in the treatment of hyperammonemia associated with inborn errors of urea synthesis (2, 3) or liver failure (4). Clinical experience indicates that acute or long-term treatment with high doses of NaPA (250–550 mg/kg/day) is well tolerated by both infants and adults, is essentially free of adverse effects, and is effective in reducing plasma glutamine levels. These characteristics should be of value in cancer intervention, considering the unique dependence of tumor cells on circulating glutamine (5).

Glutamine is the major nitrogen source for nucleic acid and protein synthesis and a substrate for energy in rapidly dividing normal and tumor cells (5, 6). In contrast to normal tissues, tumor cells typically operate at limiting levels of glutamine availability due to its decreased synthesis, increased utilization, and accelerated catabolism. Consequently, tumor cells are significantly more sensitive to further glutamine depletion than their normal counterparts, rendering glutamine an attractive therapeutic target. Along this line, promising antineoplastic activities have been demonstrated with the glutamine-depleting enzyme glutaminase and the glutamine antimetabolites 6-diazo-5-oxo-L-norleucine (DON), and L(αS.5S) α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin) (5, 7). Unfortunately, clinical usefulness has been limited by the unacceptable toxicities of DON and acivicin (possibly due to irreversible inhibition of glutamine-utilizing enzymes) and the development

of neutralizing antibodies to the bacterial glutaminase. Phenylacetate, which depletes glutamine without an apparent affect on glutamine-utilizing enzymes, was first proposed by Neish (8) as a potential antitumor agent; however, no data were provided. We report here that phenylacetate, used at pharmacologically attainable concentrations, can arrest the growth and induce differentiation of cultured premalignant and malignant cells through nontoxic mechanisms.

Materials and Methods

Cell Cultures and Reagents. HL-60 cells were provided by T. R. Breitman (National Cancer Institute, Bethesda, MD) and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco Laboratories), antibiotics, and 2 mM L-glutamine, unless otherwise specified. C3H 10T½ cells were from the American Type Culture Collection, and the growth medium was Dulbecco's modified Eagle's medium with the above supplements. To determine the effect of drugs on proliferation, HL-60 (2×10^5 cells/ml) and 10T½ (1×10^5 cells/60-mm dish) were seeded, and the drugs were added 20–24 h later. After 4 days, the adherent cells were detached with trypsin/EDTA, and the cell number was determined using a hemocytometer. Cell viability was determined by trypan blue exclusion. For glutamine-starvation studies, the fetal calf serum was stored at 4°C for 2–4 weeks prior to use, in order to reduce its glutamine levels to less than 0.6 mM (9). Since the serum concentration in the growth medium was 10%, the final concentration of glutamine did not exceed 0.06 mM if glutamine was not supplemented. Phenylacetic acid (Sigma, St. Louis, MO) and PAG (BRI, Houston, TX) were dissolved in distilled water, brought to pH 7.0 by the addition of NaOH, and stored in aliquots at −20°C until they were used. DON, acivicin, β-all-*trans*-retinoic acid, 5AzadC, TPA, and 4-hydroxyphenylacetate were purchased from Sigma. γ-Interferon was from Genentech, Inc.

Characterization of HL-60 Cell Differentiation. Oxidase activity of differentiated cells was evaluated by the NBT reduction assay (10). The direction of differentiation toward granulocytes *versus* monocytes was determined by a microscopic evaluation of cells stained with Wright's stain (Fisher Diagnostics) and an analysis of nonspecific esterase activity (the assay kit was from Sigma).

Adipocyte Conversion. For quantitative analysis, the 10T½ cells were plated at clonal densities (500–1500 cells in 100-mm dishes) in the presence or absence of drugs, and the number of colonies containing adipocytes with large lipid droplets was scored 2 weeks later. Conversion into mature adipocytes was evident only after the formation of dense colonies or monolayers with cell-cell contact. Therefore, in some experiments the cells were grown to confluency before treatment was initiated and observed for at least one additional week. Adipocytosis was determined by microscopic evaluation of cultures fixed with 37% formaldehyde and stained with Oil-Red O. The stained intracellular lipids were then extracted with butanol, and their absorbance at 510 nm was determined using Titertek Multiskan MC (Flow Laboratories). Blocking experiments involved the simultaneous treatment of cells with NaPA (5 mM) and TPA (10–100 nM). To distinguish cells at nonterminal and terminal states of differentiation, NaPA treatment was discontinued after 7 days, and the cultures were exposed to TPA (100 nM) for 3–6 days and observed for loss of accumulated lipids. This TPA treatment has been reported to bring about the loss of lipid droplets in nonterminally differentiated but not in terminally differentiated adipocytes (11).

Tumorigenesis Studies. Cells (3–4 × 10⁵) were plated in 75-cm² dishes, and 5AzadC (0.1 μM) was added to the growth medium at 20 and 48 h after plating. The cells were then subcultured in the absence of the nucleoside analogue for an additional 7 weeks. Cells treated with NaPA were subcultured in the continuous presence of NaPA. Treated and control cells were transplanted s.c. (1 × 10⁶/site) into female athymic nude mice, 4–5 weeks old (Division of Cancer Treatment, National Cancer Institute Animal Program, Frederick Cancer Research Facility), and the animals were observed for tumor growth at the site of injection.

Northern Blot Analysis. Cytoplasmic RNA was prepared from cultures at the logarithmic phase of growth (unless otherwise specified) and separated on 1% agarose-formaldehyde gels. RNA isolation, gel electrophoresis, transfer of RNA onto Nytran membranes (Schleicher & Schuell), hybridization with radiolabeled DNA probes, and autoradiography (Kodak X-ray film XAR5) were described (12). The probes were aP2 complementary DNA (13), myc third exon (Oncor, Gaithersburg MD), and HLA-A3 HindIII/EcoRI fragment (14). Probes were labeled with [³²P]dCTP (NEN) using a random primed DNA labeling kit (Boehringer Mannheim, Germany).

Results and Discussion

The Effect of Phenylacetate on Cell Growth and Differentiation. The experimental system included two in vitro differentiation models: (a) human promyelocytic leukemia cell line HL-60, representing uncommitted precursor cells that can be induced to differentiate along the myeloid or monocytic lineage (10); and (b) immortalized embryonic mesenchymal C3H 10T½ cells, which can differentiate into myocytes, adipocytes, or chondrocytes (15). NaPA treatment of HL60 and 10T½ cultures resulted in the dose-dependent inhibition of cell proliferation (Fig. 1), the inhibitory concentration causing 50% reduction in cell proliferation being 6 and 9 mM, respectively. No cytotoxicity was observed with doses twice as high. In addition to cytostasis, NaPA induced granulocyte differentiation in the HL-60 cultures (Fig. 2) and adipocyte conversion in 10T½ (Fig. 3). Differentiation in both cell lines was time dependent, efficient at 5–10 mM, and apparently irreversible upon cessation of treatment. Pharmacokinetics studies in children with urea cycle disorders (2, 3) revealed that phenylacetate plasma levels of 3–6 mM can be achieved with no significant adverse effects. In one case involving a Reye's syndrome patient, phenylacetate blood levels reached 19.2 mM; as with the lower standard doses, there was no evidence of toxic effects.[4]

In the leukemic HL-60 model, after 7 days of treatment with NaPA (5 mM), the cultures were composed of early-stage myelocytes and metamyelocytes (50–60%) as well as banded and segmented neutrophils (30–40%) capable of NBT reduction. The oxidase activity, characteristic of the more mature forms of human bone marrow granulocytes (10), persisted after treatment was discontinued; after 4 days there was a decrease of approximately 10% in the fraction of NBT-positive cells, which might be due to resumed proliferation of a less mature subpopulation. NaPA activity in the leukemic cells could be enhanced by the addition of low doses of retinoic acid (10 nM) (Fig. 2) or γ-interferon (300 IU/ml) (not shown). In confluent 10T½ cultures treated with 5 mM NaPA, more than 90% of the cells differentiated into adipocytes within 4–6 days. Higher drug concentrations further increased the frequency of differentiation as well as the size of lipid droplets in each cell (Fig. 3). Adipocytosis could be prevented by the simultaneous addition of 10–100 nM TPA, an activator of protein kinase C. However,
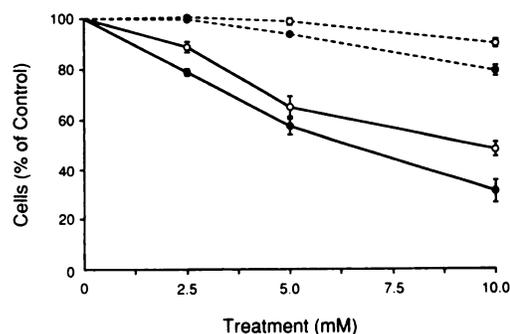
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[4] S. Brusilow, personal communication.

Fig. 1. The effect of NaPA on cell proliferation. Logaritmically growing HL-60 (●) and 10T½ (○) cells were treated for 4 days with NaPA (——) or PAG (– – –). Points, mean (n = 4); bars, SD. Cell viability was over 95% in all cases.
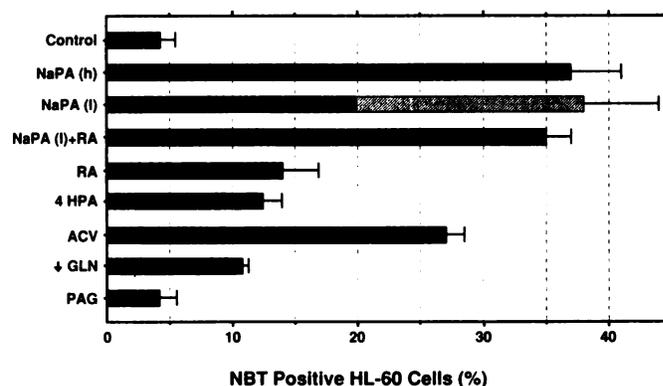

NBT Positive HL-60 Cells (%)

Fig. 2. Induction of HL-60 cell differentiation. The number of NBT-positive cells was determined after 4 (■) or 7 (▨) days of treatment. Control, control untreated cells; NaPA (h), 10 mM; NaPA (l), 5 mM; RA, retinoic acid, 10 nM; 4HPA, 4-hydroxyphenylacetate, 10 mM; ACV, acivicin, 3 μg/ml (the effect of acivicin was similar to that of 30 μg DON/ml); ↓Gln, ≤0.06 mM (see "Materials and Methods"); PAG, 6 mM. Cell viability was over 95% in all cases, except for DON and acivicin (75% and 63%, respectively).

this tumor-promoting agent, known to reverse nonterminal adipocytosis (10), was not effective if added 1 week after the initiation of NaPA treatment. The rapid, remarkably efficient, and stable adipocyte conversion by NaPA is reminiscent of the effect of some growth hormones and contrasts the low frequency of differentiation by genotoxic chemotherapy (11, 15, 16). It should be noted that the profound effect of phenylacetate on cell biology is not limited to animal and human tissues; this metabolite of phenylalanine has been shown to play a fundamental role in regulating the growth of such diverge organisms as bacteria (17), fungi (18), and plants (19).

Is Glutamine a Target of NaPA Antitumor Activity in Vitro? The mechanisms by which NaPA affects tumor cell biology are not known. One possible mechanism is glutamine depletion. In agreement with previous reports (20), glutamine starvation alone was sufficient to inhibit cell proliferation (not shown) and increase the number of NBT-positive cells in HL-60 cultures (Fig. 2). Similar results were obtained with the glutamine antagonists DON (10–30 μg/ml) and acivicin (1–3 μg/ml) (Fig. 2; Ref. 21). Furthermore, while the effect of NaPA could be mimicked by treatment with the analogue 4-hydroxyphenylacetate, also known to conjugate glutamine (1), the glutamine-conjugated form, i.e., PAG (6 mM), had no significant effect on cell growth, NBT reduction, or gene expression (see Figs. 1, 2, and 4). Despite the above findings, the role of glutamine in this experimental model is not clear. Although it is established that phenylacetate binds glutamine in the mitochondria of human
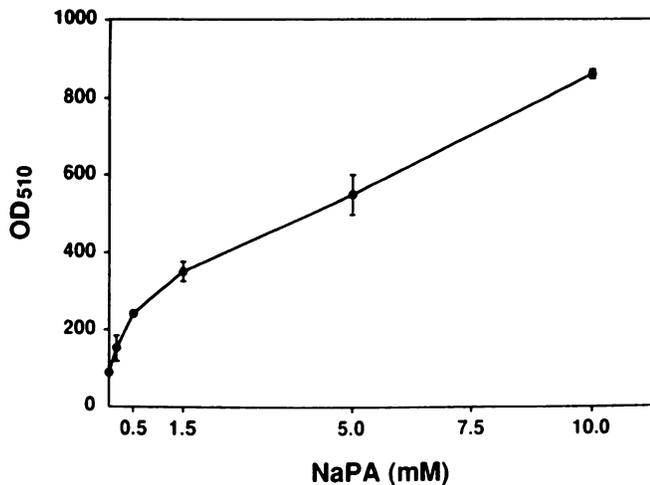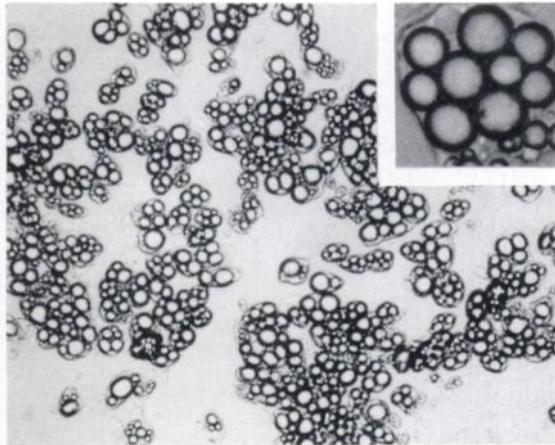
of extracellular glutamine or inhibition of glutamine-utilizing enzymes by DON and acivicin caused a transient maturation into monocytes. Moreover, glutamine conjugation by NaPA is limited to humans and higher primates; in rodents NaPA binds glycine (1). Accordingly, the effect of NaPA on the mouse 10T½ cells could not be mimicked by glutamine starvation or by treatment with the glutamine antagonists DON and acivicin, suggesting that NaPA may affect tumor cells through mechanisms that do not involve glutamine metabolism.

Modulation of Gene Expression by NaPA. Northern blot analyses of HL-60 and 10T½ cells revealed marked changes in gene expression occurring shortly after NaPA treatment. Expression of the adipocyte-specific *aP2* gene (13) was induced within 24 h in confluent 10T½ cultures (not shown), reaching maximal mRNA levels by 72 h of NaPA treatment (Fig. 4*A*). In HL-60, cell transformation has been linked to *myc* amplification and overexpression, and differentiation typically requires down-regulation of *myc* expression (reviewed in Ref. 10). As shown in Fig. 4*B*, NaPA caused rapid decline in the amounts of *myc* mRNA, which occurred within 4 h of treatment, preceding the phenotypic changes detectable by 48 h after treatment
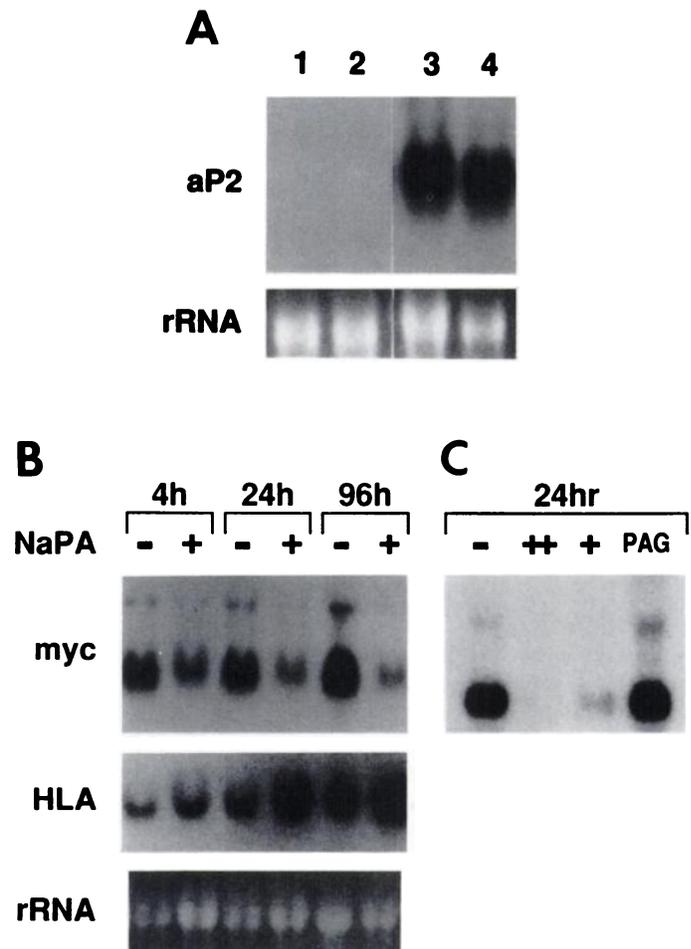

Fig. 3. Adipocyte conversion in 10T½ cultures. Confluent cultures were treated with NaPA for 7 days. *Top*, untreated control. × 100. *Middle*, cells treated with 5 mM NaPA. × 100. The *empty spaces* represent areas where mature adipocytes have detached. *Insert*, a single cell with multiple lipid droplets. × 400. *Bottom*, quantitation of adipocytosis. Lipids stained with Oil-Red O were extracted with butanol, and the absorbance at 510 nm was determined. Increased lipid accumulation was evident with NaPA concentrations ≥0.2 mM.

liver and kidney cells, whether or not a similar enzymatic reaction takes place in other cells such as the leukemic HL-60 remains to be determined. In any case, glutamine depletion alone could not explain some of the molecular and phenotypic changes induced by NaPA. While HL-60 cells treated with NaPA converted primarily into granulocytes, a mere depletion

Fig. 4. Modulation of gene expression in NaPA-treated cells. Cytoplasmic RNA (20 µg/lane) was subjected to Northern blot analysis using ^{32}P-labeled specific DNA probes. Ethidium bromide-stained 28S rRNA indicates the relative amounts of total RNA in each lane. *A*, induction of *aP2* expression in 10T½ cells. RNA from confluent cultures treated for 72 h. *Lane 1*, untreated control; *Lane 2*, PAG, 6 mM; *Lanes 3* and *4*, NaPA, 5 and 10 mM, respectively. *B*, kinetics of *myc* inhibition and HLA-A induction in HL-60. Cells at the logarithmic phase of growth were treated with 5 mM NaPA for the specified duration (+); −, untreated controls. *C*, dose dependency and specificity of effect. HL-60 cells were treated with 10 mM (++) or 5 mM (+) NaPA or 6 mM PAG.

Table 1 *Tumorigenicity of C3H 10T1/2 cells in athymic mice*

Treatment[a]	Tumors		
	Incidence (positive/ injected mice)	Diameter (mm ± SD)	Time (weeks)
None	0/8	0	13
5AzadC	8/8	5.5 ± 2.5	8
NaPA	0/8	0	13

[a] Cells were treated in culture with 0.1 μg 5AzadC/ml or with 10 mM NaPA as described in "Materials and Methods" and then injected s.c. (1 × 10⁶ cells/ site) into mice. The results were scored at the indicated time after transplantation.

(approximately two cell cycles). Similar kinetics of *myc* inhibition have been reported for other differentiation agents such as retinoids and 1,25-dihydroxyvitamin D_3 (10). By contrast, a decline in *myc* expression in acivicin-treated HL-60 occurred only after 48–72 h of treatment, a delayed effect thought to be mediated by drug-induced, autocrine interleukin 1β and tumor necrosis factor α (22). The rapid effect of NaPA indicates that down-regulation of oncogene expression may be responsible in part for the growth arrest and induction of differentiation in HL-60. Furthermore, NaPA treatment of the leukemic cells was associated with time- and dose-dependent accumulation of HLA-A mRNA (Fig. 4*B*) coding for class I major histocompatibility antigen. The latter is of special interest since increased HLA expression has been shown to enhance the immunogenicity of tumors *in vivo* (23).

NaPA Does Not Cause Tumor Progression. Studies in plants showed that high concentrations of NaPA can inhibit DNA methylation (24), an epigenetic mechanism implicated in the control of gene expression and cell phenotype. Hypomethylation can lead to cell differentiation. However, it can also promote oncogenesis and the evolution of cells with metastatic capabilities, as demonstrated for the hypomethylating chemotherapeutic drug 5-aza-2'-deoxycytidine (5AzadC) (12). Comparative analysis using the highly susceptible 10T½ cells as a model, revealed that NaPA, unlike the cytosine analogue did not cause neoplastic transformation. As previously shown (11, 14), transient treatment of actively growing 10T½ cells with 0.1 μM 5AzadC resulted in differentiation into myotubes, chondrocytes, and adipocytes; however, these cultures also developed foci of neoplastically transformed cells (frequency of about 7 × 10⁻⁴), which eventually became capable of tumor formation in athymic mice (Table 1). By contrast, actively replicating 10T½ cultures treated for 7 weeks with 5–10 mM NaPA differentiated solely into adipocytes, forming neither neoplastic foci *in vitro* nor tumors in recipient mice. Preliminary data show that NaPA can actually prevent spontaneous or 5AzadC-induced neoplastic transformation,[5] suggesting a potential role in cancer prevention. The antitumor activities, lack of toxicity, and easy administration (oral) would qualify NaPA as a chemopreventive drug.

Conclusions. Our data indicate that NaPA can induce differentiation in cultured malignant and premalignant cells at concentrations that have already been achieved in people with no significant adverse effects. The phenotypic changes observed *in vitro* were associated with the modulation of expression of genes involved in the control of cell growth, differentiation, and immunogenicity. In humans, NaPA could affect tumor growth also through depletion of circulating glutamine, the amino acid required by most tumor cells for energy metabolism and macromolecular synthesis. An antitumor agent that exploits glutamine as a therapeutic target would be expected to block the phenotypic expression of a wide variety of malignant cells,

regardless of their origin or etiology. Indeed, ongoing studies show that NaPA can promote hemoglobin biosynthesis in erythroleukemia cells and cause growth arrest and reversal of malignant properties in cell lines established from solid tumors including astrocytoma, melanoma, and hormone-refractory prostate adenocarcinoma.[6] In all cases, NaPA suppressed tumor cell proliferation while sparing actively growing human diploid skin fibroblasts.

Sodium phenylacetate is an investigational new drug approved for human use by the U.S. Food and Drug Administration. The drug has already been established as safe and effective in the treatment of hyperammonemia (2–4); we propose that its use may be extended to cancer prevention and therapy.

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