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CYTOSTATIC ACTIVITY OF PHENYLACETATE AND DERIVATIVES AGAINST TUMOR CELLS

CORRELATION WITH LIPOPHILICITY AND INHIBITION OF PROTEIN PRENYLATION

W. ROBERT HUDGINS,* SONSOLES SHACK,* CHARLES E. MYERS† and DVORIT SAMID*‡

*Clinical Pharmacology Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892; and †Cancer Center, University of Virginia, Charlottesville, VA 22908, U.S.A.

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Abstract—The aromatic fatty acid phenylacetate, a common metabolite of phenylalanine, shows promise as a relatively non-toxic drug for cancer treatment. This slowly metabolized fatty acid alters tumor cell lipid metabolism causing, among other effects, inhibition of protein prenylation critical to malignant growth. In pursuit of more potent analogues, we have examined the activity of related compounds against tumor cell lines established from patients with advanced prostatic carcinoma, glioblastomas, and malignant melanoma. Like phenylacetate, derivatives containing α-carbon or ring substitutions induced cytostasis and phenotypic reversion at non-toxic concentrations. Potency was correlated with the degree of calculated lipophilicity of the aromatic fatty acid, and the extent of inhibition of protein prenylation. Remarkably, a parallel cytostatic activity was reported in embryonic plant cells, which respond to phenylacetate and its analogues in the same concentration range and the same rank order of lipophilicity. These data suggest that phenylacetate and its analogues may act through common mechanisms to inhibit the growth of vastly divergent, undifferentiated cell types, and provide a basis for the development of new agents for the treatment of human malignancies.

Key words: aromatic fatty acids; lipophilicity; cytostasis; phenotypic reversion; prostate cancer; glioblastoma; melanoma; plants

Phenylacetate, an aromatic fatty acid, was identified recently as a relatively non-toxic antitumor agent, with demonstrable activity in experimental models [1] and in humans [2]. Preclinical studies show that this drug and its analogue, phenylbutyrate, have broad spectrum activity, inducing cytostasis and differentiation in various hematopoietic and solid tumors [1, 3–9]. Phenylacetate promotes maturation of human leukemic cells [1, 3] and brings about reversal of malignancy of hormone-refractory prostatic carcinoma [4], glioblastoma [5, 6], neuroblastoma [7], rhabdomyosarcoma [8], and malignant melanoma [9]. The marked changes in tumor biology were associated with alterations in expression of genes implicated in tumor growth, invasion, angiogenesis, and immunogenicity. Systemic treatment of rats bearing intracranial gliomas results in significant tumor suppression with no apparent toxicity to the host [5, 6]. More importantly, clinical experience obtained during phenylacetate treatment of cancer [2] and of urea cycle disorders [10, 11] indicated that phenylacetate concentrations producing significant antitumor effects *in vitro* (millimolar) can be achieved in humans without significant adverse effects. The antitumor activity, easy administration (oral or i.v.), and lack of significant adverse effects, make phenylacetate an attractive agent for clinical use in the treatment of some hematopoietic and solid neoplasms, including those that do not respond to conventional cytotoxic therapies.

It has been known since 1906 that long chain aromatic fatty acids are subject to fatty acid metabolism and can be β-oxidized repeatedly, but the aromatic ring itself resists oxidation [12]. Consequently, phenylacetate is metabolically resistant, having a half-life in the patient of more than 4 hr [2], and its analogue, phenylbutyrate, a half-life of 1–2 hr [13], compared with nonaromatic, butyric acid, which has a half-life of only 6 min. Preserving the aromatic nucleus and the carboxyl group while making other modifications to phenylacetate might provide more potent analogues of the parent compound. The direction of our search for active analogues was inspired, among other considerations, by experience with plants in which phenylacetate serves as an endogenous growth regulator, stimulating growth of embryonic plant tissue at micromolar concentrations and inhibiting it at millimolar levels [14]. Studies in plants revealed that increasing the lipophilicity of a phenylacetate analogue (as measured by its octanol-water partition coefficient) enhanced its growth-regulatory activity [15]. We show here that for these analogues enhanced potency in inducing cytostasis and phenotypic reversion in prostate carcinoma, glioblastoma, and melanoma cultures was correlated with increased drug lipophilicity.

Despite its known capacity to mimic fatty acids, the mechanism by which phenylacetate selectively inhibits growth and promotes differentiation in tumor cells is not certain. Recent studies demonstrated that phenylacetate, possibly because of its resemblance to MVA§, inhibits cholesterol synthesis and protein prenylation in glioblas-

‡ Corresponding author: Dr. Dvorit Samid, Clinical Pharmacology Branch, National Cancer Institute, Bldg. 10, Room 12C103, 9000 Rockville Pike, Bethesda, MD 20892. Tel. (301) 496-3390; FAX (301) 402-1997.

§ Abbreviations: MVA, mevalonic acid; CLOGP, calculated logarithm of the octanol–water partition coefficient; and IC_{50} and IC_5, inhibitory concentration causing 50 and 5% reduction in cell proliferation, respectively.

toma cells [5], which like most cancer cells are dependent on intracellular synthesis of isoprenoids for survival. When we examined the capacity of the analogues to block protein prenylation, we found that their prenylation inhibitory activity correlated with their cytostatic activity. Taken together, these studies indicate that cytostatic activity, phenotypic reversion, lipophilicity, and inhibition of protein prenylation are interrelated phenomena on which the potency of analogues of phenylacetate can be evaluated.

MATERIALS AND METHODS

Cell cultures

Studies included the following human tumor cell lines: (a) hormone-refractory prostatic carcinomas PC3 and DU145, purchased from the American Type Culture Collection (ATCC, Rockville, MD); (b) glioblastoma U87 (ATCC); and (c) melanoma 1011, provided by J. Fidler (M. D. Anderson, Houston, TX) and J. Weber (NCI, Bethesda, MD), respectively. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Gibco Laboratories), antibiotics, and 2 mM L-glutamine. Diploid human foreskin FS4 fibroblasts (ATCC), human umbilical vein endothelial cells (HUVC), and normal melanocytes were used for comparison. The HUVC cells, isolated from freshly obtained cords, were provided by D. Grant and H. Kleinman (NIH, Bethesda, MD). Primary melanocytes, provided by J. Weber, were cultured in Melanocyte Basal Medium (Clonetics, San Diego, CA).

Antitumor agents

Sodium phenylacetate and phenylbutyrate were from the Elan Pharmaceutical Corp., Gainesville, GA. In some experiments, phenylacetic acid and phenylbutyric acid, obtained from Aldrich (Milwaukee, WI), were neutralized and used for comparison of biological activity. 4-Iodophenylacetate, 4-iodophenylbutyrate and 4-chlorophenylbutyrate were synthesized by the Sandmeyer procedure [16, 17] from the corresponding 4-amino-phenyl fatty acids. The halogenated products were extracted from the acidic reaction mixtures with diethyl ether, which was then taken to dryness. The residue was dissolved in boiling hexane, and the crystals that formed on cooling were collected by suction filtration. The product was recrystallized from hexane until the reported melting points were obtained. Amides of phenylacetate and phenylbutyrate were produced by heating the sodium salts with a small excess of thionylchloride followed by the addition of ice-cold concentrated ammonia. The amides were purified by recrystallization from boiling water. The identity of synthesized compounds was verified by melting point determination and by mass spectroscopy. All commercially available derivatives were purchased from Aldrich or Sigma (St. Louis, MO), depending on availability. Tested compounds were all dissolved in distilled water, brought to pH 7.0 by the addition of NaOH as needed, and stored in aliquots at −20° until used.

Calculation of relative drug lipophilicities

Estimation of the contribution of lipophilicity to the biological activity of a molecule was based on its CLOGP. This was determined for each compound using the BLOGP program of Bodor *et al.* (BLOGP version 1.0, Center for Drug Discovery, University of Florida).

Quantitation of cell growth and viability

Growth rates were determined by an enzymatic assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) as substrate [18]. Assays were performed in quadruplicate at four different drug concentrations in 96-well plates (Costar, Boston, MA). The IC_{50} values were determined as described previously [1]. The cytostatic activity determined in the enzymatic assay was confirmed in an alternative assay using cell enumeration with a hemocytometer following detachment with trypsin-EDTA. Cell viability was assessed by trypan blue exclusion. These two assays produced essentially the same results.

Colony formation in semi-solid agar

For analysis of anchorage-independent growth, cells were harvested with trypsin-EDTA and resuspended at 1.0×10^4 cells/mL in growth medium containing 0.36% agar (Difco, Detroit, MI). Two milliliters of cell suspension was added to 60-mm plates (Costar), which were pre-coated with 4 mL of solid agar (0.9%). Tested drugs were added at different concentrations in triplicate assays, and colonies composed of 30 or more cells were counted after 3 weeks. The minimum concentration at which colony formation was inhibited 80–100% was scored.

Growth on Matrigel

Cells were first treated with drugs in plastic tissue culture dishes for 4–6 days, and then replated (5×10^4 cells/well) onto 16-mm dishes (Costar) coated with 250 μL of 10 mg/mL Matrigel, a reconstituted basement membrane (Collaborative Research, Bedford, MA). Drugs were either added to the dishes or omitted in order to determine the reversibility of effect. Net-like formation characteristic of invasive cells occurred within 12 hr, while invasion into the Matrigel was evident after 6–9 days. Over a range of four different concentrations, the minimum concentration totally inhibiting net formation was scored.

Drug uptake studies

Cells were plated in 6-well tissue culture dishes (Costar) at 5×10^5 cells/dish. The growth medium was replaced after 24 hr with 750 μL of fresh medium containing 4.5×10^5 dpm of either [^{14}C]phenylacetic acid (3.4 mCi/mmol, Sigma) of [^{14}C]naphthylacetic acid (5.4 mCi/mmol, Sigma), and the cultures were incubated for 10–180 min at 37°. Labeling was terminated by placing plates on ice. Cells were then washed twice with 5 mL of ice-cold PBS and detached by scraping; the radioactivity retained by cells was determined using liquid scintillation. Blank values were determined by incubating the radiolabeled compounds in an empty dish.

Analysis of protein prenylation

Protein prenylation was determined as described previously [5]. Briefly, melanoma 1011 cells were grown for 24 hr in the presence of phenylacetate or an analogue. The concentration used for each sample was adjusted to be non-lethal during the 24 hr of treatment (about 2–4 × IC_{50}). At 8 hr after starting treatment, labeling was begun using [^{14}C]MVA (16 μCi/mL;

15 μCi/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO). Labeled proteins were reduced with mercaptoethanol and resolved on 10% polyacrylamide. The resulting gel was stained with Coomassie Blue and autoradiographed.

RESULTS

Correlation between drug lipophilicity and the growth-inhibitory effect of phenylacetate and its analogues

The structures of tested aromatic fatty acids are shown in Fig. 1. The growth inhibitory effects of these compounds on prostatic carcinoma, glioblastoma, and melanoma cell lines are expressed as IC_{50} and compared with drug lipophilicity determined using the CLOGP program. As seen in Tables 1 and 2, there was a strong relationship between cytostasis and lipophilicity. Except where noted in the tables differences were significant at $P = 0.01$ (Student's *t*-test, one-sided). In agreement with previous observations with phenylacetate [3], the cytostatic effects of its analogues were selective, as higher drug concentrations were needed to affect significantly the proliferation of normal endothelial cells, skin fibroblasts, or primary melanocytes. No cytotoxicity (i.e. decline in cell viability as indicated by trypan blue staining) occurred during 4–6 days of continuous treatment with the tested compounds. Reduced proliferative capacity of prostate carcinoma PC3 and DU145 treated with phenylacetate was associated with cell accumulation in the G_0/G_1 phase of the cell cycle (as determined by cell sorting on prostate cancer cells treated with bromodeoxyuridine and propidium iodide, unpublished results); the effects of the analogs are yet to be determined. To check the possibility that the cytostatic activity may have resulted from an impurity, we determined the IC_{50} values of phenylacetate and phenylbutyrate from two different sources: one supplying the 99% pure acids, the other pharmaceutical grade sodium salts. The IC_{50} values were characteristic of the compounds and independent of their sources (data not shown).

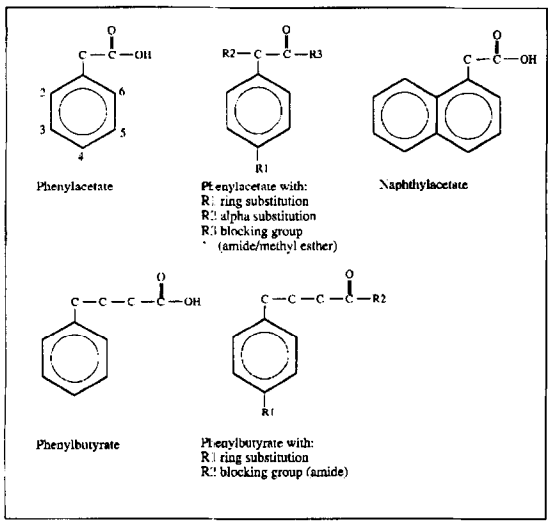

Fig. 1. Structures of phenylacetate and analogs. The compounds represented by these generalized structures are listed in Tables 1 and 2.

Further analysis of structure-activity relationships was based on the method of Hansch and Anderson [19] used for the correlation of the anesthetic and metabolic effects of barbiturates with their octanol-water partition coefficients. Adaptation of this method assumes that, if the relationship is simple, it will follow the equation: $\log 1/C = \text{slope} \times \log P + K$ (where P is the partition coefficient and K is the constant). Plotting the $\log 1/IC_{50}$ values obtained with prostatic cells versus drug CLOGP of compounds from Table 2, we found that the best fit line (Fig. 2) was described by the equation: $\log 1/IC_{50} = 0.89$ CLOGP + 0.57 ($r = 0.887$). The slope of this line (0.89) was in the range of values found for the anesthetic potencies of a series of barbiturate analogues [19]. Hansch and colleagues [15] also studied the effects of phenylacetate and several derivatives on embryonic plant growth. As shown in Fig. 2, the concentration range and rank order of inhibition of plant growth by phenylacetate analogues were comparable to the inhibition of growth of prostatic cancer cells by this same series of compounds. Compared with derivatives containing ring or α-carbon substitutions, those with blocked carboxyl groups were less effective. The methyl ester of phenylacetate was about half as active as the free acid (IC_{50} in DU145 prostatic cells 8.8 mM vs 4.1 mM for phenylacetate). The amide forms were also less active than the parent compounds in this experimental system, with IC_{50} values of 2.0 mM for phenylbutyramide versus 0.82 mM for phenylbutyrate, and 4.8 mM for phenylacetamide versus 4.1 mM for phenylacetate.

Drug uptake

One possible function of increasing lipophilicity is an increasing ease with which aromatic fatty acids can enter into and cross the plasma membrane as well as the membranes of other organelles. The rate of phenylacetate uptake by tumor cells was compared with that of the more hydrophobic analog, naphthylacetate. After 10 min, relative to phenylacetate more than twice as much naphthylacetate had entered the glioblastoma U87 cells (uptake of phenylacetate was 41% that of naphthylacetate), indicating that its movement through the plasma membrane was more than twice as fast as phenylacetate. After 20 min, the amount of naphthylacetate taken up by the cells was only 26% greater than that of phenylacetate and at 180 min the intracellular levels of both compounds were nearly equal, suggesting that at this time the more rapid influx of naphthylacetic acid was balanced by an equally rapid efflux. There was little further uptake, and the concentrations of phenylacetate inside and outside the cells were about equal, indicating that these cells do not actively accumulate much aromatic fatty acid.

Phenotypic reversion

In addition to causing selective cytostasis, phenylacetate induces malignant cells to undergo reversion to a more benign phenotype [1, 3–5]. The effect of analogs on tumor biology was tested using as a model the hormone-refractory prostatic PC3 cells originally derived from a bone metastasis. PC3 cells exhibit several growth characteristics *in vitro* that correlate with their malignant behavior *in vivo*, including anchorage-independent growth (i.e. colony formation in semi-solid agar), and the capacity to invade and degrade membranes forming "net"-like structures when plated on a reconstituted

Table 1. Phenylacetate (PA) and analogues containing alkyl-chain substitutions: Relationship of IC_{50} to CLOGP

Rx	CLOGP	IC_{50} (mM) Prostate carcinoma (PC3)	Glioblastoma (U87)	Melanoma (1011)	Normal cells
PA	2.05	5.2 ±0.1	4.8 ±0.1	4.9 ±0.1	11.7 ±0.5*
α-Methoxy-PA	2.17	6.3 ±0.1	7.2 ±0.3	6.4 ±0.2	ND†
α-Methyl-PA	2.42	4.4 ±0.1	4.6 ±0.1‡	3.7 ±0.1	12.4 ±0.4*
α-Ethyl-PA	2.77	2.6 ±0.2	1.72 ± 0.05	2.2 ±0.1	9.4 ±0.5*
PB§	2.89	0.82 ± 0.04	2.0 ±0.2	0.9 ±0.1	2.8 ±0.1‖
4-Chloro-PB	3.30	0.61 ± 0.08	ND	0.81 ± 0.05	1.10 ± 0.05‖
4-Iodo-PB	3.85	0.28 ± 0.04	0.27 ± 0.02	0.22 ± 0.03	0.41 ± 0.01‖

Except where noted, differences were significant at $P = 0.01$ (Student's t-test, one-sided).
* FS4 fibroblasts.
† ND, not determined.
‡ Not significantly different from PA at $P < 0.01$.
§ PB, phenylbutyrate.
‖ Melanocytes.

basement membrane (Matrigel) [4]. The abilities of phenylacetate and representative analogs to bring about the loss of such properties are summarized in Fig. 3A (colony growth) and Fig. 3B (Matrigel invasion). Similar to the cytostatic effect, ability to induce reversion to a nonmalignant phenotype was correlated with the calculated lipophilicity of the drugs ($r = 0.81$, inhibition of growth in soft agar, and $r = 0.94$, inhibition of Matrigel invasion). Of the tested compounds, naphthylacetate and derivatives of phenylbutyrate and phenylacetate with iodo- and chlorine substitutions were found to be the most active on a molar basis. The relative efficacy of the compounds in suppressing anchorage-independent growth was confirmed using U87 glioblastoma cells (data not shown).

Inhibition of protein prenylation

Phenylacetate inhibits cholesterol synthesis and protein prenylation in glioblastoma cells during the first 24 hr of treatment, under conditions that do not affect overall protein and DNA synthesis [5]. Now we have examined whether prenylation is also inhibited in melanoma cells and whether the analogues of phenylacetate are also effective prenylation inhibitors. As shown in Fig. 4, all the analogues inhibited the incorporation of [^{14}C]MVA into melanoma cell proteins including those having the apparent M_r of G-proteins (M_r = 21,000–26,000). The most lipophilic analogue, 4-Iodophenylbutyrate, was the most potent inhibitor of prenylation, while the least lipophilic agent, phenylacetate, was the weakest, on a molar basis. Densitometric analysis of the M_r = 21,000–26,000 bands normalized for protein load gave values of 27% of control at 10 mM phenylacetate; 39% of control at 3 mM phenylbutyrate; 15% at 3 mM 4-Chlorophenylacetate; 12% at 2 mM 4-Chlorophenylbutyrate; 5% at 1 mM 4-Iodophenylbutyrate; and 14% at 3 mM naphthylacetate.

DISCUSSION

The comparative activities of phenylacetate and its analogues against a number of tumor cell lines suggest

Table 2. Phenylacetate (PA) and analogues containing ring substitutions: Relationship of IC_{50} to CLOGP

Rx	CLOGP	IC_{50} (mM) Prostate carcinoma (PC3)	Glioblastoma (U87)	Melanoma (1011)	Normal cells
4-Hydroxy-PA	1.78	8.3 ±0.2	14.0 ±0.7	11.1 ±0.3	ND*
PA	2.05	5.2 ±0.1	4.8 ±0.1	4.9 ±0.1	11.7 ± 0.5†
4-Fluoro-PA	2.17	2.8 ±0.1	4.4 ±0.4‡	2.7 ±0.2	ND
2-Methyl-PA	2.43	2.5 ±0.1	ND	ND	ND
3-Methyl-PA	2.45	2.2 ±0.2	ND	ND	ND
4-Methyl-PA	2.47	2.0 ±0.2	ND	ND	ND
4-Chloro-PA	2.48	0.93 ± 0.03	1.10 ± 0.07	1.1 ±0.1	3.0 ± 0.1†
3-Chloro-PA	2.54	1.5 ±0.2	1.8 ±0.1	1.45 ± 0.05	6.8 ± 0.5†
2-Chloro-PA	2.56	2.0 ±0.1	2.8 ±0.2	2.6 ±0.3	ND
2,6-Dichloro-PA	2.87	0.9 ±0.1	1.0 ±0.2	1.07 ± 0.05	ND
4-Iodo-PA	3.12	0.6 ±0.1	0.9 ±0.1	1.2 ±0.2	ND
1-Naphthylacetate	3.16	0.83 ± 0.05	0.95 ± 0.05	0.87 ± 0.07	3.6 ± 0.2†

Except where noted, differences were significant at $P = 0.01$ (Student's t-test, one-sided).
* ND, not determined.
† FS4 fibroblasts.
‡ Not significantly different from PA at $P < 0.01$.

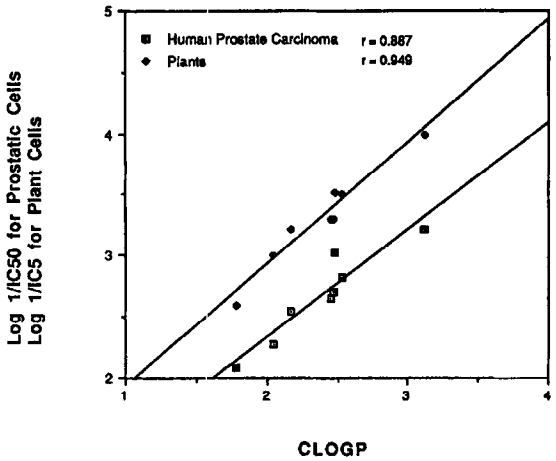

Fig. 2. Relationship between lipophilicity and the cytostasis induced by phenylacetate (PA) derivatives in prostate carcinoma cells and in embryonic plant tissues. The log $1/IC_{50}$ values for prostatic cells (calculated from data presented in Table 1) were compared with the $1/IC_5$ for rapidly developing plant tissues (data provided in Ref. 19). Tested compounds, listed in an increasing order of their CLOGPs, included 4-hydroxy-PA, PA, 4-fluoro-PA, 3-methyl-PA, 4-methyl-PA, 4-chloro-PA, 3-chloro-PA, and 4-iodo-PA.

that these compounds may form a new class of therapeutic agents whose effectiveness varies with structure. Improved anticancer activity might be achieved if factors controlling their action were understood, and toward this end we have compared the effects of systematic changes in structure with changes in activity. The outstanding results of this study are the discovery that: (a) there is a simple relationship between the lipophilicity of a phenylacetate derivative and its activity against human tumor cells, (b) the analogues of phenylacetate are effective inhibitors of protein prenylation, and (c) the relative potency observed with human neoplasms is similar to that documented in plants, indicating that the role of the aromatic fatty acids in growth regulation has been conserved in evolution.

Lipophilicity should affect the ease with which these aromatic fatty acids enter into and cross the lipid bilayer of cell membranes. In agreement, cellular entry by the more hydrophobic compound, naphthylacetate, was faster than that of phenylacetate. At equilibrium, however, there were no differences between the intracellular and the extracellular concentration of either compound. Apparently, the rates of drug uptake are balanced by proportional rates of efflux, and the overall capacity of the cell to retain such compounds is not much greater than that of the extracellular milieu.

Although there is a good correlation between drug potency and lipophilicity (see Fig. 2), small deviations within the phenylacetate-related series may give some clues regarding mechanisms of action. Halogen substitution *para* to the alkylcarboxyl group was found to increase potency more than that in the *ortho* position ($P < 0.01$) by a factor of two, suggesting that orientation of the hydrophobic substituent may be important. At the *para* position, chlorine had twice the impact on efficacy of the methyl group despite nearly equal contributions to CLOGP, indicating that electronegativity may affect growth inhibitory interactions. While α-ethylphenylacetic acid, in which the carboxyl group is crowded by the adjacent ethyl group, was more potent than the parent compound, the slightly more lipophilic analog α-methoxyphenylacetic acid was less active. Other parameters such as addition of an aromatic ring to phenylacetate, or an increase in the distance between the aromatic nucleus and the carboxyl group did not cause anomalous enhancement or interference in biological activity (naphthylacetate and phenylbutyrate were about as active as would be expected on the basis of their lipophilicity). Selectivity was reduced for chlorophenylbutyrate and iodophenylbutyrate, but not for phenylbutyrate or for the β-oxidation product of chlorophenylbutyrate, chlorophenylacetate. It is possible that β-oxidation intermediates of some of the substituted phenylbutyrates are active and less selective for tumor cells. Further studies would be needed to determine the *in vivo* safety or toxicity of these ring-substituted phenylbutyrates. The importance of a free carboxyl group is unclear. The amide forms of phenylacetate and phenylbutyrate, in which the carboxylic group is blocked, were less cytostatic compared with the parental compounds and failed to induce cell differentiation (unpublished data). Moreover, phenylacetylglutamine has no detectable effect on cell growth and maturation [3–5]. It appears, therefore, that a free carboxyl group may be essential for some aspects of the antitumor activity of phenylacetate and derivatives.

Despite a large body of knowledge implicating phenylacetate and analogs in growth control throughout phylogeny [14, 20, 21], little is known regarding their mode of action. In plants, phenylacetate and naphthylacetate are endogenous growth hormones (auxins) known to stimulate proliferation at micromolar concentrations, while inhibiting growth at millimolar levels [14, 15]. As growth inhibitors (but not stimulators), the effect of phenylacetate analogues on rapidly developing embryonic plant tissues, like that on human tumor cells, is a simple function of their lipophilicities [15]. This similarity in potency in plants and mammals, summarized in Fig. 2, suggests that some of the underlying mechanisms of negative growth control may be similar as well.

We show here that the aromatic fatty acids interfered with protein post-translational processing by inhibiting the MVA pathway of cholesterol synthesis. MVA is a precursor of several isopentenyl moieties required for progression through the cell cycle, and of prenyl groups that modify a small set of critical proteins [22]. The latter include plasma membrane G and G-like proteins (e.g. ras) involved in mitogenic signal transduction (M_r 20–26 kDa), and nuclear envelope lamins that play a key role in mitosis (44–74 kDa). The aromatic fatty acids can conjugate with coenzyme-A, enter the pathway to chain elongation, and interfere with lipid metabolism, in general [23]. Furthermore, compounds such as phenylacetate can assume a conformation resembling mevalonate pyrophosphate and inhibit MVA utilization specifically. Phenylacetate activity against poorly differentiated tissues, such as human glioblastoma cells [5] and the developing fetal brain [24], is associated with inhibition of MVA decarboxylation and a decline in protein isoprenylation. In this work, we showed that the effects on prenylation are not limited to brain cells or to phenylacetate; melanoma cells treated with phenylacetate had a marked decline in protein prenylation. Several analogues of phenylacetate were even more potent inhibitors of protein prenylation. This effect, like cytostatic activity,

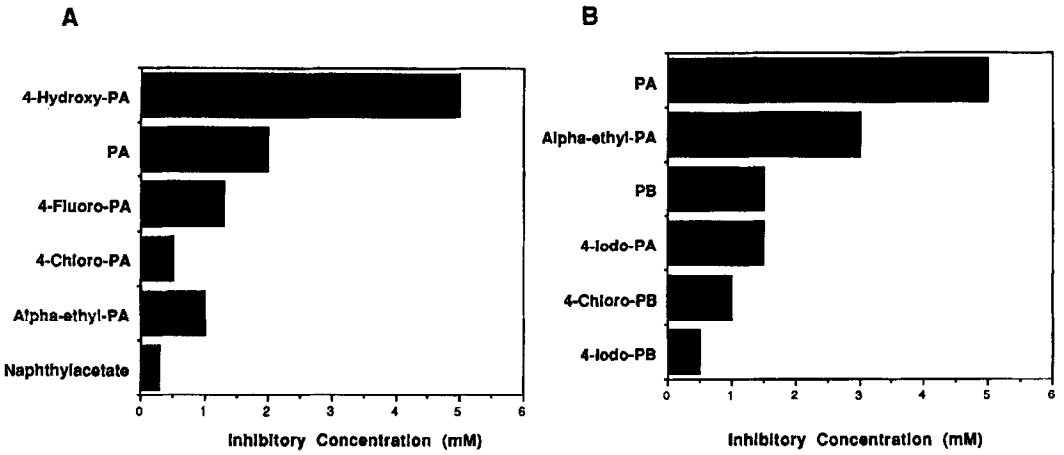

Fig. 3. Phenotypic reversion induced by phenylacetate (PA) and derivatives. The malignant prostatic PC3 cells were treated as described in Materials and Methods. Data indicate the relative potency of tested compounds in significantly inhibiting (≥80%) PC3 anchorage-independence (A) and completely blocking Matrigel invasion (B). Phenylacetate and analogs are presented in an increasing order of CLOGP (top to bottom). CLOGP values are provided in Tables 1 and 2. The effect on anchorage-dependency was confirmed with U87 cells (not shown). PB = phenylbutyrate.

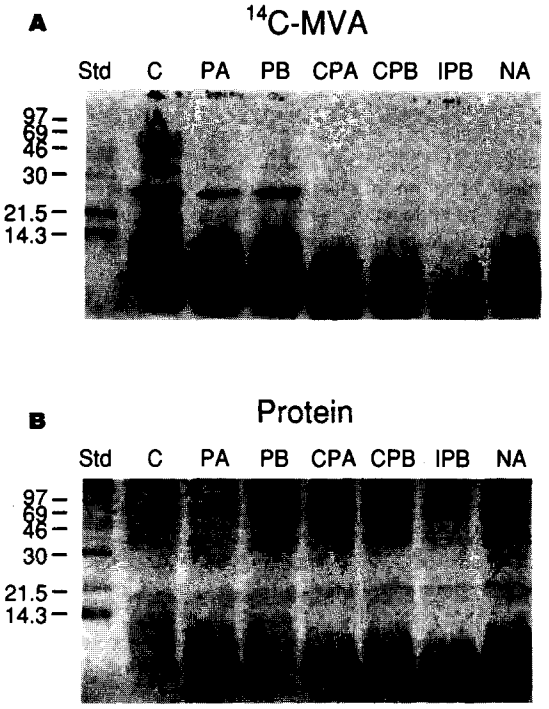

Fig. 4. Inhibition of protein prenylation by phenylacetate analogues. (A) Autoradiogram of protein from melanoma (1011) cells labeled for 24 hr with 16 μCi [^{14}C]MVA in the presence of 10 mM phenylacetate (PA), 3 mM phenylbutyrate (PB), 3 mM 4-Chlorophenylacetate (CPA), 2 mM 4-Chlorophenylbutyrate (CPB), 1 mM 4-Iodophenylbutyrate (IPB), or 3 mM naphthylacetate (NA). (B) Coomassie staining of the same gel.

changed with the lipophilicity of the analogues. The suppression of prenylation was not specific to any of the labeled proteins. Some affected proteins were in the M_r range of G-like proteins. Studies focusing on immunoprecipitable ras protein from cell membranes demonstrated that it was decreased by pretreatment with phenylacetate and phenylbutyrate.* Rapidly developing plant tissues are also highly dependent on intracellular MVA metabolism for cell replication [25]. Inhibition of MVA utilization by phenylacetate-related compounds could thus be responsible, in part, for their cytostatic effect documented in such highly divergent organisms.

In addition to affecting post-translational processing of critical proteins, phenylacetate and its analogues may alter gene expression through: (a) inhibition of DNA methylation, an epigenetic mechanism controlling the transcription of various eukaryotic genes, and (b) activation of transcription factor(s). Phenylacetate inhibits DNA methylation in plant [26] and mammalian cells [27], and both phenylacetate and phenylbutyrate were shown to activate the expression of otherwise dormant methylation-dependent genes [3, 13, 28, 29]. Also, we now have evidence that phenylacetate, phenylbutyrate and several analogs activate the human peroxisome proliferator activated receptor (PPAR), which functions as a transcriptional factor (unpublished data). PPAR is a member of a steroid nuclear receptor superfamily, the ligands of which are carboxylic acids and include well characterized differentiation inducers such as retinoids [30].

In conclusion, phenylacetate and analogs appear to represent a new class of pleiotropic growth regulators that may alter tumor cell biology by affecting gene expression at both the transcriptional and post-transcriptional levels. Phenylacetate and phenylbutyrate have already been established as safe and effective in the treatment of hyperammonemia [10, 11], and both drugs are now in clinical trials for the treatment of adults with cancer. Phase I studies with phenylacetate confirmed that millimolar levels can be achieved in the plasma and cerebrospinal fluid with no significant toxicities, and result in clinical improvement in patients with high-grade gliomas and hormone-refractory prostate cancer [2, 31]. However, rather large doses (300 mg/kg/day or more)

* Danesi R, Samid D and Myers CE, Manuscript submitted for publication.

are required to achieve therapeutic levels. The identified relationship between lipophilicity of analogs, their capacity to inhibit protein prenylation, and their selective antitumor activity in experimental models should lead to the identification of highly effective and safe antitumor agents suitable for clinical application.

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