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Phenylacetate Inhibits Protein Isoprenylation and Growth of the Androgen-Independent LNCaP Prostate Cancer Cells Transfected with the T24 Ha-*ras* Oncogene

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SUMMARY

The refractoriness of prostate cancer to androgen suppression is the landmark of clinically aggressive disease. In this study, the androgen-dependent LNCaP prostate cancer cells were transfected with the mutated c-Ha-*ras* gene from the T24 human bladder cancer. The derivative clone overexpressing T24-*ras* (LNCaP$^{T24\text{-}ras}$) proliferated in androgen-depleted medium and showed increased growth. Protein isoprenylation and p21*ras* farnesylation in LNCaP$^{T24\text{-}ras}$ cells were tested in the presence of phenylacetate to document a possible relationship with the drug-induced inhibition of cell proliferation. Phenylacetate is a differentiation inducer that down-regulates *in vitro* the expression of the *myc* oncogene and activates the human peroxisome proliferator-activated nuclear receptor involved in cell growth regulation. The drug inhibited protein isoprenylation and p21*ras* farnesylation in LNCaP$^{T24\text{-}ras}$ cells; IC_{50} values were 3.1 and 3.3 mM, respectively, compared with controls. The drug reduced the cellular levels of endogenous farnesyl-*PP* (mean IC_{50} = 3.5 mM) and inhibited activation of the p21*ras* downstream target, p42^{MAPK}/ERK2. LNCaP$^{T24\text{-}ras}$ was more sensitive than the parental line to both growth inhibition (mean IC_{50} = 3.01 and 7.1 mM, respectively) and apoptosis by phenylacetate. Exogenous farnesyl- and geranylgeranyl-*PP* indeed reduced the effects of the drug on proliferation and apoptosis in LNCaP$^{T24\text{-}ras}$ cells. In conclusion, the inhibition of protein isoprenylation and p21*ras* farnesylation by phenylacetate resulted in increased chemosensitivity of the androgen-independent LNCaP$^{T24\text{-}ras}$ cells compared with LNCaP, and this effect might contribute to the pharmacological activity of the drug.

Pharmacological suppression of the mitogenic activity of androgens is the mainstay for management of patients with advanced prostate cancer, a major cause of deaths in men due to cancer (1). However, tumor recurrence is characterized by androgen independence and rapidly progressive disease that is not affected by current treatment. The lack of valid therapeutic options for hormone-resistant prostate cancer has encouraged an extensive search at molecular levels to identify the mechanisms of disease progression and to find specific targets for effective therapy. The transition of prostate cancer to androgen independence is a poorly understood event. Previous research on the relationship between oncogene activation and prostate cancer has demonstrated that the v-Ki-*ras* oncogene is able to transform human prostate epithelial cells (2), and androgen-independent growth is observed after transfection of the human prostate cancer cells LNCaP with the v-Ha-*ras* oncogene (3). Furthermore, enhanced expression of the p21*ras* protein has been associated with increasing histological grade in human prostate neoplasms (4).

The function of a group of cellular proteins, including p21*ras*, is dependent on the attachment of isoprenoids derived from the mevalonic acid through a thioether linkage to a cysteine-containing consensus sequence at the —COOH terminus (5). Further processing includes the removal of three terminal amino acid residues and α-carboxyl-methyl esterification of the cysteine (5). Post-translational modifications of p21*ras* proteins include the attachment of a farnesyl moiety that is necessary for their biological activity (5). The isoprenoid farnesyl-*PP* is a particularly important intermediate in the mevalonate pathway; in addition to its role in the

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ABBREVIATIONS: FBS, fetal bovine serum; FPTase, farnesyl/protein transferase; NP-40, nonidet P-40; SDS, sodium dodecyl sulfate; DHT, 5α-dihydrotestosterone; MTS, 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TLC, thin layer chromatography.

processing of *ras*, it is used to synthesize cholesterol (6). Much of the interest in lipid modification has arisen out of the potential for pharmacological intervention; in particular, it would be desirable to target the post-translational modifications of oncogenic proteins.

Phenylacetate, an inducer of cell differentiation, suppresses prostate cancer cell proliferation *in vitro* (7). The drug inhibits the enzyme mevalonate-5-*PP* decarboxylase (8) as well as cholesterol synthesis (9). Phenylacetate is presently under clinical investigation for the treatment of advanced tumors, and a phase I trial has provided preliminary evidence that the drug has activity in patients with metastatic, hormone-refractory prostate cancer (10). For this reason, the aim of the current study was to investigate the role of protein isoprenylation inhibition in the antiproliferative effect of phenylacetate. The model used was a subline derived from the androgen-dependent human prostate cancer cells LNCaP after transfection with the mutated c-Ha-*ras* gene from the human bladder cancer T24. The derivative cell line LNCaP$^{T24\text{-}ras}$ shows androgen-independent growth and allowed us to study the ability of phenylacetate to target the post-translational modification of the activated *ras* oncoprotein.

Experimental Procedures

Reagents and drugs. RPMI-1640 and L-glutamine were obtained from GIBCO-BRL (Gaithersburg, MD), and FBS was obtained from HyClone (Logan, UT). [^{3}H]*RS*-mevalonolactone (50-60 Ci/mmol), [^{3}H]isopentenyl-*PP*, [^{3}H]farnesyl-*PP*, [^{3}H]geranylgeranyl-*PP* (30-60 Ci/mmol), farnesyl-*PP*, and geranylgeranyl-*PP* were from American Radiolabeled Chemicals (St. Louis, MO). The v-Ha-*ras* (Y13-259) and pan-*ras* valine-12 (DWP) antibodies were from Oncogene Science (Uniondale, NY); the antibodies to the β subunit of FPTase and to the extracellular signal-regulated kinase p42^{MAPK}/ERK2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Enlightening autoradiography enhancer and the bicinchoninic acid protein assay kit were from DuPont-NEN (Boston, MA) and Pierce (Rockford, IL), respectively. GammaBind Plus Sepharose was from Pharmacia (Uppsala, Sweden). Phenylacetate (sodium salt) was provided by Elan Pharmaceutical Research Corp. (Gainesville, GA). Lovastatin was from Biomol (Plymouth Meetings, PA); the water-soluble form was obtained as described previously (11). Nonidet P-40, protease inhibitors, and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell line and T24 Ha-*ras* transfection. The human androgen-dependent prostate cancer cell line LNCaP (12) was obtained from American Type Culture Collection (Rockville, MD) and cultivated in RPMI-1640 containing 10% heat-inactivated FBS and 2 mM L-glutamine. The *ras* expression vector pHO6T1 was a generous gift from Dr. A. D. Spandidos (Hellenic Anticancer Institute, Athens, Greece) and has been described previously (13). pHO6T1 was obtained by inserting the 6.6-kb T24 Ha-*ras* oncogene and the aminoglycoside phosphotransferase gene into the *Bam*HI site of pHO6 vector. The activated *ras* oncogene from the T24 human bladder cancer contains a glycine-to-valine substitution as a result of a point mutation at the 12th codon of the gene (13). Transient expression of the T24 Ha-*ras* oncogene was obtained by introducing pHO6T1 into LNCaP cells through electroporation (Cell-Porator, GIBCO-BRL). Cells were pulsed in the presence of 10 μg of pHO6T1 and cloned through selection with 0.5 mg/ml G418 (geneticin) for 14 days. Clones were expanded and analyzed for p21*ras* production through immunoblotting and androgen-independent growth (see below).

Immunoblot analysis of cellular proteins. LNCaP and LNCaP$^{T24\text{-}ras}$ cells were solubilized in lysis buffer (10 μl/ml NP-40, 50 mM Tris, pH 7.6, 2 mM EDTA, 100 mM NaCl, 20 μg/ml phenylmethylsulfonyl fluoride, and 5 μg/ml concentration each of antipain, pepstatin, and aprotinin) for 30 min at 4°. Lysates were centrifuged for 30 min at 14,000 rpm, and the protein concentration in the detergent-solubilized cells was measured. Aliquots of 100 μg of proteins were boiled in SDS-sample buffer (50 mM Tris, pH 6.8, 20 mg/ml SDS, 100 mM dithiothreitol, 100 μl/ml glycerol, and 0.25 mg/ml bromphenol blue), separated on a 4-20% SDS-polyacrylamide gel, and blotted onto Immobilon-P (Millipore, Bedford, MA) with a Multiphor II NovaBlot cell (Pharmacia, Piscataway, NJ). Blots were probed with the p21*ras* antibody (DWP, 1:50) and detected with the use of horseradish peroxidase-conjugated secondary antibody (ECL Western detection kit, Amersham, Arlington Heights, IL). Ten clones were analyzed; the subclone expressing the highest amount of mutated p21*ras* (named LNCaP$^{T24\text{-}ras}$) was expanded and used throughout the study. The effect of phenylacetate on FPTase levels was evaluated through the use of immunoblotting analysis of the β subunit of FPTase in detergent-solubilized LNCaP$^{T24\text{-}ras}$ cells after drug treatment at 2-10 mM for 24 hr. FPTase is the enzyme involved in the transfer of the farnesyl moiety to p21*ras*, and the β subunit is a 46-kDa peptide specific to FTPase (14). Blots were probed with the antibody (1:500) and detected as reported above. As a marker of the activation of the p21*ras* signal transduction pathway, the cellular kinase p42^{MAPK}/ERK2 was analyzed in LNCaP$^{T24\text{-}ras}$ cells treated with phenylacetate (3-10 mM) for 48 hr. p42^{MAPK}/ERK2 immunoblotting was performed as described previously (15); blots were probed with the antibody (1:150), and detection was performed as detailed above. Band densities were measured by video imaging densitometry as reported in Analysis of data.

Analysis of LNCaP$^{T24\text{-}ras}$ growth characteristics. The proliferation of parental and LNCaP$^{T24\text{-}ras}$ cells in steroid-depleted medium was evaluated in 96-well plates containing 2.5×10^3 cells/well in 100 μl of RPMI 1640 supplemented with 10% charcoal-stripped FBS and incubated for 24-96 hr. In separate wells, the androgen agonist DHT 10^{-9} M was added to the medium. Cell growth was measured by the reduction of tetrazolium salt MTS (Promega, Madison, WI). At the end of the incubation, 30 μl of MTS/phenazine methosulfate (20:1, v/v) was added to each well, and the plates were incubated for 2 hr at 37°. Cell viability was evaluated through absorbance reading at 490 nm in a microplate reader (Bio-Rad, Melville, NY).

Cell cycle analysis was performed in exponentially growing parental and LNCaP$^{T24\text{-}ras}$ cells. Cultures were washed with phosphate-buffered saline, pH 7.4, and harvested, and cells (2×10^6) were lysed at 4° for 2 hr in 1 mg/ml sodium citrate, 3 μl/ml NP-40, 50 μg/ml propidium iodide, and 100 μg/ml boiled RNase A. DNA analysis was performed with a FACStar cytofluorimeter (Becton Dickinson, San Jose, CA). Cell cycle distribution data were computed with the CellFIT software (Becton Dickinson, San Jose, CA), and the results of triplicate experiments are presented as the percentage of cells in each cycle phase.

Measurement of protein isoprenylation. Post-translational protein isoprenylation was evaluated as reported previously (16). Briefly, LNCaP and LNCaP$^{T24\text{-}ras}$ cells were treated with phenylacetate (2-10 mM) for 6 hr and then labeled with [^{3}H]*RS*-mevalonolactone (100 μCi/ml) for 18 hr. The HMG-CoA reductase inhibitor lovastatin (30 μM) was added 6 hr before [^{3}H]*RS*-mevalonolactone to inhibit endogenous synthesis of mevalonic acid. Detergent-solubilized cells were obtained as detailed in Immunoblot analysis of cellular proteins. Aliquots of 100 μg of proteins were boiled in SDS-sample buffer and separated on 12.5% SDS-polyacrylamide gel. Gels were stained with Coomassie Brilliant blue and equilibrated for 30 min in fluorography enhancer, and dried gels were fluorographed for 4-12 days. Band intensities were quantified through video imaging densitometry as reported in Analysis of data, and the IC_{50} of protein prenylation was calculated. Protein isoprenylation was also evaluated by labeling cells with the isoprenoids [^{3}H]farnesyl-*PP* and [^{3}H]geranylgeranyl-*PP* in the presence of phenylacetate. LNCaP and

LNCaP$^{T24\text{-}ras}$ cells were exposed to the drug at 4 mM for 6 hr and then labeled with [^{3}H]farnesyl-*PP* and [^{3}H]geranylgeranyl-*PP* (35 μCi/ml each) for 18 hr. Proteins from detergent-solubilized cells were size-fractionated on SDS-polyacrylamide gels and fluorographed as reported previously (16).

Measurement of p21*ras* farnesylation. Detergent-solubilized LNCaP$^{T24\text{-}ras}$ cells treated with phenylacetate (1-10 mM) and labeled with [^{3}H]*RS*-mevalonolactone were obtained as described in Measurement of protein isoprenylation. As an alternative labeling procedure, cells were incubated with [^{3}H]farnesyl-*PP* 50 μCi/ml and treated with 2-10 mM phenylacetate. The immunoprecipitation of p21*ras* was performed as described previously (16). Briefly, protein concentration in the detergent-solubilized cells was adjusted to 3 mg/ml, and samples were mixed with the anti-p21 *ras* antibody Y13-259 (20 μl/ml of cell extract) at 4° for 6 hr. Immune complexes were precipitated for 12 hr at 4° through the addition of 70 μl of a 15% suspension of protein G/Sepharose beads in lysis buffer. The immunoprecipitates were collected through centrifugation at 14,000 rpm at 4° and washed with 50 mM Tris, pH 6.8. Pellets were dissolved in 20 μl of SDS-sample buffer and boiled. SDS-polyacrylamide gel electrophoresis, fluorography, and video imaging densitometry were performed as described in Measurement of protein isoprenylation and Analysis of data.

TLC of polyisoprenyl-*PP*. LNCaP$^{T24\text{-}ras}$ cells were treated with phenylacetate (1-10 mM) and then labeled with [^{3}H]*RS*-mevalonolactone as reported in Measurement of protein isoprenylation. Cells were solubilized in lysis buffer, and the residual particulate material was removed through centrifugation at 4° for 30 min at 14,000 rpm. Extraction and dephosphorylation of cell lysates were performed as reported previously (17). Lipids were separated through reverse-phase TLC with LKC-18 plates (Whatman, Maidstone, UK) developed with acetone/water (7:1). Plates were sprayed with fluorography enhancer and exposed to a Kodak XOMAT-AR film at −70° for 14-18 days. The positions of [^{3}H]farnesol and [^{3}H]geranylgeraniol were identified through comparison with authentic standards. The radioactive spots corresponding to [^{3}H]farnesol and [^{3}H]geranylgeraniol were scraped from the TLC plates and counted with liquid scintillation. The IC_{50} values of phenylacetate on [^{3}H]farnesol and [^{3}H]geranylgeraniol production by LNCaP$^{T24\text{-}ras}$ cells compared with untreated cultures were calculated as reported in Analysis of data.

Assay of cell growth inhibition by phenylacetate. Parental and LNCaP$^{T24\text{-}ras}$ cells were plated at 1×10^3 cells/35-mm wells in 1 ml of medium used for cell propagation; 24 hr later, phenylacetate (0.1-10 mM) was added. Plates were incubated for 144 hr; then, cell proliferation was evaluated by counting colonies of >50 cells with a phase-contrast Leitz DM IL inverted microscope (Leitz, Heerbrugge, Germany). The protective effect of isoprenoids against phenylacetate growth inhibition was evaluated by adding farnesyl-*PP* and geranylgeranyl-*PP* (3 μM each) to the culture medium every other day from the first day of drug treatment, and clonogenic survival was evaluated by colony counting. The IC_{50} of phenylacetate on cell growth was obtained as reported in Analysis of data.

Assay of apoptosis by phenylacetate. Quantitative assay of apoptosis in LNCaP and LNCaP$^{T24\text{-}ras}$ cells was carried out with the Cell Death Detection enzyme-linked immunosorbent assay (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's instructions. This method is based on the photometric sandwich immunoassay of cytoplasmic histone-associated DNA fragments. Cells (1×10^4) were seeded into 96-well plates and treated with 2-10 mM phenylacetate for 24 hr; the protective effect of isoprenoids on drug-induced apoptosis was also evaluated by adding farnesyl-*PP* (3 μM) and geranylgeranyl-*PP* (3 μM) to the culture medium with phenylacetate. Results are given as enrichment factor; i.e., the specific enrichment of oligonucleosomes produced by apoptotic cells is calculated as follows: absorbance (A_{405nm}/A_{490nm}) of treated cells divided by absorbance (A_{405nm}/A_{490nm}) of control cells.

Analysis of data. Film densities from protein prenylation and immunoblotting assays were quantified through video imaging densitometry with the Kontron Imaging system KS300 (Kontron Elektronik, Eching, Germany) connected to a JVC TK-1280E color video camera (JVC, Tokyo, Japan). Results of treatments were expressed as percentage of absorbance of the fluorogram signals compared with controls. The IC_{50} values of phenylacetate on protein isoprenylation, p21*ras* farnesylation, farnesyl-*PP* and geranylgeranyl-*PP* production, and cell growth were obtained with nonlinear regression fitting. Results were subjected to analysis of variance followed by the Student-Newman-Keuls test; statistical significance was taken at values of $p < 0.05$.

Results

Cell line transfection, p21*ras* production, and growth characteristics. LNCaP cells were transfected with the vector pHO6T1 carrying the c-Ha-*ras* oncogene from the T24 human bladder cancer and selected in G418-containing medium. Cells were analyzed for the presence of p21*ras* protein by immunoblotting; 10 clones were identified, and 1 (C8) was found to produce the highest amount of the mutated oncoprotein p21*ras* (Fig. 1). Therefore, the results reported in the current study refer to the LNCaP$^{T24\text{-}ras}$ subclone C8. In the majority of derivative cell lines, immature p21*ras* was shown in the immunoblots (Fig. 1).

No detectable differences in cellular morphology were observed between parental and transfected cells. However, large clumps of cells loosely attached to the surface of culture flasks were frequently observed in LNCaP$^{T24\text{-}ras}$ cells, whereas this finding was uncommon in LNCaP cells. The percentage of LNCaP$^{T24\text{-}ras}$ cells in G_0/G_1 phase was reduced compared with the parental LNCaP (58.5 ± 1.2% versus 71.3 ± 2.5%, $p < 0.05$), whereas the percentage of cell population in S (28.2 ± 3.4% versus 15.2 ± 0.9%, $p < 0.05$) and G_2/M phases (23.3 ± 1.4% versus 13.5 ± 1.2%, $p < 0.05$) was increased. The acquisition of an androgen-independent phenotype by LNCaP$^{T24\text{-}ras}$ was demonstrated by its ability to proliferate in steroid-depleted medium, whereas LNCaP cells did not grow in the same condition (Fig. 2). DHT 10^{-9} M restored the growth of parental LNCaP but had no effect on LNCaP$^{T24\text{-}ras}$ (Fig. 2).

Inhibition of protein prenylation and p21*ras* farnesylation by phenylacetate. To test whether drug treatment affected isoprenylation of proteins in LNCaP and LNCaP$^{T24\text{-}ras}$, extracts from cells treated with 2-10 mM phenylacetate and labeled with 100 μCi/ml [^{3}H]*RS*-mevalonolactone were resolved with the use of SDS-polyacrylamide gel electrophoresis, and isoprenylated proteins were visualized with the use of gel fluorography (Fig. 3). The radioactivity derived from [^{3}H]*RS*-mevalonolactone labeling of untreated cells was concentrated in bands corresponding to molecular masses of 43 and 21-26 kDa (Fig. 3), as well as at

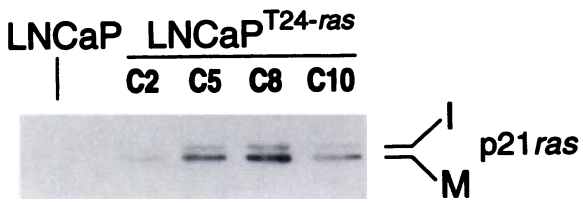

Fig. 1. Expression of T24-*ras* protein in prostate cancer cells. LNCaP and LNCaP$^{T24\text{-}ras}$ cells were analyzed through immunoblotting with the antibody to the mutated p21*ras*. *C2*, *C5*, *C8*, and *C10*, different subclones obtained after T24-*ras* oncogene transfer in LNCaP cells. *I*, Immature, nonprenylated *ras*; *M*, mature, prenylated *ras*.

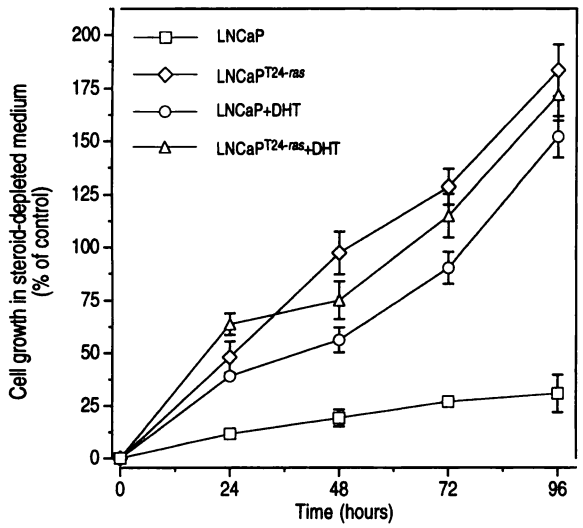

Fig. 2. LNCaP and LNCaP$^{T24\text{-}ras}$ prostate cancer cell proliferation in steroid-depleted medium. Cell growth was estimated on the basis of the bioreduction of the MTS compound; in separate plates, DHT 10^{-9} M was added to the cell culture medium to evaluate its effect on proliferation. Values are mean ± standard error from triplicate experiments. *Bars*, standard error.

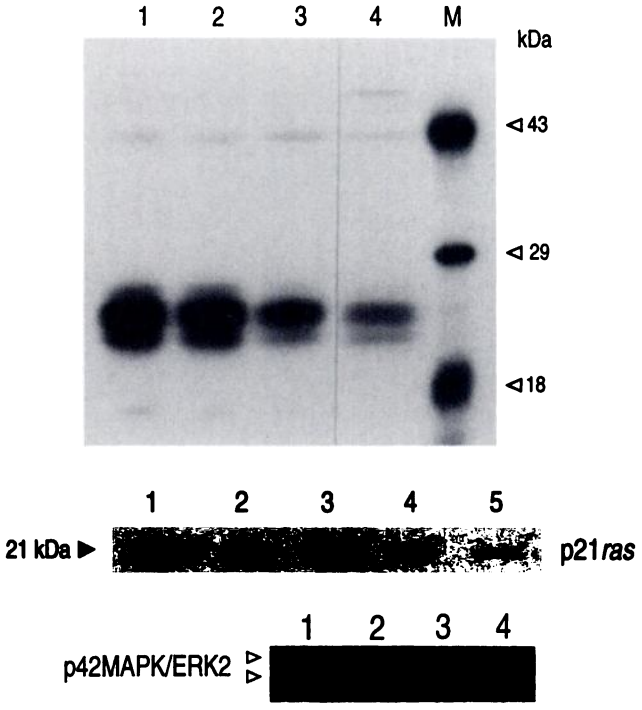

Fig. 3. *Top*, inhibition of protein isoprenylation by phenylacetate. LNCaP$^{T24\text{-}ras}$ received vehicle (*lane 1*) or 2, 4, and 10 mM phenylacetate (*lanes 2-4*) and were labeled with [^{3}H]*RS*-mevalonolactone. *Right*, protein markers (*M*). Quantification of this fluorogram indicated that the cumulative density of *lanes 2-4* was 79.8%, 39.6%, and 27.9%, respectively, of that of *lane 1*. For comparison, fluorographic analysis of LNCaP cells demonstrated that the mean cumulative density of protein bands after treatment with 2, 4, and 10 mM phenylacetate was 84.5%, 45.9%, and 38.6%, respectively, of control. *Middle*, inhibition of p21*ras* farnesylation by phenylacetate. Cells received vehicle (*lane 1*) or 1, 2, 4, and 10 mM phenylacetate (*lanes 2-5*) and were labeled with [^{3}H]*RS*-mevalonolactone. p21*ras* was immunoprecipitated, and the gels were fluorographed. Quantification of this fluorogram indicated that the density of *lanes 2-5* was 79.5%, 75.7%, 30.8%, and 14.7%, respectively, of that of *lane 1*. *Bottom*, inhibition of p42^{MAPK}/ERK2 activation by phenylacetate in LNCaP$^{T24\text{-}ras}$ cells. Immunoblotting of p42^{MAPK}/ERK2 shows the unphosphorylated and inactive lower band compared with the phosphorylated, active upper band. *Lane 1*, control. *Lanes 2-4*, 3, 6, and 10 mM phenylacetate. A slightly lower amount of protein was transferred on the membrane in *lane 4*.

the dye front (not shown). Cells treated with phenylacetate exhibited a dose-responsive decrease in the intensity of the 43- and 21-26-kDa protein labeling (Fig. 3). Image analysis of films demonstrated the progressive reduction in the absorbance of protein bands from treated cells compared with controls; IC_{50} of phenylacetate was 3.6 ± 0.7 mM in LNCaP and 3.1 ± 0.6 mM in LNCaP$^{T24\text{-}ras}$ cells. Statistical analysis of the absorbance values demonstrated that protein prenylation was significantly reduced by phenylacetate as low as 2 mM in both cell lines ($p < 0.05$ versus controls). Drug concentrations of 2-4 mM are clinically achievable in humans without serious toxicity (10). In addition, the radioimmune precipitation analysis of p21*ras* from LNCaP$^{T24\text{-}ras}$ cells labeled with 100 μCi/ml of [^{3}H]*RS*-mevalonolactone demonstrated that the farnesylation of the protein was also decreased in a dose-dependent fashion by phenylacetate 1-10 mM (Fig. 3); the calculated IC_{50} was 3.3 ± 0.6 mM. Treatment did not change the amount of p21*ras* in cells, as demonstrated by Western blot analysis (data not shown). In addition, 3-10 mM phenylacetate reduced the amount of the active, phosphorylated form of the p21*ras* downstream cellular target, p42^{MAPK}/ERK2, a mitogen-activated protein kinase, in LNCaP$^{T24\text{-}ras}$ cells (Fig. 3). Detectable levels of p42^{MAPK}/ERK2 were not shown in parental LNCaP cells.

The inhibitory effect of phenylacetate on protein isoprenylation was reversed by cultivating cells with 35 μCi/ml each of [^{3}H]farnesyl-*PP* and [^{3}H]geranylgeranyl-*PP*. In LNCaP$^{T24\text{-}ras}$ cells incubated with [^{3}H]polyisoprenyl-*PP* and phenylacetate 4 mM, the pattern of protein labeling was almost identical to that generated by incubation of cells with 100 μCi/ml of [^{3}H]*RS*-mevalonolactone without phenylacetate (Fig. 4). Equivalent results were obtained in LNCaP parental cells. In accordance with this finding, the drug did not inhibit the transfer of the farnesyl moiety to p21*ras* in LNCaP$^{T24\text{-}ras}$ because the amount of protein labeled with exogenous [^{3}H]farnesyl-*PP* was increased by phenylacetate (Fig. 4). This finding suggests that p21*ras* farnesylation can be restored by farnesyl-*PP* supplementation in the culture medium. Compared with the effect produced by the highest concentration of the drug, the half-maximal increase in farnesylated p21*ras*, as calculated through image analysis, was obtained with 3.1 ± 0.7 mM phenylacetate. No significant changes in the levels of the FPTase β subunit in LNCaP$^{T24\text{-}ras}$ cells could be demonstrated during drug treatment (Fig. 4). However, phenylacetate produced a dose-responsive reduction in the amount of farnesyl-*PP* and geranylgeranyl-*PP* synthesized by cells, as demonstrated by the decrease in [^{3}H]farnesol and [^{3}H]geranylgeraniol extracted from LNCaP$^{T24\text{-}ras}$ cells and measured through TLC (Fig. 5). The mean IC_{50} values for [^{3}H]farnesol and [^{3}H]geranylgeraniol production were 3.5 and 3.4 mM, respectively. In LNCaP cells, these values were 3.8 and 3.7 mM, respectively. This effect seems to account for the inhibition of p21*ras* farnesylation on treatment with phenylacetate. This finding, together with the evidence that the drug enhanced the incorporation of [^{3}H]isopentenyl-*PP* in cells (data not shown), suggests that the level of inhibition of the mevalonate pathway is upstream the step catalyzed by the FPTase.

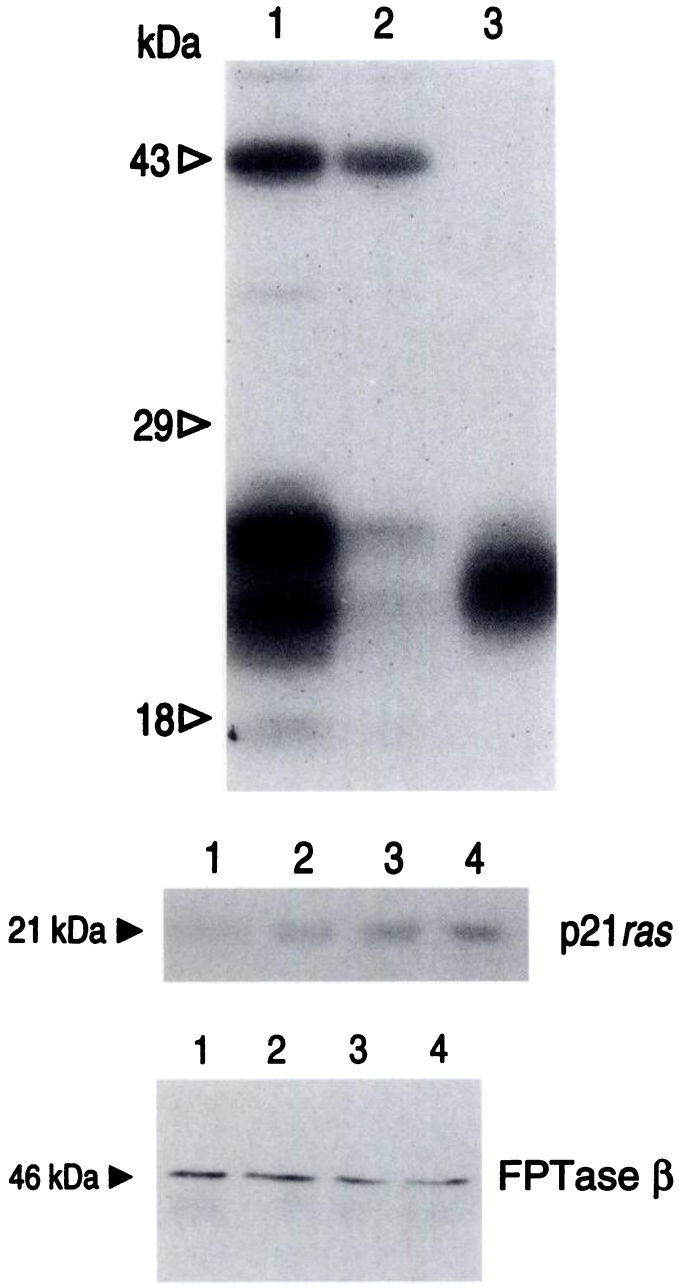

Fig. 4. *Top*, incorporation of [^{3}H]farnesyl- and [^{3}H]geranylgeranyl-moieties in LNCaP$^{T24\text{-}ras}$ proteins. LNCaP$^{T24\text{-}ras}$ cells were labeled with [^{3}H]*RS*-mevalonolactone (*lane 1*) or treated with 4 mM phenylacetate and then labeled with [^{3}H]farnesyl-*PP* (*lane 2*) and [^{3}H]geranylgeranyl-*PP* (*lane 3*). Quantification of this fluorogram indicated that the cumulative density of protein bands in [^{3}H]farnesyl- and [^{3}H]geranylgeranyl-*PP*-labeled LNCaP$^{T24\text{-}ras}$ cells was 85% of that obtained in cells labeled with [^{3}H]*RS*-mevalonolactone only. *Middle*, enhanced incorporation of [^{3}H]farnesyl moiety in p21*ras* by phenylacetate. LNCaP$^{T24\text{-}ras}$ cells received no treatment (*lane 1*) or 2, 4, and 10 mM phenylacetate (*lanes 2-4*) and were labeled with [^{3}H]farnesyl-*PP*. p21*ras* was immunoprecipitated, and the gels were fluorographed. Quantification of this fluorogram indicated that the density of *lanes 2-4* was 130.1%, 175.2%, and 209.2%, respectively, of that of *lane 1*. *Bottom*, immunoblot analysis of the FPTase β subunit. LNCaP$^{T24\text{-}ras}$ cells received vehicle (*lane 1*) or 2, 4, and 10 mM phenylacetate (*lanes 2-4*). Cells were analyzed through immunoblotting with the antibody to the FPTase β subunit.

Cell growth inhibition and apoptosis by phenylacetate. A dose-dependent inhibition of prostate cancer cell growth was obtained after treatment with phenylacetate for

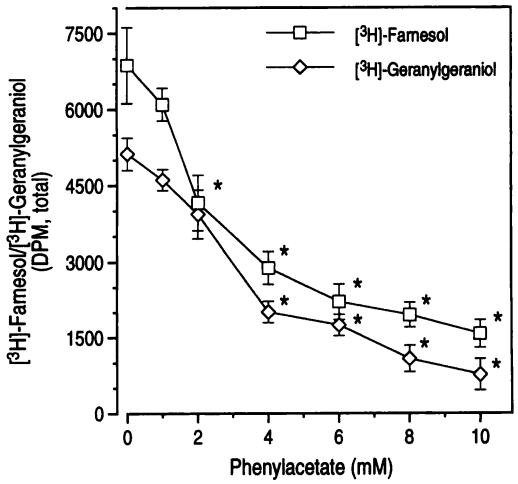

Fig. 5. Inhibition of [^{3}H]farnesyl-*PP* and [^{3}H]geranylgeranyl-*PP* production by phenylacetate. LNCaP$^{T24\text{-}ras}$ cells were treated with phenylacetate and labeled with of [^{3}H]*RS*-mevalonolactone. Dephosphorylated lipids were separated by TLC; [^{3}H]farnesol and [^{3}H]geranylgeraniol were recovered and counted by liquid scintillation. Values are mean ± standard error of triplicate experiments. *, $p < 0.05$ versus no treatment. *Bars*, standard error.

144 hr, as evaluated with the clonogenic survival assay. This effect was more pronounced in the LNCaP$^{T24\text{-}ras}$ cells compared with the parental LNCaP cells (Fig. 6); the IC_{50} values of phenylacetate were 3.01 ± 0.4 and 7.1 ± 0.8 mM, respectively, with the between-group mean difference being signif-

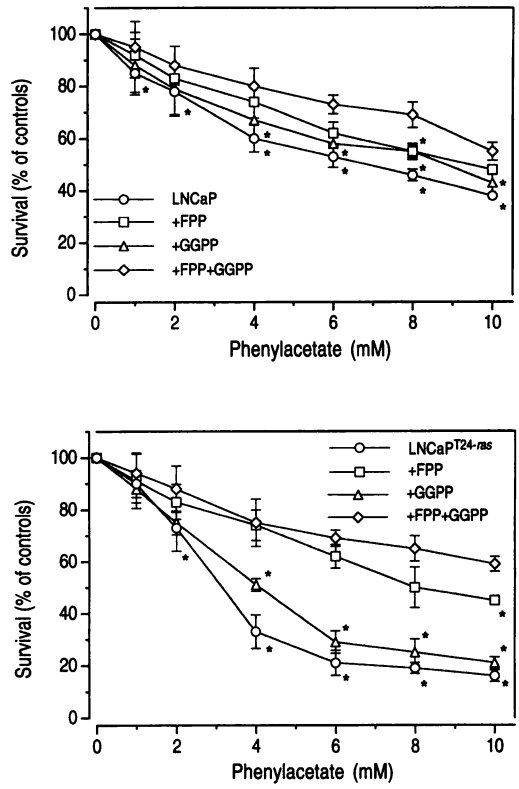

Fig. 6. Inhibition of prostate cancer cell growth by phenylacetate. LNCaP (*top*) and LNCaP$^{T24\text{-}ras}$ cells (*bottom*) were treated with phenylacetate for 144 hr. In separate plates, farnesyl-*PP* (*FPP*) and geranylgeranyl-*PP* (*GGPP*) (3 μM each) were added to the culture medium, and cell proliferation was measured with the clonogenic survival assay. Values are mean ± standard error of triplicate experiments. *Bars*, standard error. *, $p < 0.05$ versus phenylacetate.

icant ($p < 0.05$). The supplementation of cell culture medium with farnesyl-*PP* and geranylgeranyl-*PP* (3 μM each) significantly protected LNCaP and LNCaP$^{T24\text{-}ras}$ cells from the growth inhibition of phenylacetate. The maximum protection was obtained with the combination of both polyisoprenyl-*PP* compounds (mean IC_{50} = >10 mM in both cell lines) (Fig. 6). If they were added separately to the culture medium, the resulting effect was reduced (Fig. 6), and the mean calculated IC_{50} values with farnesyl-*PP* and geranylgeranyl-*PP* were 7.9 and 4.1 mM in LNCaP$^{T24\text{-}ras}$ and 9.5 and 8.5 mM in LNCaP, respectively. Farnesyl-*PP* or geranylgeranyl-*PP* of >3 μM did not provide additional protection (data not shown).

After a 24-hr treatment of LNCaP and LNCaP$^{T24\text{-}ras}$ cells with phenylacetate, apoptotic death was demonstrated at concentrations as low as 2 mM through the immunoassay of oligonucleosomes released by cells (Fig. 7). Based on the enrichment factor, LNCaP$^{T24\text{-}ras}$ cells showed an increased sensitivity to the effect of phenylacetate compared with LNCaP cells, as drug-induced apoptosis was more evident in T24-*ras*-transfected cells than in parental cells (Fig. 7). Medium supplementation with farnesyl-*PP* and geranylgeranyl-*PP* substantially protected cells from drug-induced apoptosis, with farnesyl-*PP* being more effective than geranylgeranyl-*PP* in preventing apoptosis in phenylacetate-treated cells (Fig. 7). Extension of the time of treatment to 48 hr produced a higher degree of apoptosis, with mean enrichment factor values of 14.1 and 6.8 in LNCaP$^{T24\text{-}ras}$ and LNCaP cells at 10 mM phenylacetate, respectively. Lovastatin alone inhibited LNCaP and LNCaP$^{T24\text{-}ras}$ cell growth (mean IC_{50} = 60 and 32 μM after 144 hr, respectively), and apoptosis was detectable at concentrations of >55 and >35 μM, respectively. However, 30 μM lovastatin did not significantly enhance growth inhibition or apoptosis by 2-10 mM phenylacetate (data not shown).

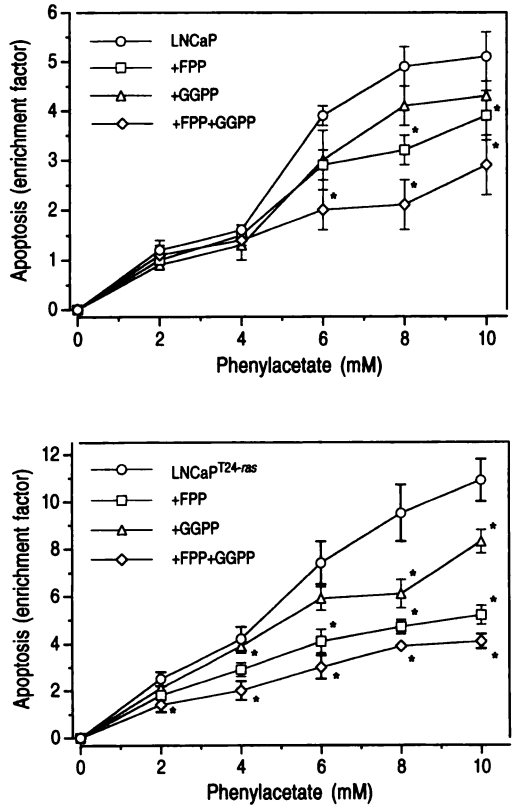

Fig. 7. Induction of apoptosis by phenylacetate. DNA fragmentation in parental (*top*) and T24 Ha-*ras*-transfected (*bottom*) LNCaP cells was evaluated with enzyme-linked immunosorbent assay of histone-tagged DNA fragments. In separate plates, farnesyl-*PP* (*FPP*) and geranylgeranyl-*PP* (*GGPP*) (3 μM each) were added to the culture medium. Results are expressed as enrichment factor. Values are mean ± standard error of triplicate experiments. *Bars*, standard error. *, $p < 0.05$ versus phenylacetate.

Discussion

Phenylacetate is an investigational chemotherapeutic agent for the treatment of solid human tumors, in particular, central nervous system neoplasms and prostate cancer. Recent studies demonstrated that phenylacetate is an inhibitor of several tumor cell lines of different histogenesis, including prostate cancer (7), malignant glioma (9), rhabdomyosarcoma (18), leukemia (19), and melanoma (20). Phenylacetate inhibits the mevalonate-5-*PP* decarboxylase (8), thus reducing the biosynthesis of isopentenyl-*PP* and cholesterol (10). However, no direct evidence has been provided of the role of the inhibition of isoprenoid biosynthesis in cell growth suppression by phenylacetate.

The biological properties conferred to prostate cancer cells through transfection with the activated T24 Ha-*ras* oncogene represent an useful model with which to study the chemotherapeutic effect of phenylacetate. Recent research on the post-translational modification of a subset of cellular proteins, including p21*ras* and other G proteins of the *ras* superfamily, has provided the clue for better understanding of the regulation of their function (5). Isoprenylation is a critical step for the subcellular localization and biological activity of signal transducing proteins, which play a pivotal role in cell growth control. Modification of proteins at the carboxyl-terminal cysteine residue(s) by the farnesyl- (C15) and geranylgeranyl- (C20) isoprenoid moieties seems to be a highly conserved post-translational process (5). Three distinct enzymes have been identified that prenylate proteins: FPTase and geranylgeranyl/protein transferase types I and II (6). Inhibitors of the HMG-CoA reductase, such as lovastatin, block the production of mevalonate and its metabolites, including farnesyl-*PP* and geranylgeranyl-*PP*, and have been shown to suppress the proliferation of many cell types (21). The data obtained in the current study suggest that the inhibition of protein isoprenylation and p21*ras* farnesylation by phenylacetate resulted in increased chemosensitivity of the transfected cell line compared with the parental cells.

Ras oncogenes may contribute to some of the biological features of prostate cancer. Neoplastic transformation of human prostate epithelial cells in culture was documented after transfection of activated Ki-*ras* (2). Poorly differentiated prostate cancer was produced at high frequency when *ras* plus *myc* oncogenes were transferred into both the mesenchymal and epithelial compartments of the urogenital sinus of transgenic mice (22). The introduction of the v-Ha-*ras* oncogene into nonmetastatic rat prostate cancer cell lines conferred a metastatic potential to some transfectants (23). Interestingly, the acquisition of hormone-independent *in vitro* growth was obtained after transfection of the hormone-sensitive LNCaP human prostate cancer cell line with the v-Ha-*ras* gene (3). This is an important finding because an-

drogen independence is the hallmark of clinically aggressive disease with unfavorable prognosis. Although studies on *ras* genes in experimental prostate cancer have been informative, data from clinical studies are controversial. Abnormal elevation in c-Ha-*ras* expression has been linked to disease progression (4), and the combination of *ras* gene mutations with human Papillomavirus infection were associated with advanced stages of the disease and higher Gleason scores (24).

In this study, T24-*ras*-transfected LNCaP cells displayed higher proliferative activity, and their steroid requirement for *in vitro* growth was greatly reduced compared with the parental cells. These findings are in agreement with a previous report (3) and indicate that a single oncogene could trigger the signalling events to bypass the hormone dependence of LNCaP cells. In addition, the results of the current study demonstrate that phenylacetate inhibits the isoprenylation of LNCaP$^{T24\text{-}ras}$ proteins and the farnesylation of p21*ras* as a possible result of the inhibition of farnesyl-*PP* production by cells. Moreover, this study provides the first evidence that prenylation inhibition plays an important role in the chemotherapeutic effect of phenylacetate because exogenous farnesyl-*PP* and geranylgeranyl-*PP* reduced the effects of the drug on cell growth, and farnesyl-*PP* was most active in LNCaP$^{T24\text{-}ras}$ cells. It should be pointed out, however, that polyisoprenyl-*PP* compounds are not simply protecting cells by restoring protein isoprenylation. Indeed, they are able to resume the biosynthesis of important products of mevalonic acid metabolism, including cholesterol.

Farnesyl-*PP* and geranylgeranyl-*PP* are bulky molecules with a highly charged phosphate group that could impair their ability to cross the cell membrane and achieve a significant intracellular concentration. It is conceivable that the phosphate group is removed before their entry into cells, where they are converted again into their pyrophosphate derivatives. Although the issue of transmembrane transfer of such molecules was not addressed in the current study, it is worth mentioning that the farnesyl-*PP* analogue α-(hydroxyfarnesyl) phosphonic acid, characterized by a stable phosphonic group, is indeed able to cross the cell membrane and inhibit p21*ras* farnesylation in whole cells (25). Therefore, it is conceivable that polyisoprenoids can cross the cell membrane to some extent as phyrophosphate derivatives or free alcohols via an unknown mechanism. Indeed, the results of the current study demonstrate that [^{3}H]farnesyl-*PP* and [^{3}H]geranylgeranyl-*PP* were able to restore prenylation in phenylacetate-treated cells, suggesting that the drug does not impair the activity of FPTase and geranylgeranyl/protein transferases. Moreover, the inhibition of p21*ras* farnesylation on treatment with phenylacetate was associated with a marked decrease in the level of the activated, phosphorylated p42^{MAPK}/ERK2 kinase in LNCaP$^{T24\text{-}ras}$ cells. This finding suggests that the inhibition of p21*ras* farnesylation might impair the ability of the oncoprotein to activate downstream cellular targets.

In this study, phenylacetate induced cell death through apoptosis, and LNCaP$^{T24\text{-}ras}$ cells were more sensitive than the parental cells. Nevertheless, farnesyl-*PP* and geranylgeranyl-*PP* protected cells against drug-triggered DNA cleavage. These results indicate that the inhibition of isoprenylated proteins, which play a specific function in cell proliferation, might be a factor contributing to the cell death, although other undetermined mechanisms cannot be ruled out. A relationship between inhibition of protein prenylation and apoptosis has been reported for the HMG-CoA reductase inhibitor lovastatin in the leukemia cell line HL-60 (26). Experimental evidence in favor of a role of inhibition of protein isoprenylation in triggering apoptosis is offered by the demonstration that isoprenylated proteins play a crucial role in the maintenance of the integrity of cellular structures, including lamins A and B (5), constituents of the nuclear lamina, and low molecular weight G proteins, including p21*rho*, which regulate cytoskeletal functions (27). Moreover, inhibition of protein isoprenylation impairs the association of lamins with the nuclear membrane (28) and induces actin depolymerization and alteration of cell morphology (11). Indeed, suppression of the mevalonate pathway in HL-60 cells blocked the membrane association of lamin A as a result of the inhibition of its post-translational modification (28). It is conceivable that the effect of reduced isoprenylation of nuclear lamin component proteins might be an augmentation of the rate or extent of apoptosis resulting from an undermining of nuclear envelope structure. However, a possible bias in the procedure used to detect apoptosis is that DNA fragmentation may not be essential to this process (29).

The molecular events of the increased sensitivity to phenylacetate of the LNCaP$^{T24\text{-}ras}$ cells compared with LNCaP cannot be elucidated on the basis of these results, and other studies have not provided a convincing explanation (30, 31). The immature form of *ras* has been found to inhibit the function of the activated GTP-bound *ras* protein (32). Although inhibitors of protein prenylation could also block the post-translational processing of other essential proteins, it has been suggested that the cell dependence on oncogenic *ras* function might render tumor cells more sensitive than normal cells to the activity of isoprenylation inhibitors (31). It is conceivable that cells committed to replication are able to recognize an imbalance between signals to grow (i.e., activated oncogenes) and signals to stop (i.e., chemotherapeutic agents), and this discordance is likely to switch cells to the suicide death program (33).

In addition to the inhibition of protein isoprenylation, phenylacetate affects gene expression in tumor cells, including transforming growth factor type β2 (7), tissue inhibitor of metalloproteinases-2, type IV collagenase, and histocompatibility locus antigen class I genes (20). The drug inhibits the activity of the urokinase plasminogen activator (7), as well as the expression of *myc* oncogene (19). Phenylacetate activates the human peroxisome proliferator-activated nuclear receptor involved in differentiation and cell growth regulation (34). These additional properties may explain the inability of pyrophosphate isoprenoids to completely reverse phenylacetate cytotoxicity.

In conclusion, results of the current study suggest that the isoprenylation inhibition is a mechanism by which phenylacetate exerts a portion of its antiproliferative effect. The finding that hormone-resistant LNCaP$^{T24\text{-}ras}$ cells displayed increased sensitivity to phenylacetate indicates that the isoprenylation inhibitors may be candidate drugs to be further evaluated for the treatment of prostate cancer refractory to antiandrogenic therapy, particularly when a mutational activation of *ras* can be demonstrated.

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