



US005605930A

United States Patent [19]**Samid**[11] **Patent Number:** **5,605,930**[45] **Date of Patent:** **Feb. 25, 1997**

[54] **COMPOSITIONS AND METHODS FOR
TREATING AND PREVENTING
PATHOLOGIES INCLUDING CANCER**

[75] Inventor: **Dvorit Samid**, Rockville, Md.[73] Assignee: **The United States of America as
represented by the Department of
Health and Human Services,**
Washington, D.C.[21] Appl. No.: **207,521**[22] Filed: **Mar. 7, 1994****Related U.S. Application Data**[63] Continuation-in-part of Ser. No. 135,661, Oct. 12, 1993,
which is a continuation-in-part of Ser. No. 779,744, Oct. 21,
1991.[51] Int. Cl.⁶ **A61K 31/21; A01N 37/00;**
A01N 47/40; A01N 47/46[52] U.S. Cl. **514/510; 514/513; 514/515;**
514/529; 514/538; 514/563; 514/567[58] Field of Search **514/538, 563,**
514/567, 510, 513, 515, 529[56] **References Cited****U.S. PATENT DOCUMENTS**

| | | | |
|-----------|---------|----------------------|---------|
| 3,973,022 | 8/1976 | Goschke | 514/311 |
| 3,976,673 | 8/1976 | Pifferi | 514/568 |
| 3,998,966 | 12/1976 | Fried et al. | 514/510 |
| 4,028,404 | 6/1977 | Bays et al. | 514/570 |
| 4,282,214 | 8/1981 | Flora et al. | 514/108 |
| 4,457,942 | 7/1984 | Brusilow . | |
| 4,470,970 | 9/1984 | Burzynski . | |
| 4,720,506 | 1/1988 | Munakata et al. | 514/538 |
| 5,204,367 | 4/1993 | Wright et al. | 514/453 |
| 5,244,922 | 9/1993 | Burzynski | 514/561 |

FOREIGN PATENT DOCUMENTS

| | | |
|-----------|---------|----------------------|
| A-0069232 | 6/1982 | European Pat. Off. . |
| POJ121610 | 7/1958 | New Zealand . |
| POJ135483 | 10/1966 | New Zealand . |
| POJ138064 | 8/1967 | New Zealand . |
| POJ137389 | 9/1967 | New Zealand . |
| POJ153383 | 8/1968 | New Zealand . |
| POJ141936 | 12/1969 | New Zealand . |
| POJ151395 | 3/1970 | New Zealand . |
| POJ153227 | 12/1970 | New Zealand . |
| POJ162983 | 3/1971 | New Zealand . |
| POJ162982 | 3/1971 | New Zealand . |
| POJ163320 | 4/1971 | New Zealand . |
| POJ158775 | 8/1971 | New Zealand . |
| POJ157388 | 12/1971 | New Zealand . |
| POJ157322 | 12/1971 | New Zealand . |
| POJ153684 | 2/1972 | New Zealand . |
| POJ175797 | 10/1974 | New Zealand . |
| POJ176382 | 1/1975 | New Zealand . |
| POJ179079 | 10/1975 | New Zealand . |
| POJ189518 | 1/1979 | New Zealand . |
| POJ192255 | 11/1979 | New Zealand . |
| POJ202921 | 12/1982 | New Zealand . |
| POJ214018 | 10/1985 | New Zealand . |
| POJ217230 | 8/1986 | New Zealand . |
| POJ217703 | 9/1986 | New Zealand . |
| POJ218235 | 11/1986 | New Zealand . |

| | | |
|-----------|---------|---------------|
| POJ218734 | 12/1986 | New Zealand . |
| POJ219560 | 3/1987 | New Zealand . |
| POJ221962 | 9/1987 | New Zealand . |
| POJ225311 | 7/1988 | New Zealand . |
| POJ229325 | 5/1989 | New Zealand . |
| POJ232250 | 1/1990 | New Zealand . |
| POJ233102 | 3/1990 | New Zealand . |
| POJ234143 | 6/1990 | New Zealand . |
| POJ235276 | 9/1990 | New Zealand . |

OTHER PUBLICATIONSNeish et al, "Phenyacetic Acid as a Potential Therapeutic
Agent for the Treatment of Human Cancer" *Experientia*, vol.
27:860-861 (1971).Smigel, K., *Non-toxic drug being tested to treat cancer and
anemias [news]*, *J. Natl. Cancer Inst.*, 84(18):1398 (Sep. 16,
1992).Ross, Philip D. and Subramanian, S., *Inhibition of sickle cell
hemoglobin gelation by some aromatic compounds*, *Bio-
chem. Biophys. Res. Commun.*, 77:1217-1223 (1977).Jones, G. L., *Anti sickling effects of Betw Di Ethylaminoet-
hylidiphenylpropyl acetate SFK-525-A*, *Pharmacologist*,
20(3):204 (1978).Erhum, Wilson O., *Acetonyl esters of hydroxybenzoic acids
as potential antisickling agents*, *Niger. J. Pharm.*,
12:285-287 (1981).Abemayor, E. et al., *Effects of retinoic acid on the in vivo
growth of human neuroblastoma cells*, *Cancer Lett. (Neth-
erlands)*, 55(1):1-5 (Nov. 19, 1990).Cinatl, J. et al., *In vitro differentiation of human neuroblas-
toma cells induced by sodium phenylacetate*, *Cancer Lett.*
(*Netherlands*), 70(1-2):15-24 (Jun. 15, 1993).Gorski, G. K. et al., *Synergistic inhibition of human rhab-
domyosarcoma cells by sodium phenylacetate and tretinoin*,
In vitro Cell. Dev. Biol., 29A:189-191 (Mar. 1993).

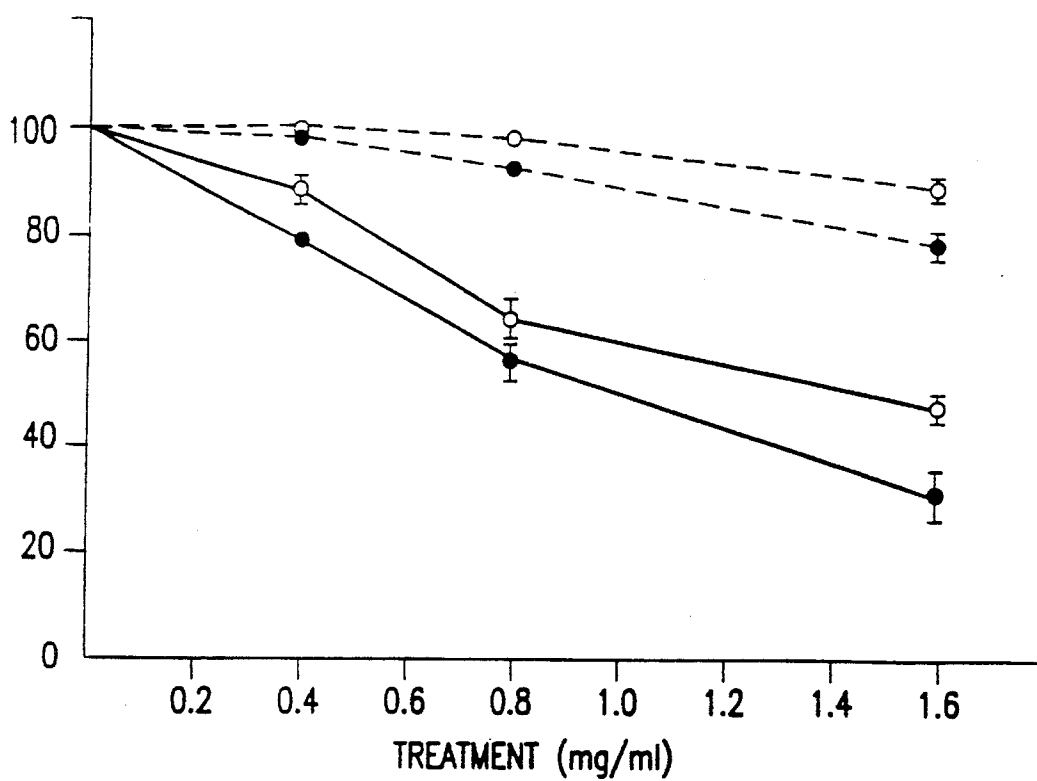
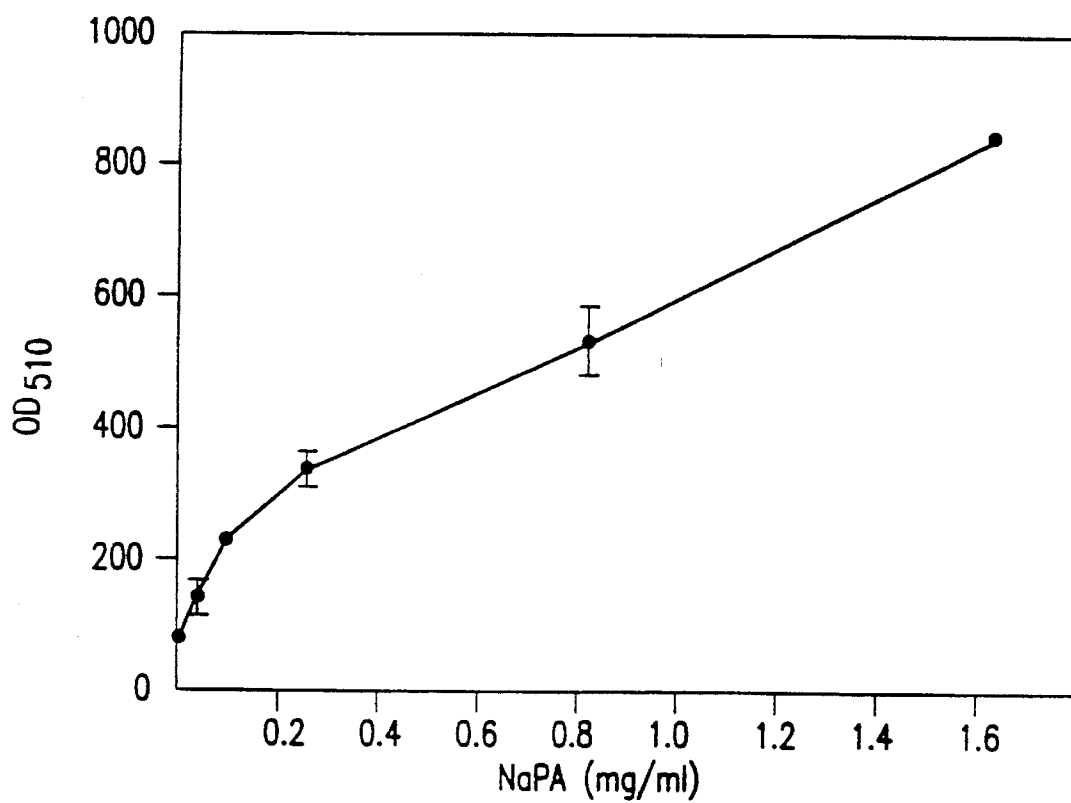
(List continued on next page.)

Primary Examiner—Nathan M. Nutter*Attorney, Agent, or Firm*—Needle & Rosenberg, P.C.[57] **ABSTRACT**

Compositions and methods of treating anemia, cancer, AIDS, or severe β -chain hemoglobinopathies by administering a therapeutically effective amount of phenylacetate or pharmaceutically acceptable derivatives thereof or derivatives thereof alone or in combination or in conjunction with other therapeutic agents including retinoids, hydroxyurea, and flavonoids. Intravesicle methods of treatment of cancers phenylacetate. Pharmacologically-acceptable salts alone or in combinations and methods of preventing AIDS and malignant conditions, and inducing cell differentiation are also aspects of this invention. A product as a combined preparation of phenylacetate and a retinoid, hydroxyurea, or flavonoid (or other mevalonate pathway inhibitor) for simultaneous, separate, or sequential use in treating a neoplastic condition in a subject. Methods of modulating lipid metabolism and/or reducing serum triglycerides in a subject using phenylacetate.

OTHER PUBLICATIONS

- Burzynski, S. R. et al., *Preclinical studies on antineoplaston AS2-1 and antineoplaston AS2-5*, *Drugs Exptl. Clin. Res.*, Supplemental 1, XII:11-16 (1986).
- Timothy J. Ley, et al., *5-Azacytidine Selectively Increases γ -globin Synthesis in a Patient with β^0 Thalassemia*, *New England Journal of Medicine*, vol. 307:1469-1475 (Dec. 8, 1982).
- Michael B. Sporn, et al., *Chemoprevention of Cancer with Retinoids*, *Federation Proceedings*, vol. 38:2528-2534 (Oct. 1979).
- Richard L. Momparler, et al., *Clinical Trial on 5-AZA-2'-Deoxycytidine in Patients with Acute Leukemia*, *Pharmac. Ther.*, vol. 30:277-286 (1985).
- Gary J. Kelloff, et al., *Chemoprevention Clinical Trials*, *Mutation Research*, vol. 267:291-295 (1992).
- I. Bernard Weinstein, *Cancer Prevention: Recent Progress and Future Opportunities*, *Cancer Research*, vol. 51:5080s-5085s (1991).
- Olli Simell, et al., *Waste Nitrogen Excretion Via Amino Acylation: Benzoate and Phenylacetate in Lysinuric Protein Intolerance*, *Pediatr. Res.*, vol. 20:1117-1121 (1986).
- Neish, et al., *Phenylacetic Acid as a Potential Therapeutic Agent for the Treatment of Human Cancer*, *Experientia*, vol. 27:860-861 (1971).
- J. A. Stamatoyannopoulos, et al., *Therapeutic Approaches to Hemoglobin Switching in Treatment of Hemoglobinopathies*, *Annu. Rev. Med.*, vol. 43:497-521 (1992).
- Marcot et al., *Chemical Abstracts*, 83, 1975, 53278s (1975).
- Leary, *Cancer Drug Also Helps in Treating Sickle Cell Anemia*, *Researchers Say*, *Atlanta Journal-Constitution*, Thursday, Aug. 20, 1992.
- Dvorit Samid, et al., *Selective Growth Arrest and Phenotypic Reversion of Prostate Cancer Cells In Vitro by Nontoxic Pharmacological Concentrations of Phenylacetate*, *The Journal of Clinical Investigation*, vol. 91:2288-2295 (1993).
- Dvorit Samid, et al., *Induction of Erythroid Differentiation and Fetal Hemoglobin Production in Human Leukemic Cells Treated with Phenylacetate*, *Journal of the American Society of Hematology*, vol. 80:1576-1581 (1992).
- Dvorit Samid, et al., *Phenylacetate: A Novel Nontoxic Inducer of Tumor Cell Differentiation*, *Cancer Research*, vol. 52:1988-1992 (1992).
- George J. Dover, et al., *Increased Fetal Hemoglobin in Patients Receiving Sodium 4-Phenylbutyrate*, *The New England Journal of Medicine*, vol. 327:569-570 (1992).
- Brusilow, S. W. and Horwich, A. L., *Urea cycle Enzymes, in Metabolic Basis of Inherited Diseases* 629-633 (C. R. Scriver ed., 1989).
- Shecter, Y. et al., *Hydroxyphenyl Acetate Derivatives Inhibit Protein Tyrosine Kinase Activity and Proliferation in Nb2 Rat Lymphoma Cells and Insulin-Induced Lipogenesis in Rat Adipocytes*, *Molecular and Cellular Endocrinology*, vol. 80, pp. 183-192 (1991).
- Samid, D. et al., *Interferon in Combination with Antitumorogenic Phenyl Derivatives: Potentiation of IFN α Activity In-Vitro*, *British J. Haematology*, vol. 79, Supp. 1, pp. 81-83 (Oct. 10, 1991).
- The Merck Index* (Susan Budavari, et al. eds., 1989).
- Burzynski, S., *Preclinical Studies on Antineoplaston AS2-1 and Antineoplaston AS2-5*, *Drugs Exptl. Clin. Res.*, 12[Supp. I], pp. 11-16 (1986).
- M. A. Smith, et al., *Retinoids in Cancer Therapy*, *Journal of Clinical Oncology*, 10:839-864 (1992).
- R. L. Stephens, M. D., et al., *Adriamycin and Cyclophosphamide Versus Hydroxyurea in Advanced Prostatic Cancer: A Randomized Southwest Oncology Group Study*, *Cancer* 53:406-410 (1984).
- Ljeune, F., et al., *Disseminated melanoma, preclinical therapeutic studies, clinical trials and patient treatment*, *Oncology* 5:390-396 (1993).
- Wuarin, L., et al., *Effects of interferon-gamma and its interaction with retinoic acid on human neuroblastoma differentiation*, *Int. J. Cancer* 48:136-141 (1991).
- Hendrix, M. J. C., et al., *Retinoic acid inhibition of human melanoma cell invasion through a reconstituted basement membrane and its relation to decreases in the expression of proteolytic enzymes and motility factor receptor*, *Cancer Research* 50:4121-4130 (1990).
- Rudling, M. J., et al., *Low density lipoprotein receptor activity in human intracranial tumors and its relation to the cholesterol requirement*, *Cancer Research* 50:483-487 (1990).
- Lando, M., et al., *Modulation of intracellular cyclic adenosine monophosphate levels and the differentiation response of human neuroblastoma cells*, *Cancer Research* 50:722-727.
- Bloedow, C. E., *Phase II studies of hydroxyurea (NSC-32065) in adults: miscellaneous tumors*, *Cancer Chemotherapy Reports No. 40*, pp. 39-41 (1964).
- Kandutsch, A. A. and Saucier, S. E., *Regulation of sterol synthesis in developing brains of normal and jimpy mice*, *Archives of Biochemistry and Biophysics* 135:201-208 (1969).
- Evans, A. E., et al., *A Review of 17 IV-S Neuroblastoma Patients at the Children's Hospital of Philadelphia*, *Cancer* 45:833-839 (1980).
- Thiele, C. J., et al., *Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma*, *Nature* 313:404-406 (1985).
- Giuffrè, L., et al., *Cyclic AMP induces differentiation in vitro of human melanoma cells*, *Cancer* 61:1132-1141 (1988).
- Nordenberg, J., et al., *Growth inhibition of murine melanoma by butyric acid and dimethylsulfoxide*, *Experimental Cell Research* 162:77-85 (1986).
- Sidell, N., et al., *Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines*, *Experimental Cell Research* 148:21-30 (1983).
- Donehower, R. C., *Hydroxyurea*, *Cancer Chemotherapy*, pp. 225-233 1993.
- Questionable methods of cancer management: hydrogen peroxide and other 'hyperoxygenation' therapies*, *Questionable Methods* 43:47-56 (1993).
- Finklestein, J. Z., et al., *13-cis-retinoic acid (NSC 122758) in the treatment of children with metastatic neuroblastoma unresponsive to conventional chemotherapy: report from the childrens cancer study group*, *Medical and Pediatric Oncology* 20:307-311 (1992).
- Sidell, N. et al., *Material and Methods*, *Exp. Cell Res.* 148:22-30 (1983).
- Nevinny, H. B. and Hall, T. C., *Chemotherapy with hydroxyurea (NSC-32065) in renal cell carcinoma*, *J. Clinical Pharmacology*, pp. 352-359 (Nov.-Dec. 1968).
- Ariel, I. M., *Therapeutic effects of hydroxyurea: experience with 118 patients with inoperable solid tumors*, *Cancer*, pp. 705-715 (Mar. 1970).
- Abemayor, E. and Sidell, N., *Human neuroblastoma cell lines as models for the in vitro study of neoplastic and neuronal cell differentiation*, *Environmental Health Perspectives* 80:3-15 (1989).

**FIG 1****FIG 3**

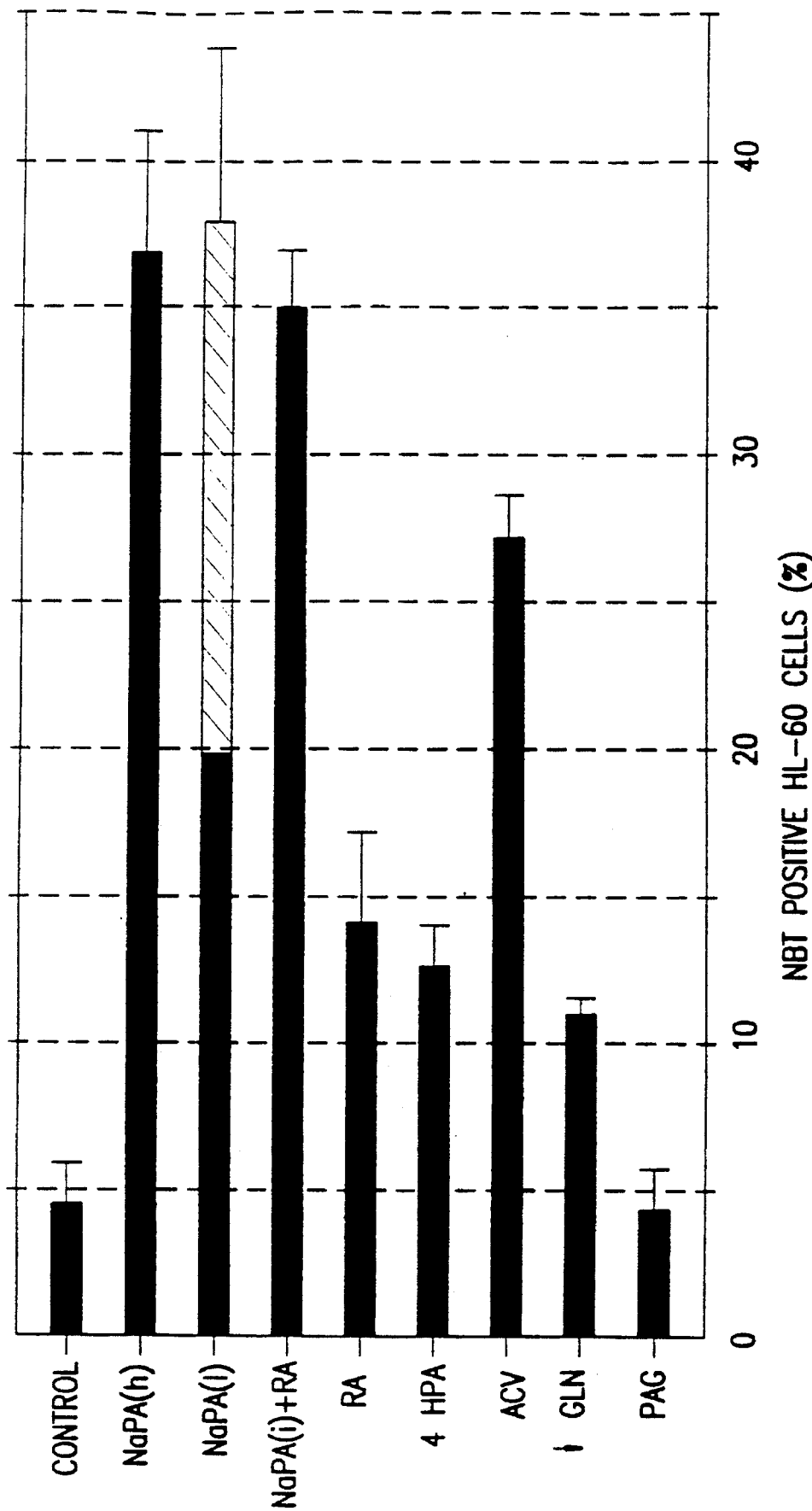
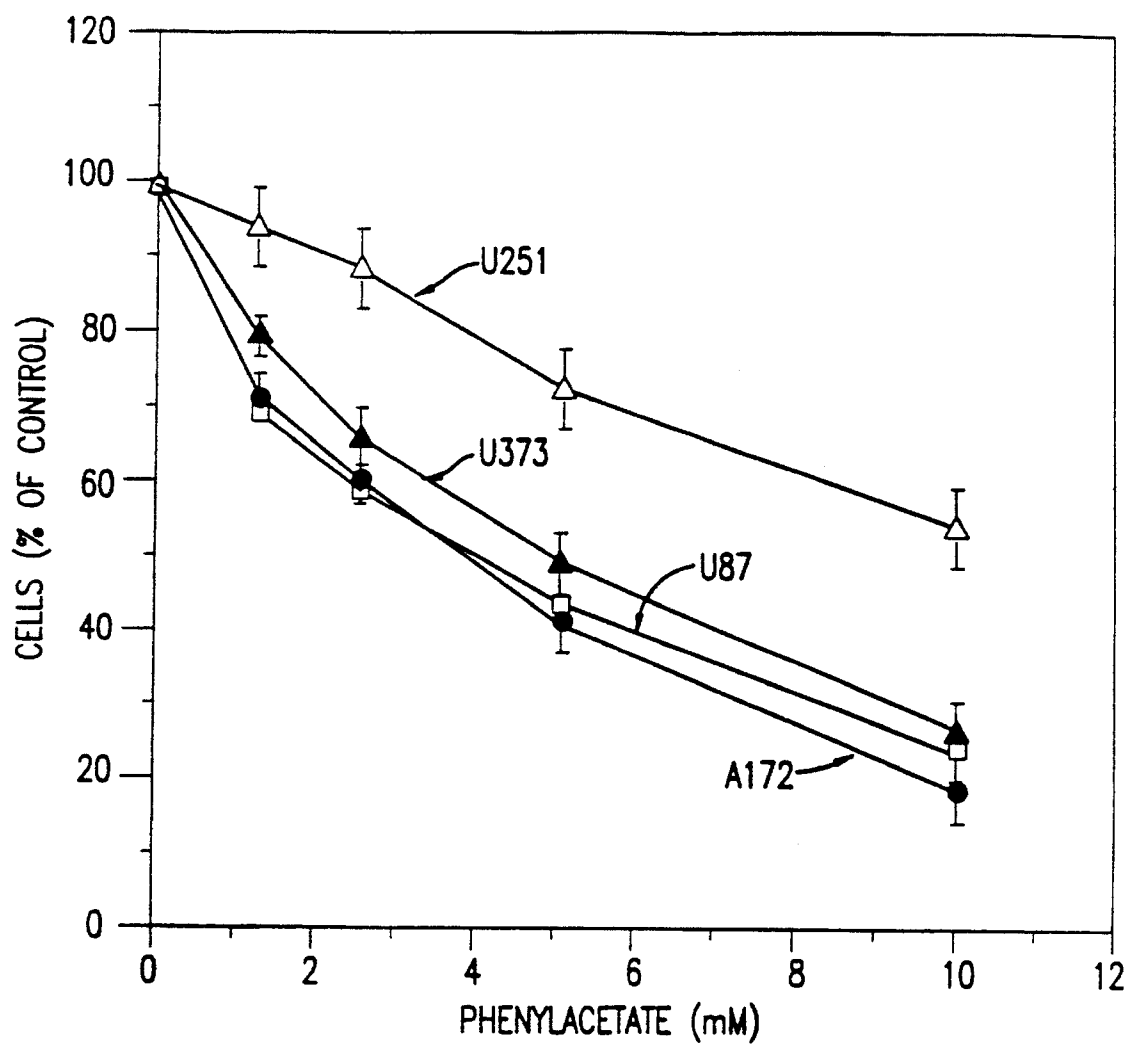


FIG 2

**FIG 4**

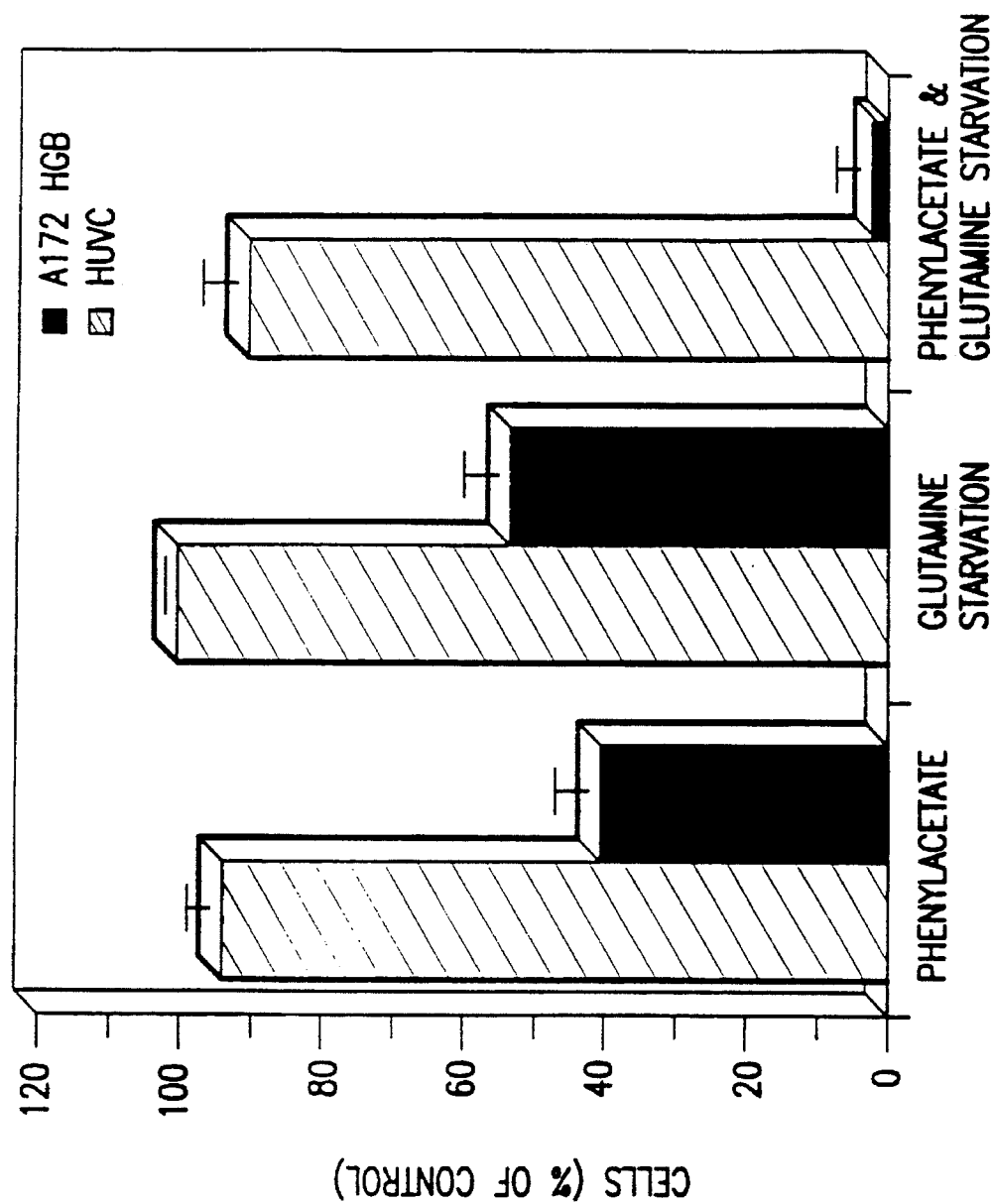
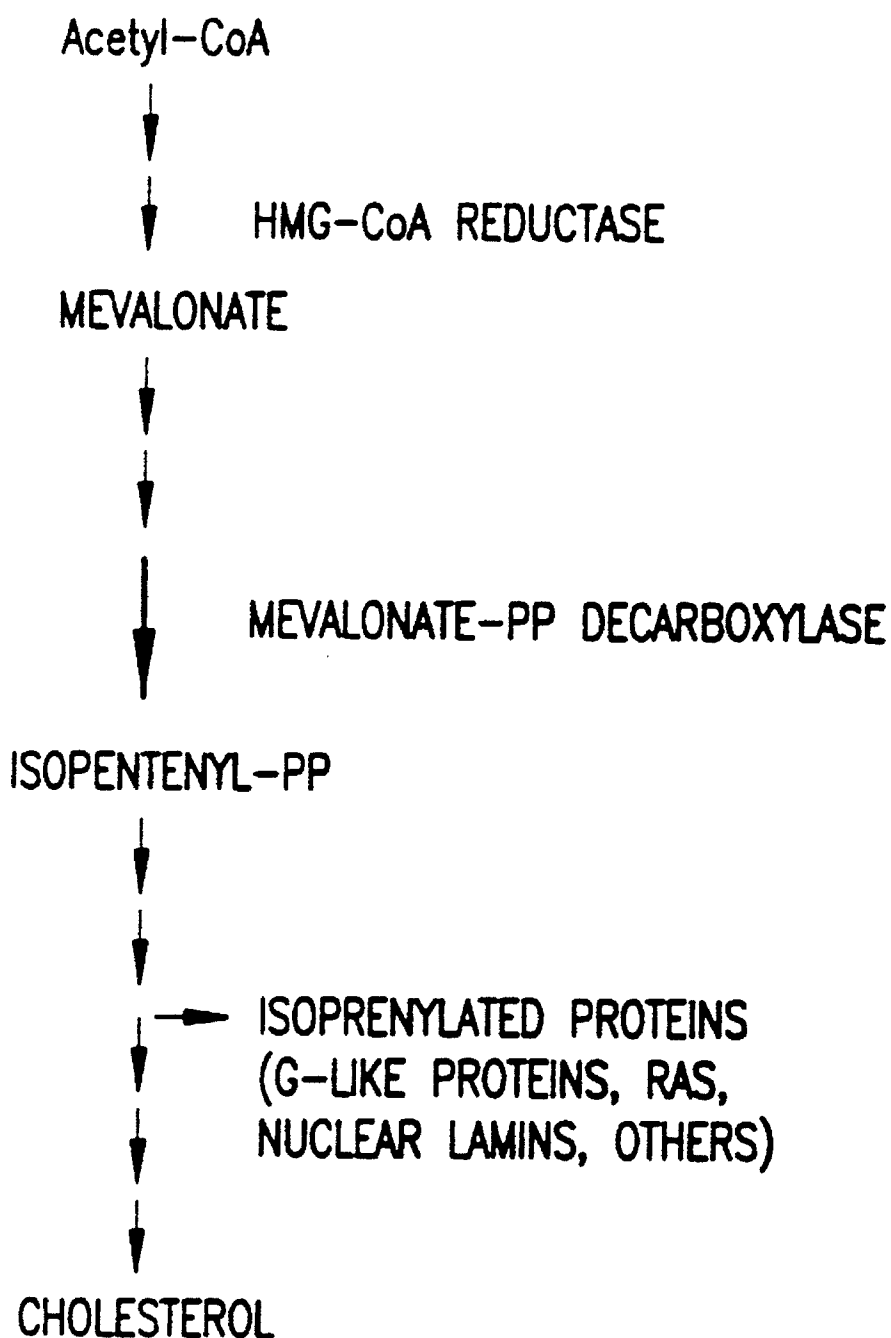


FIG 5

**FIG 6**

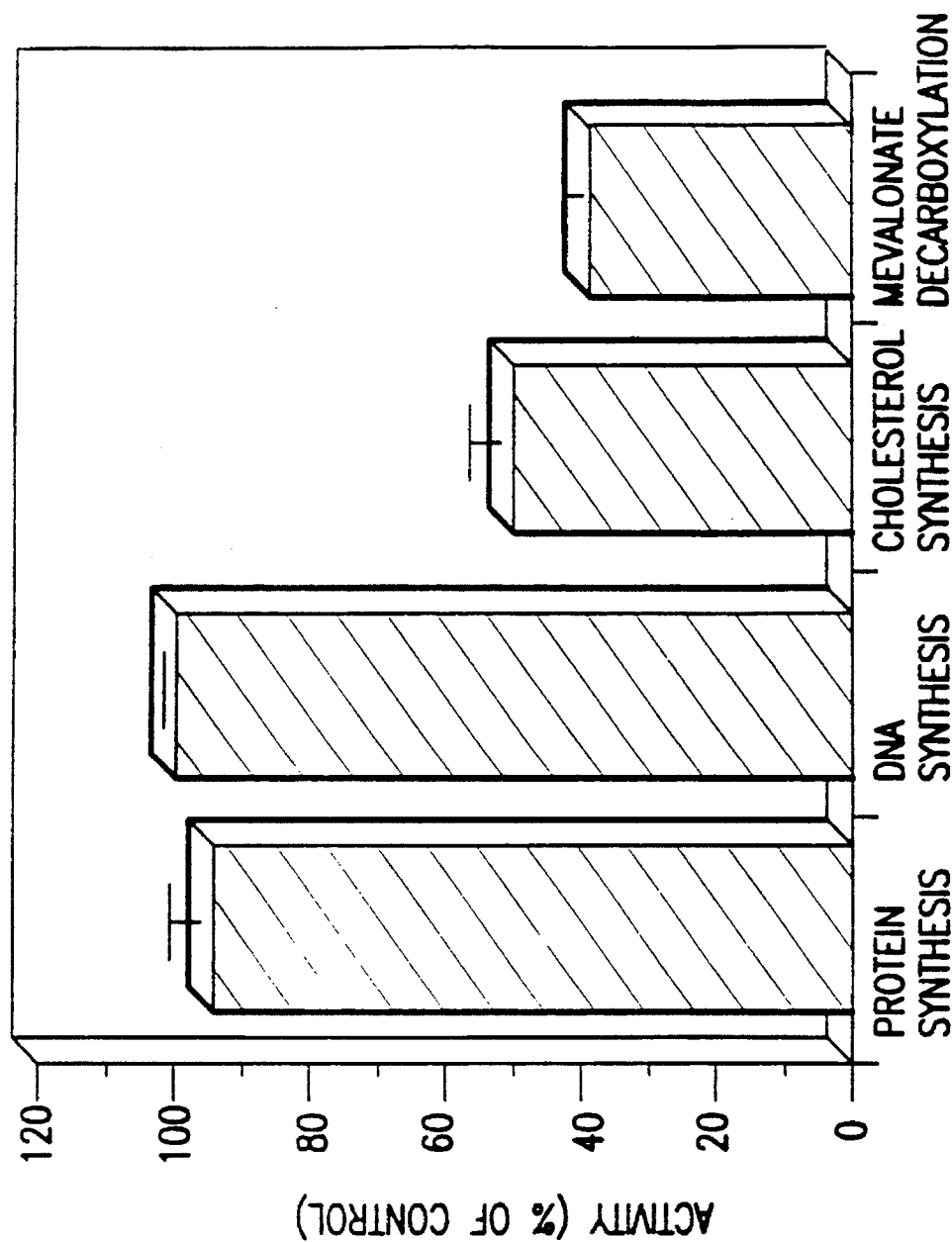


FIG 7

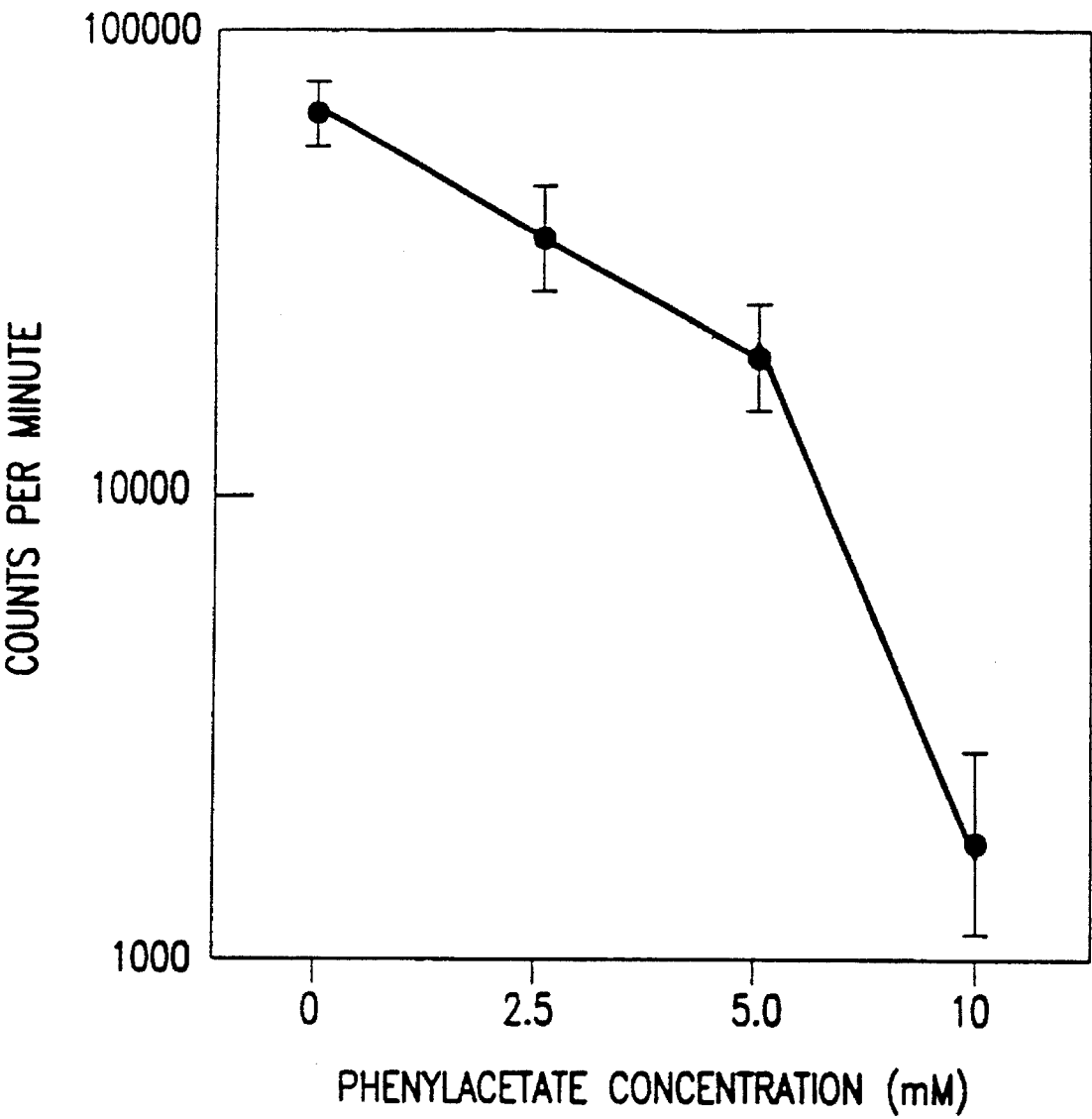


FIG 8

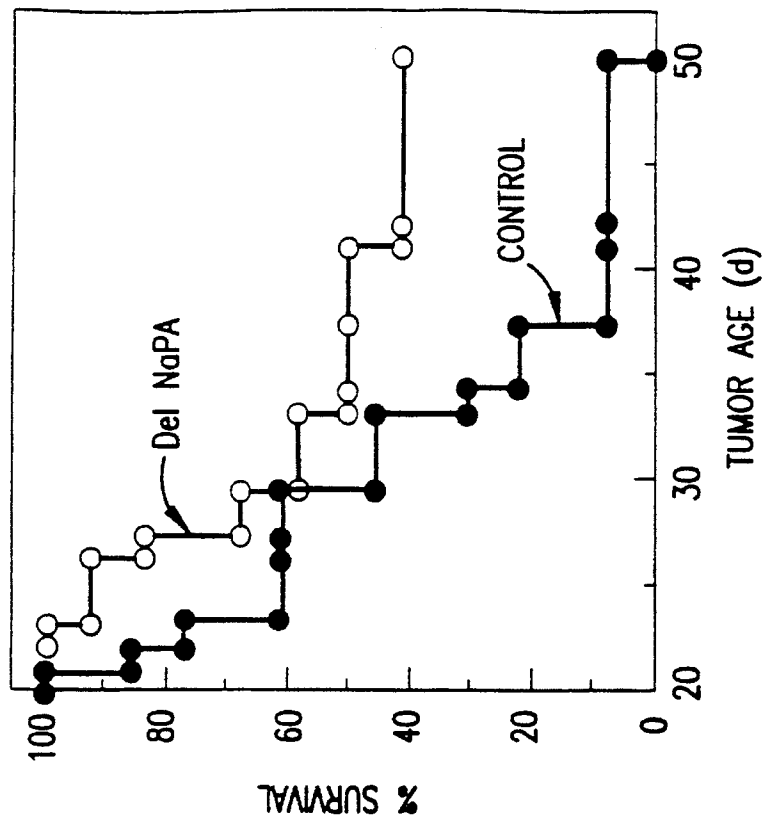


FIG 10

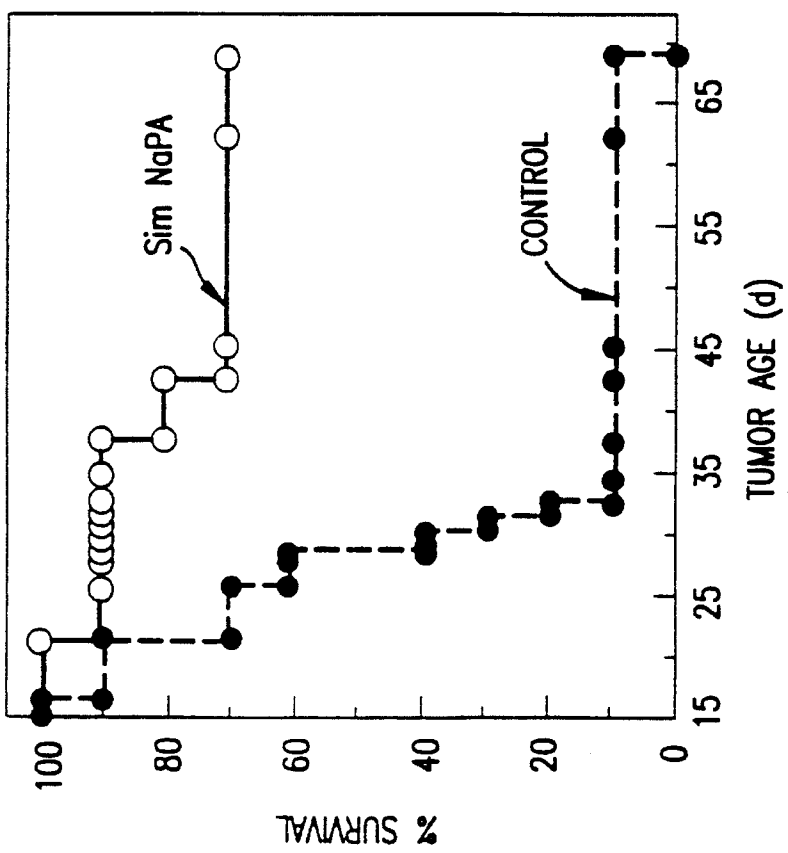


FIG 9

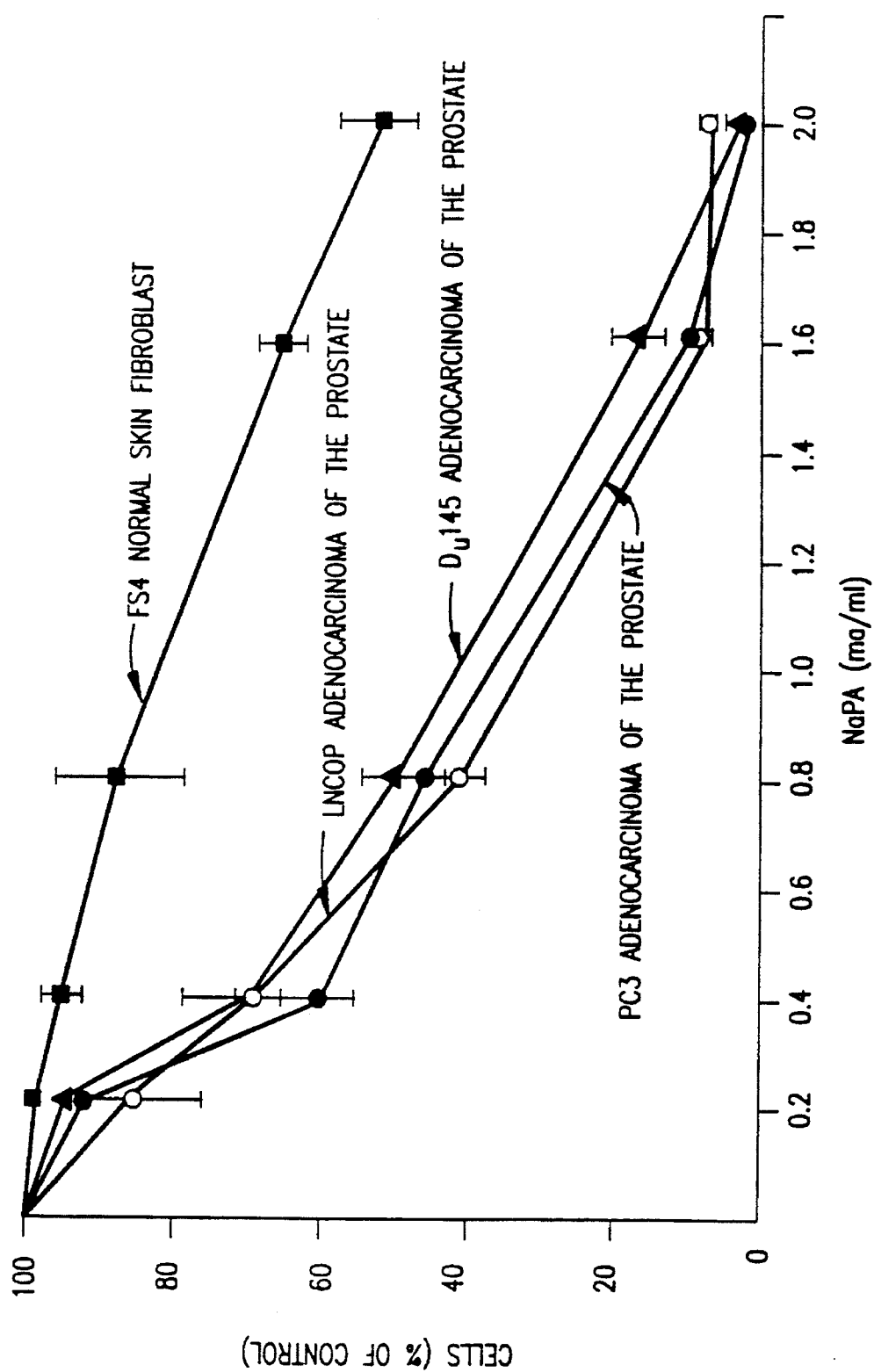
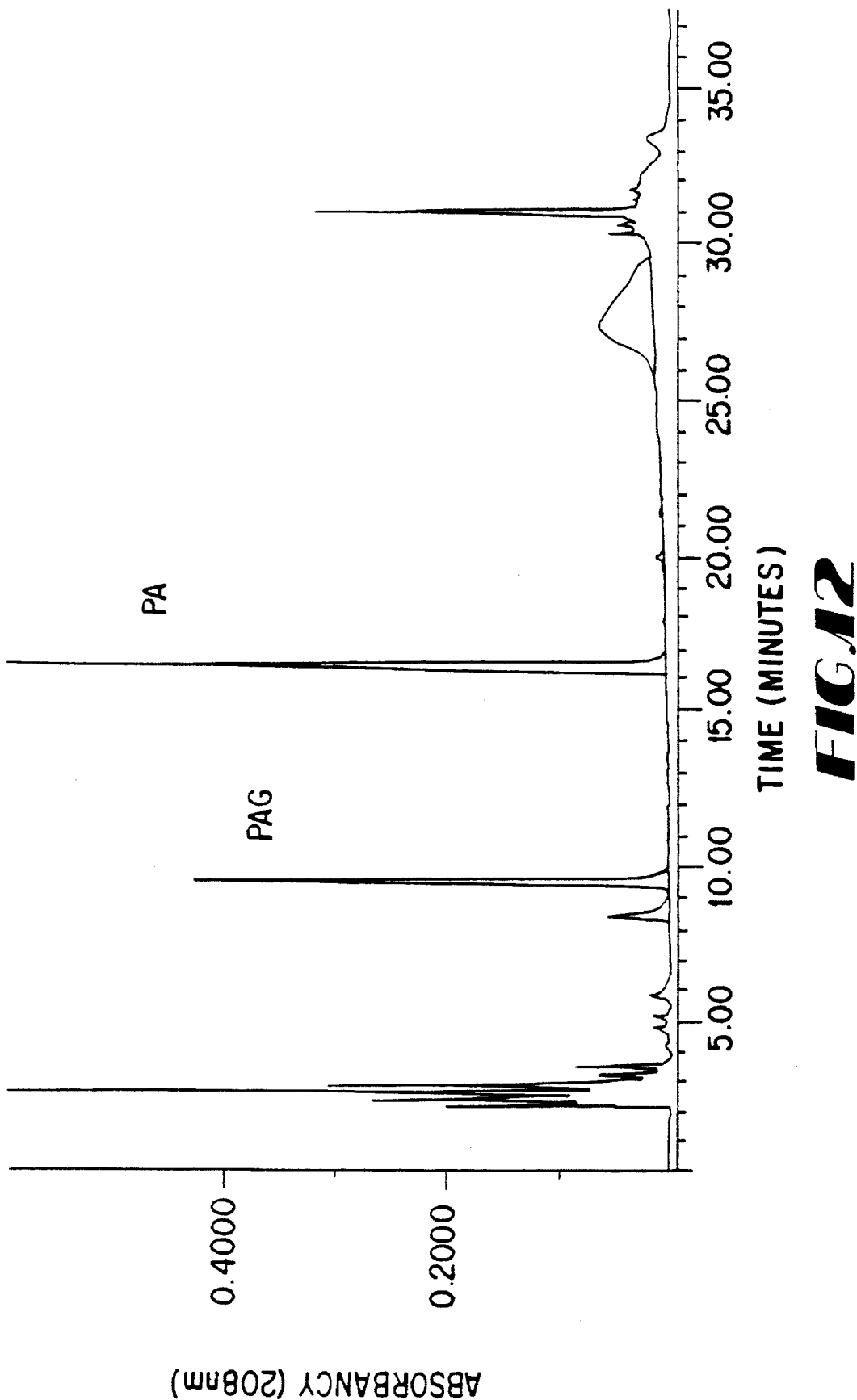


FIG. 11



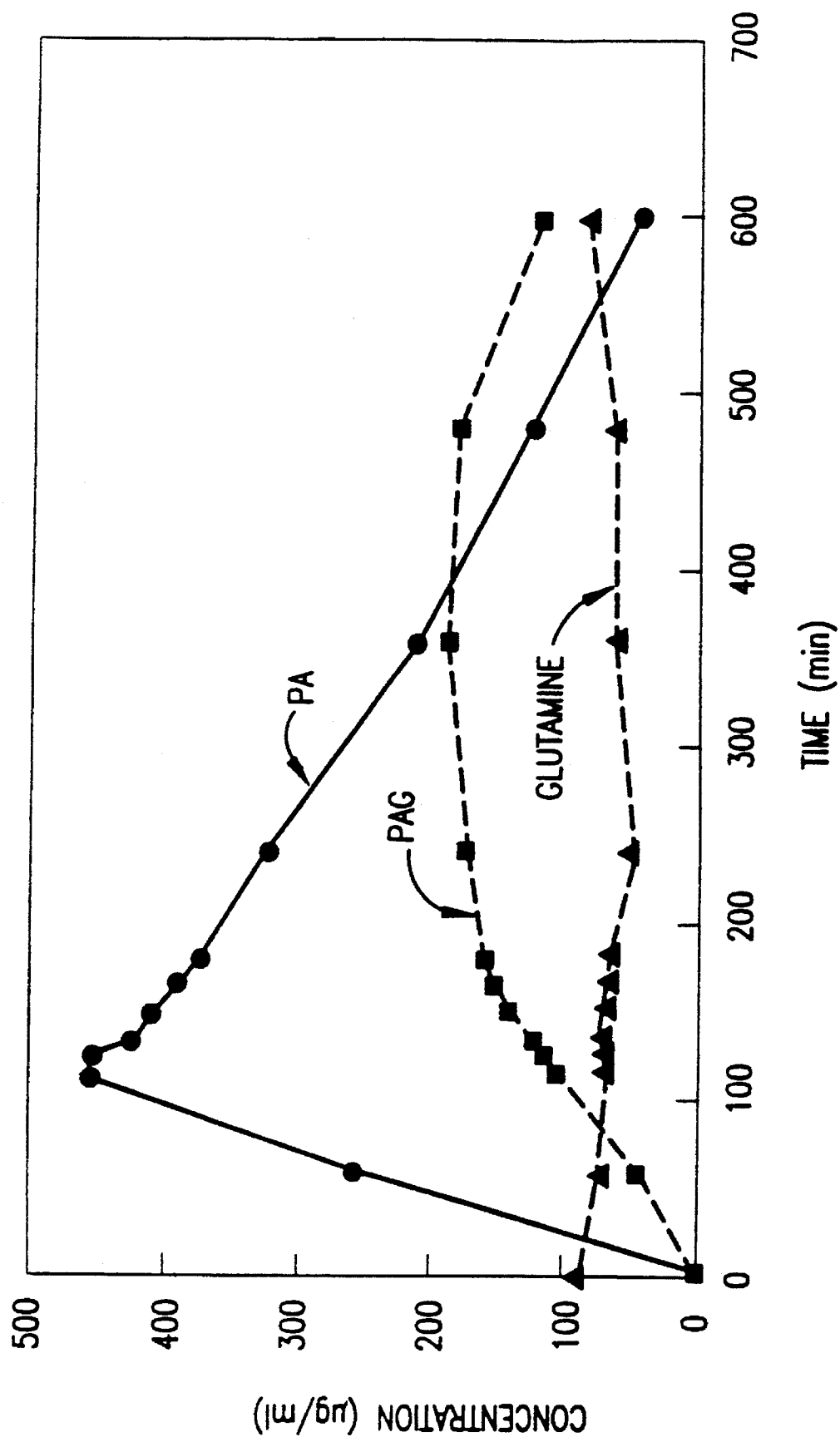


FIG 13

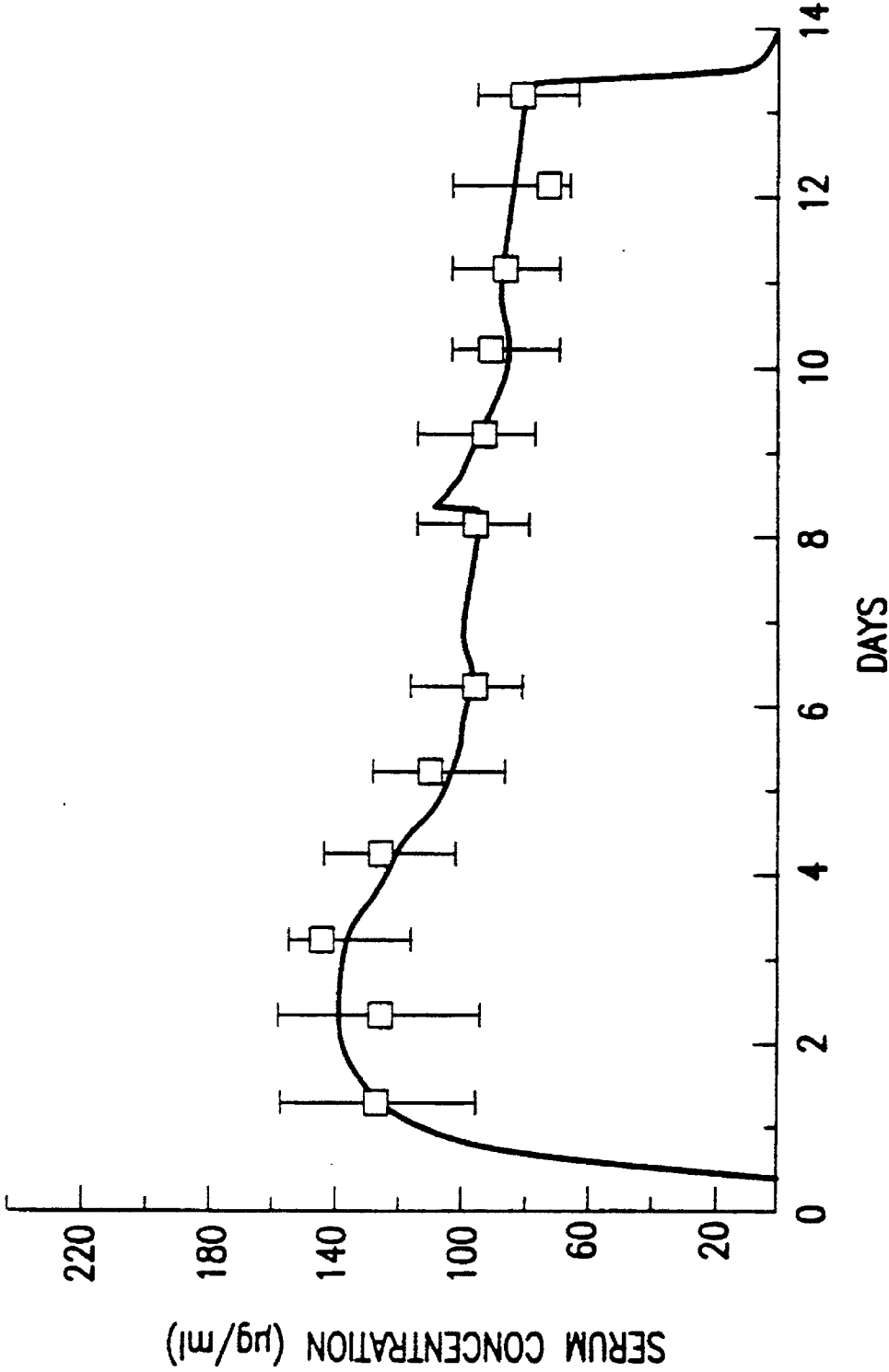


FIG. 14

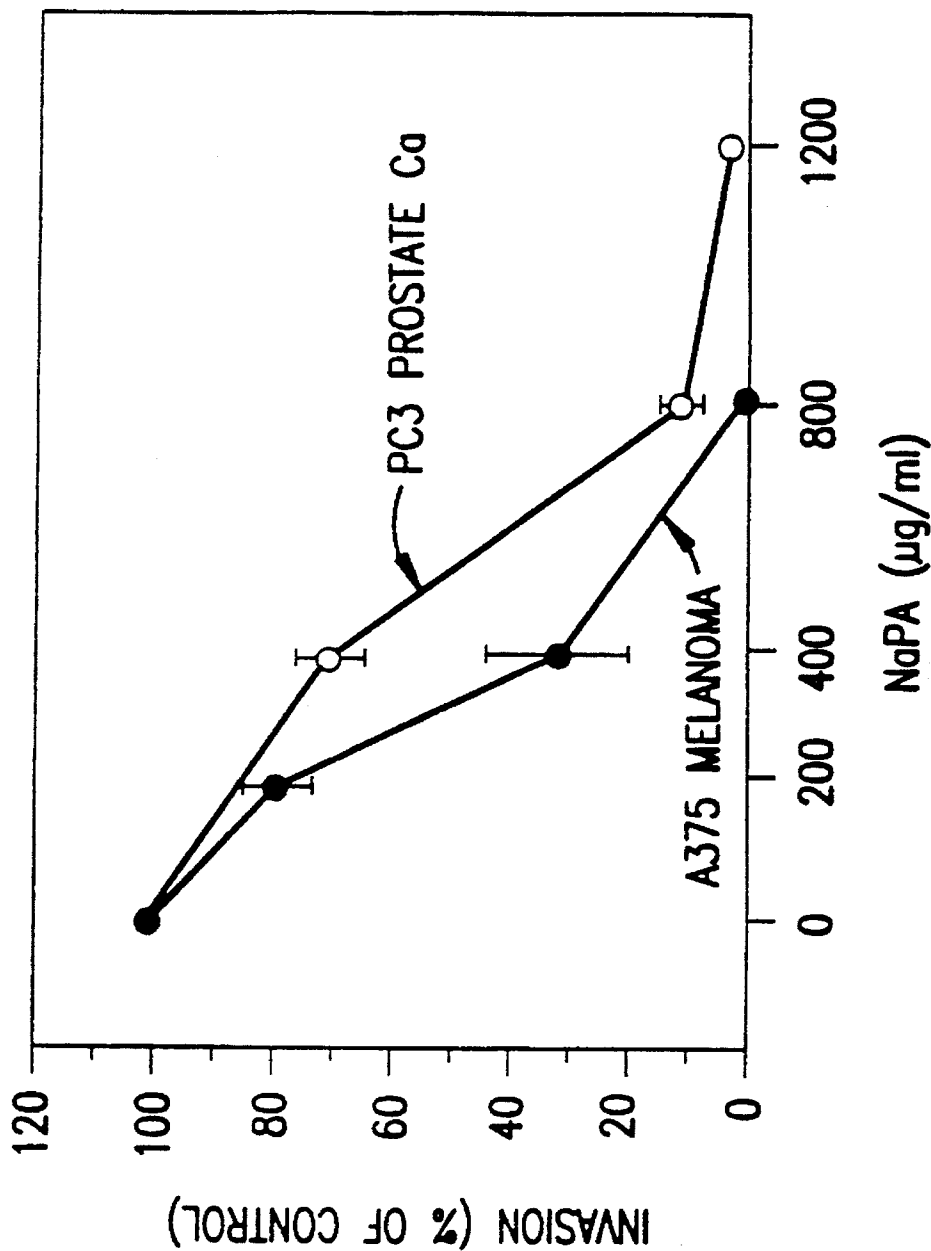


FIG 15

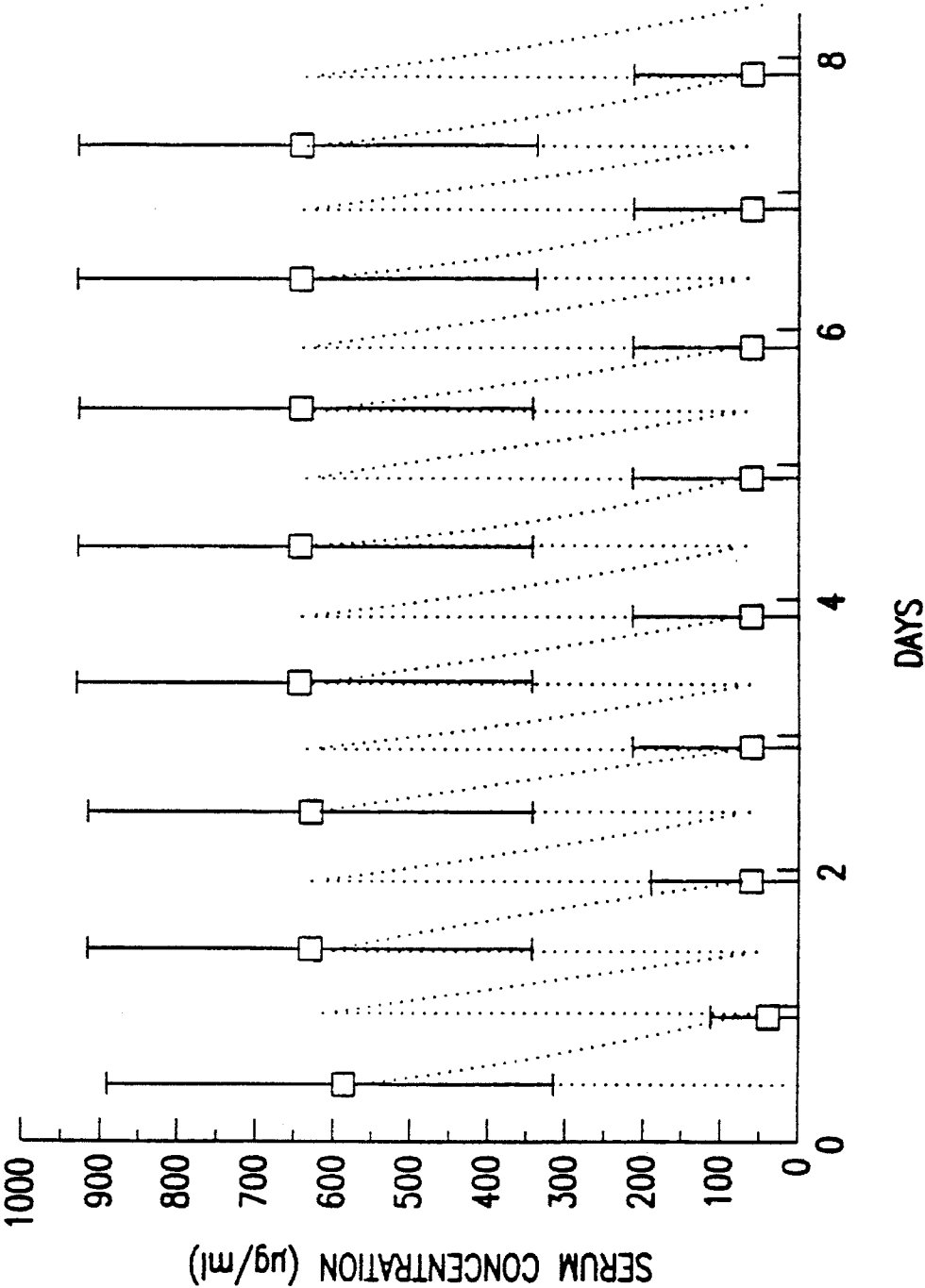


FIG 16

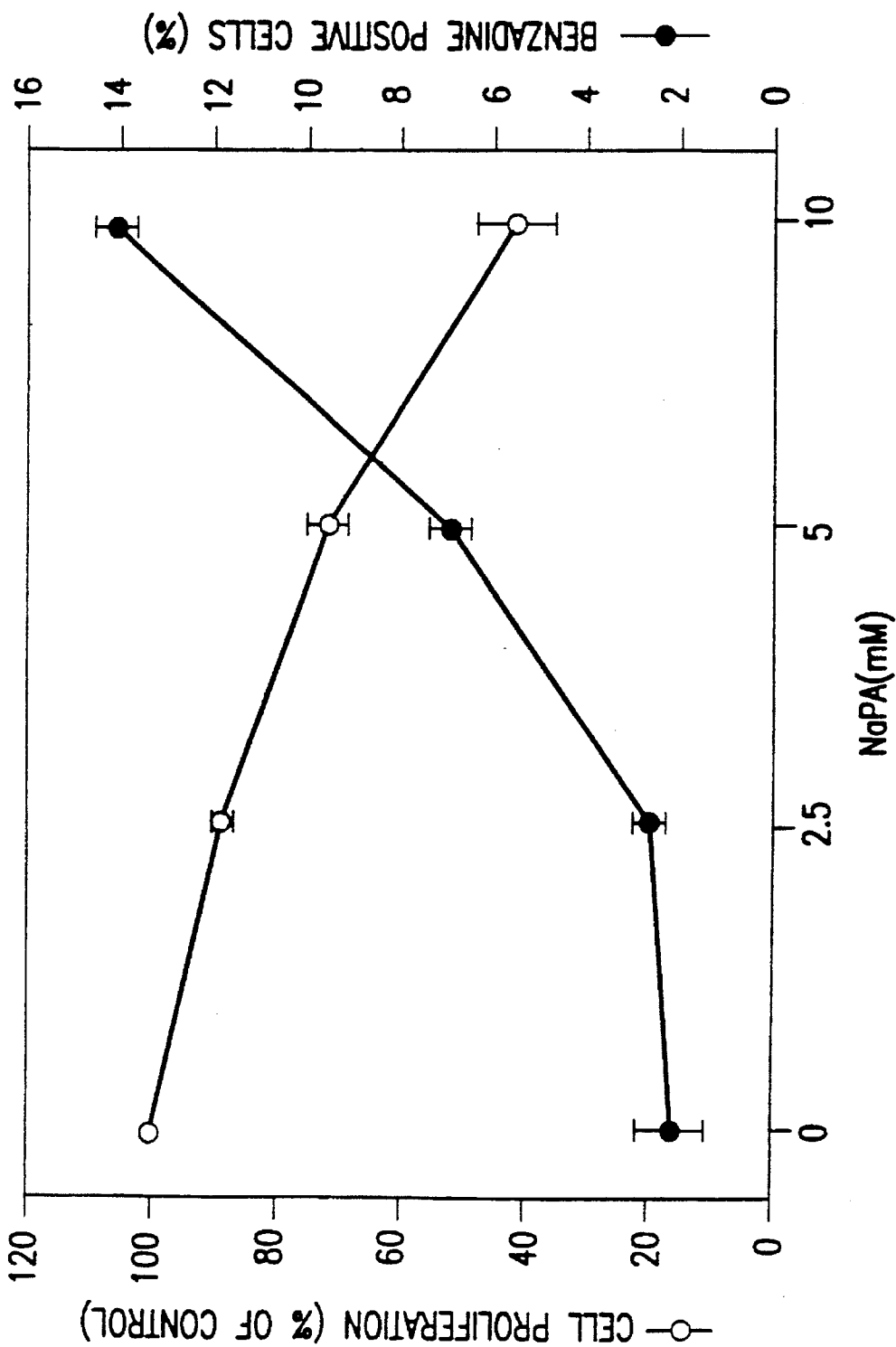


FIG. 7

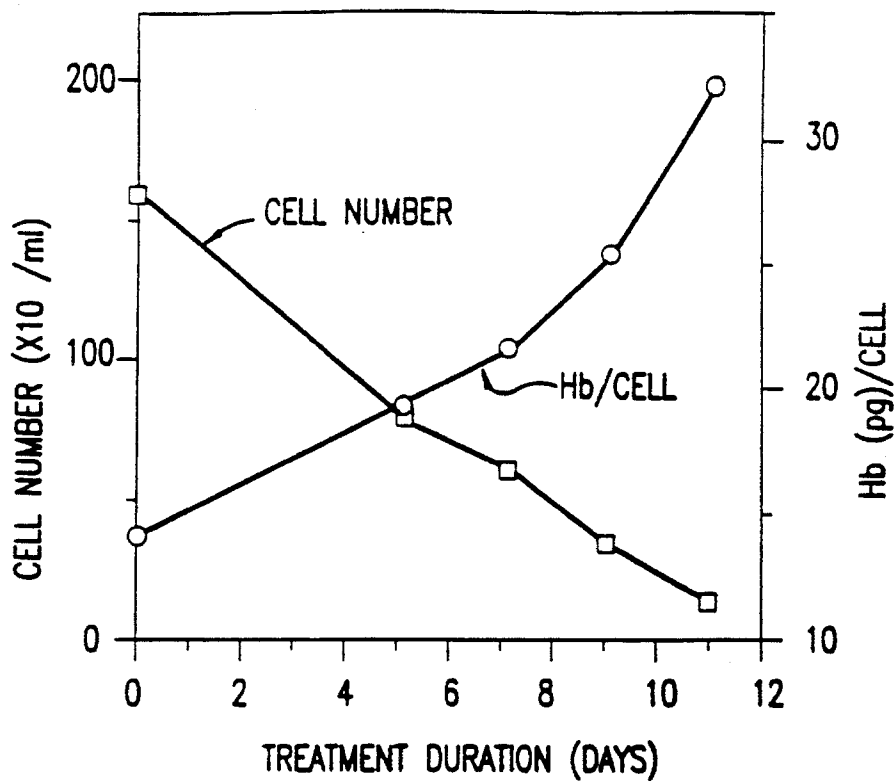


FIG 18 A

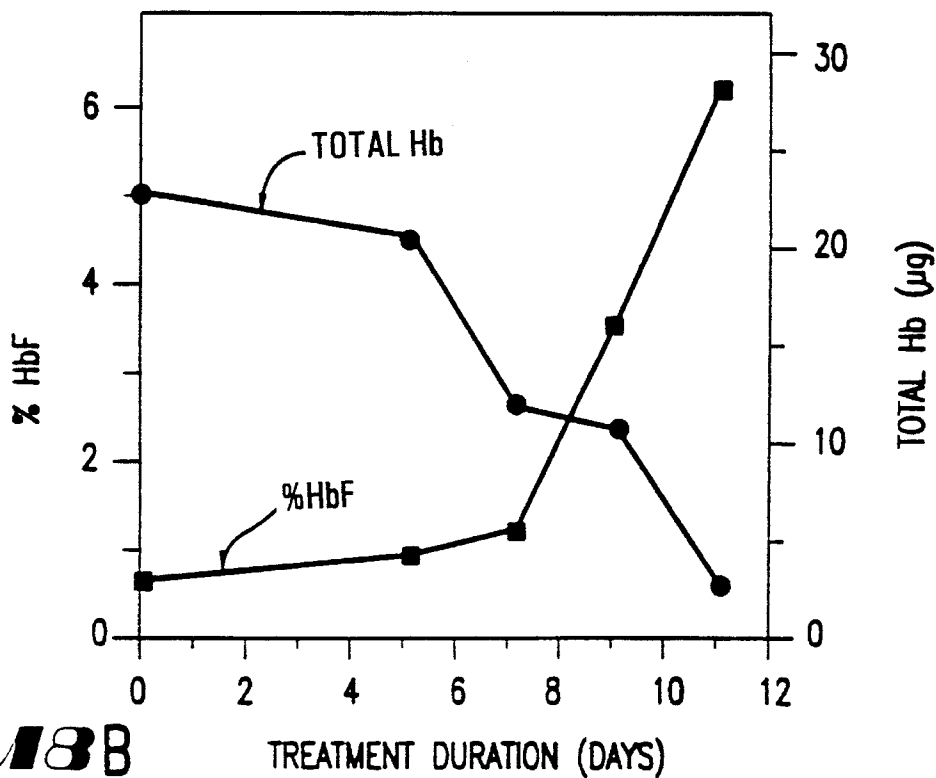


FIG 18 B

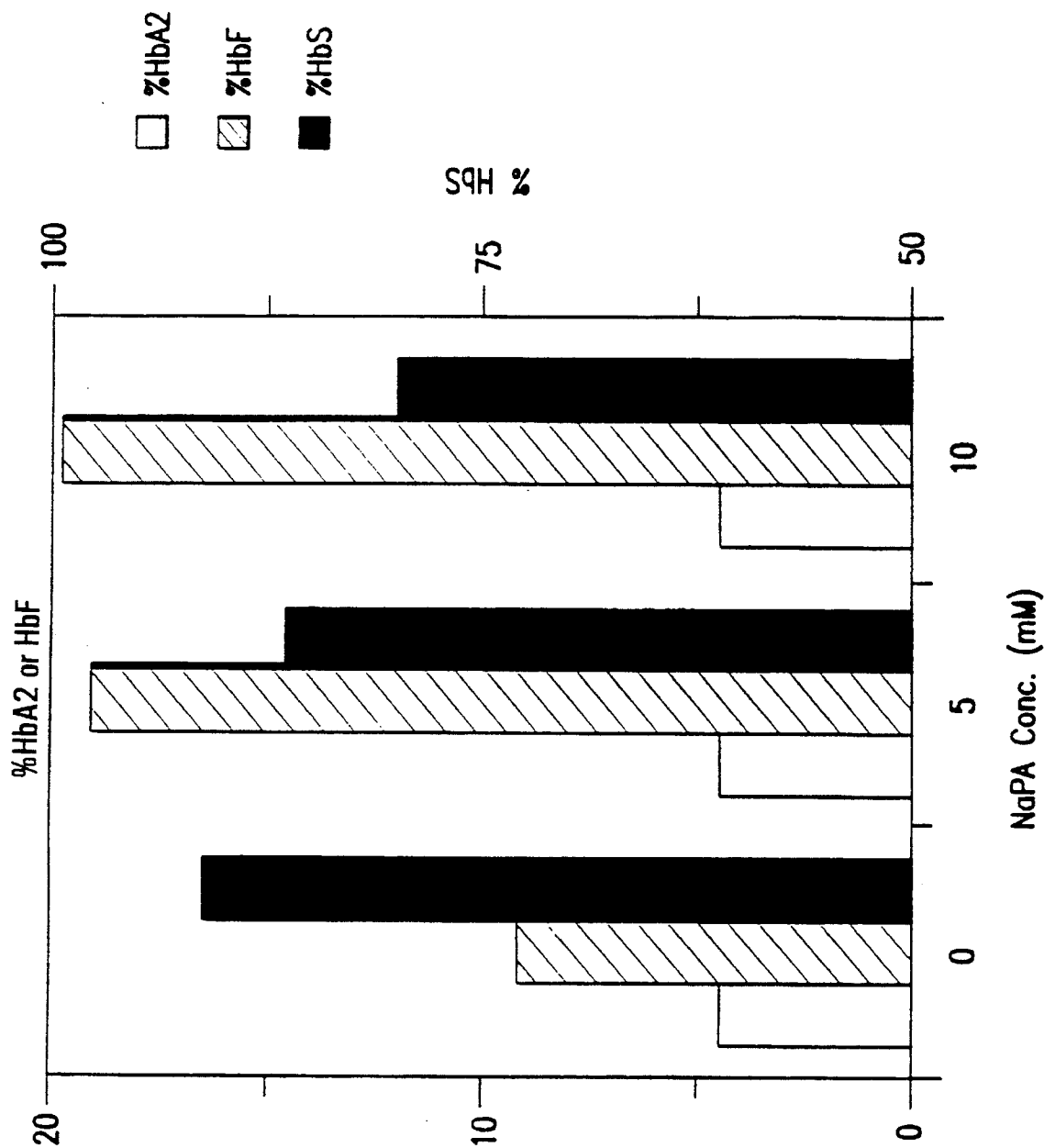


FIG. 19

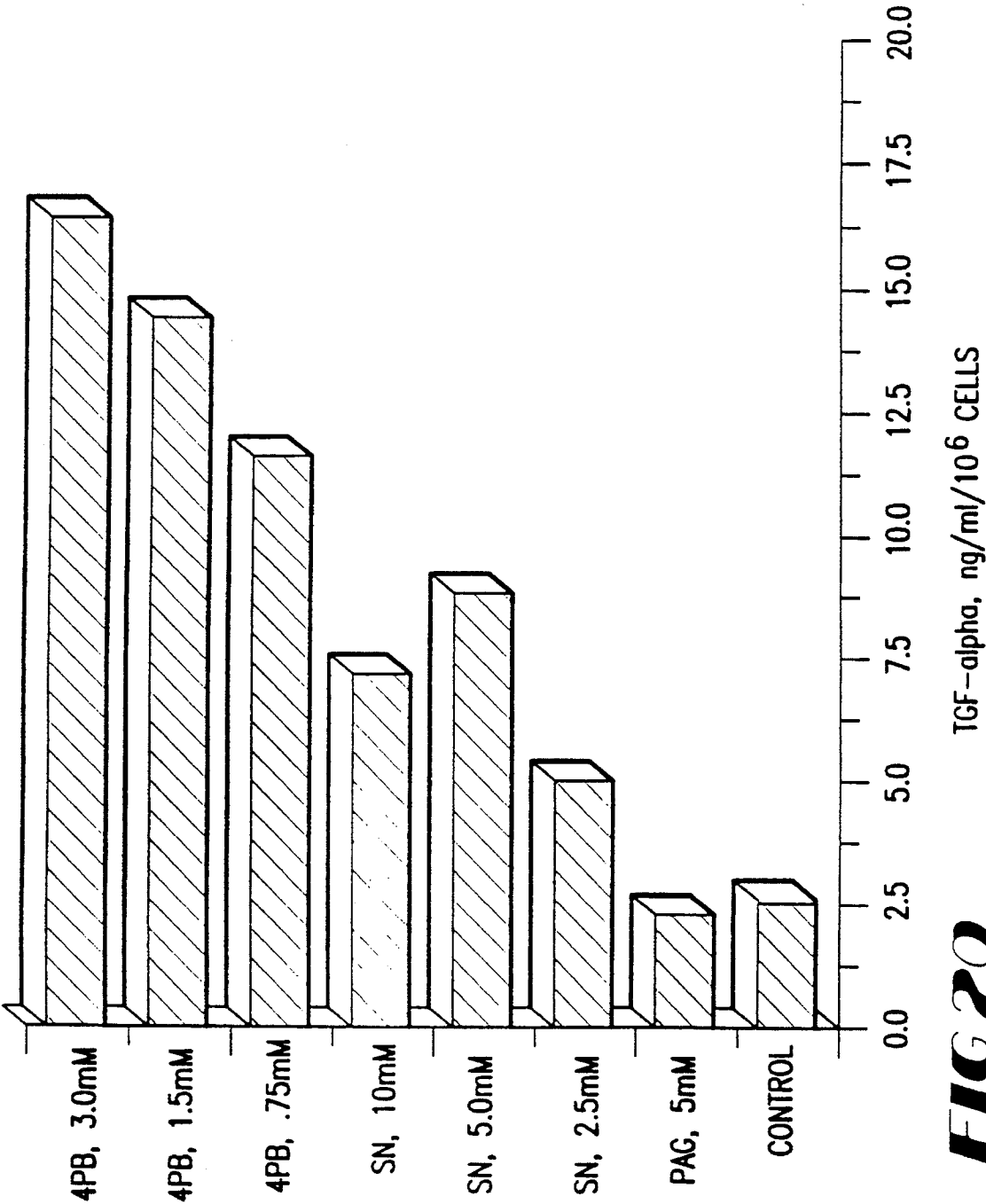


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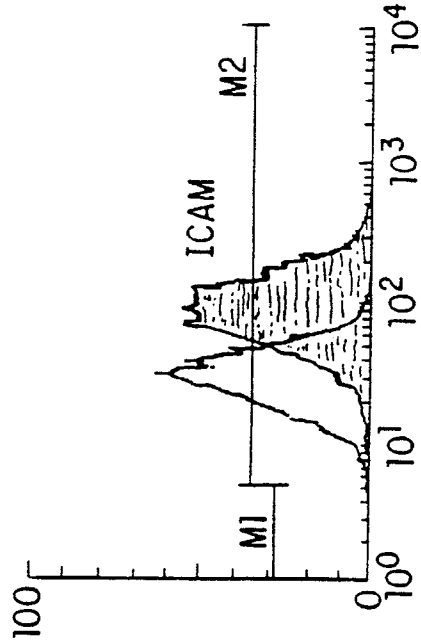


FIG 2A B

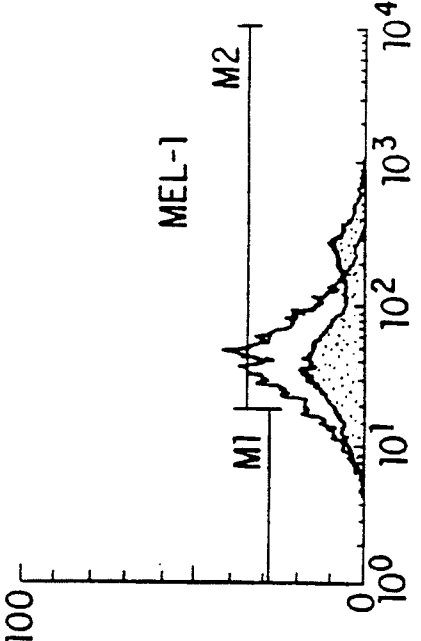


FIG 2A D

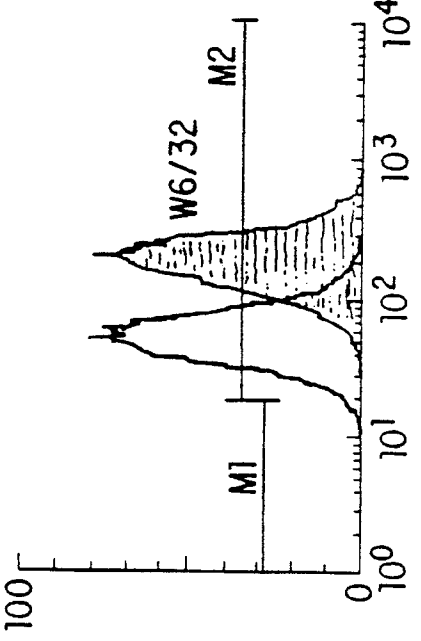


FIG 2A A

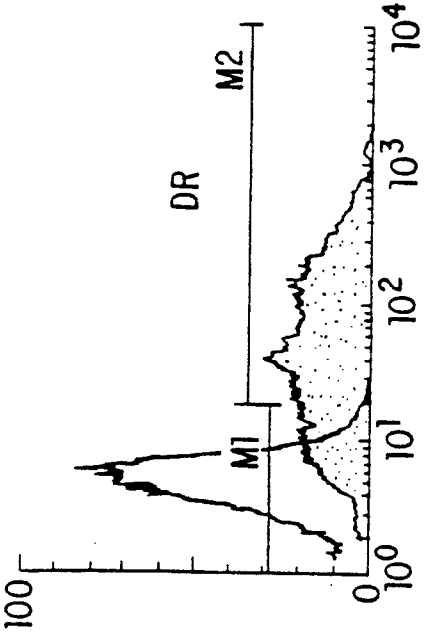


FIG 2A C

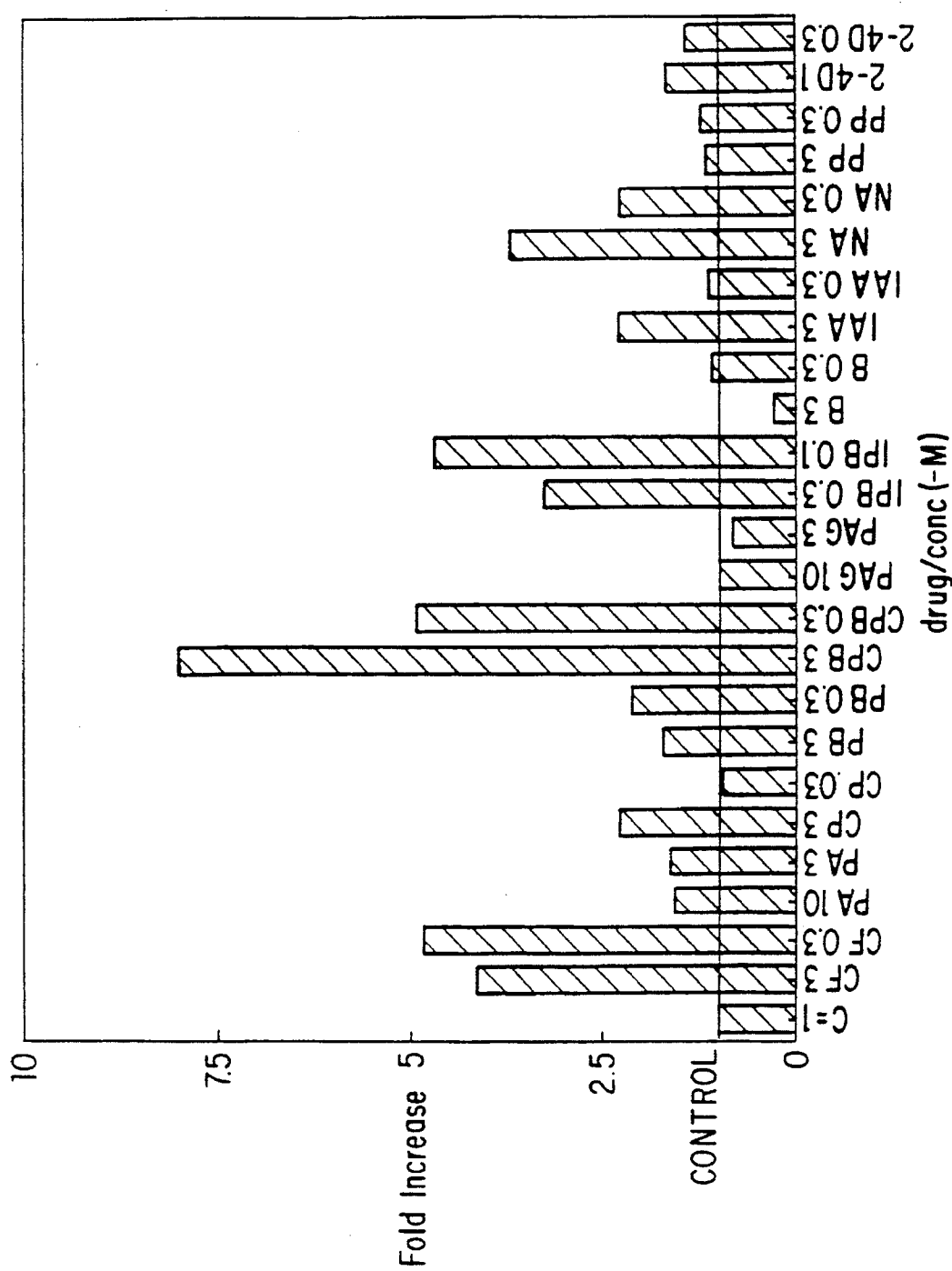
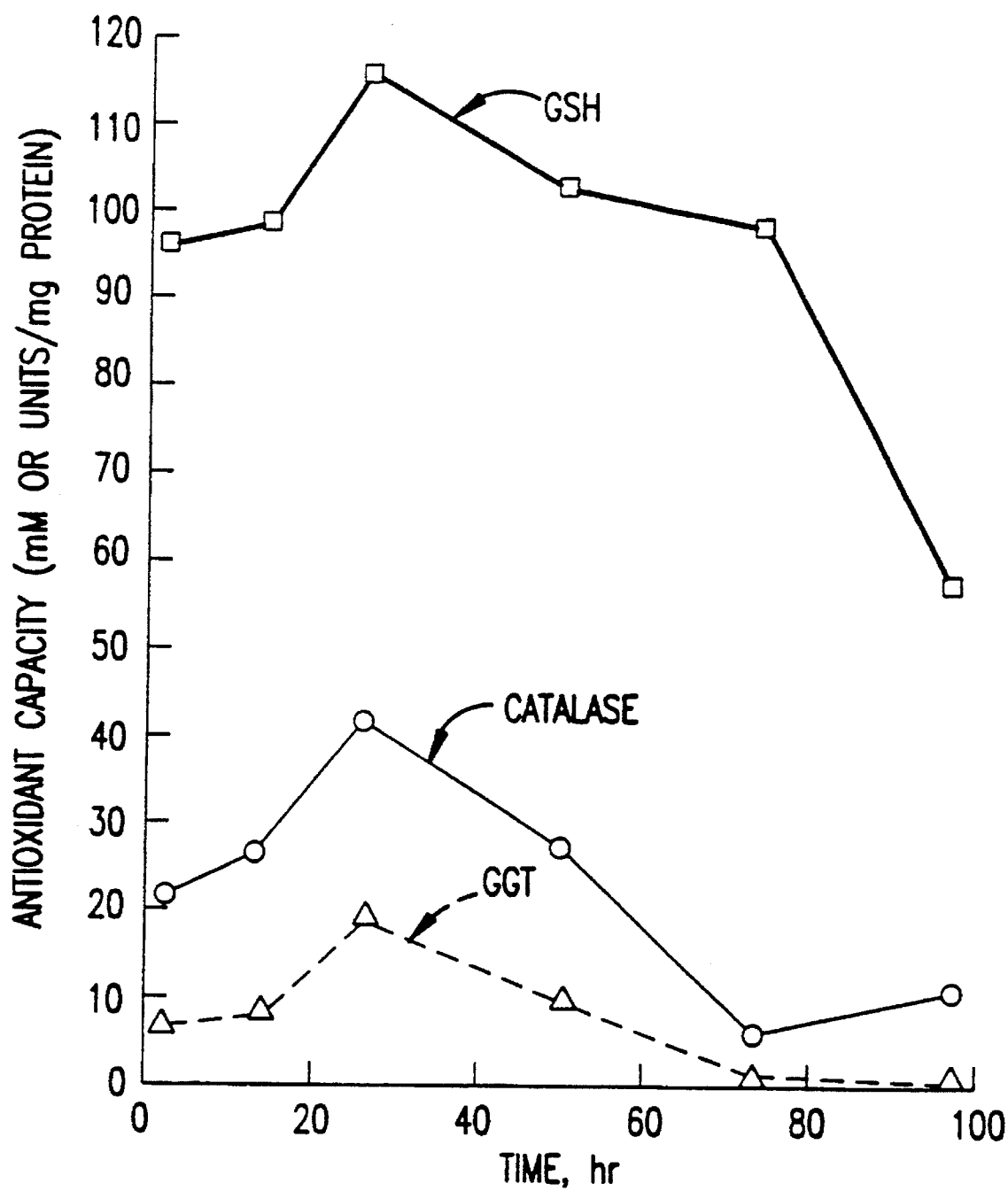
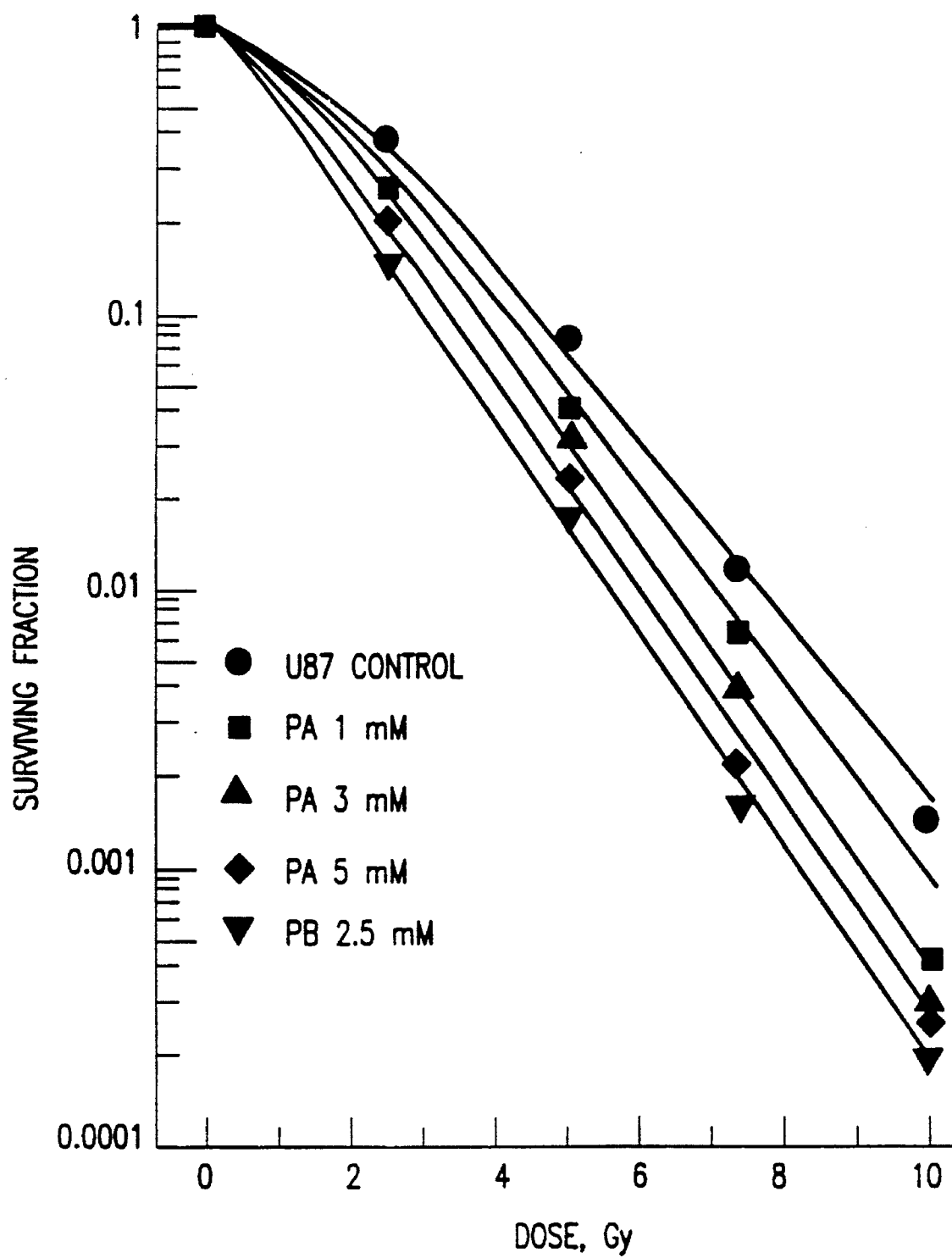


FIG 22

**FIG 23**

**FIG 24**

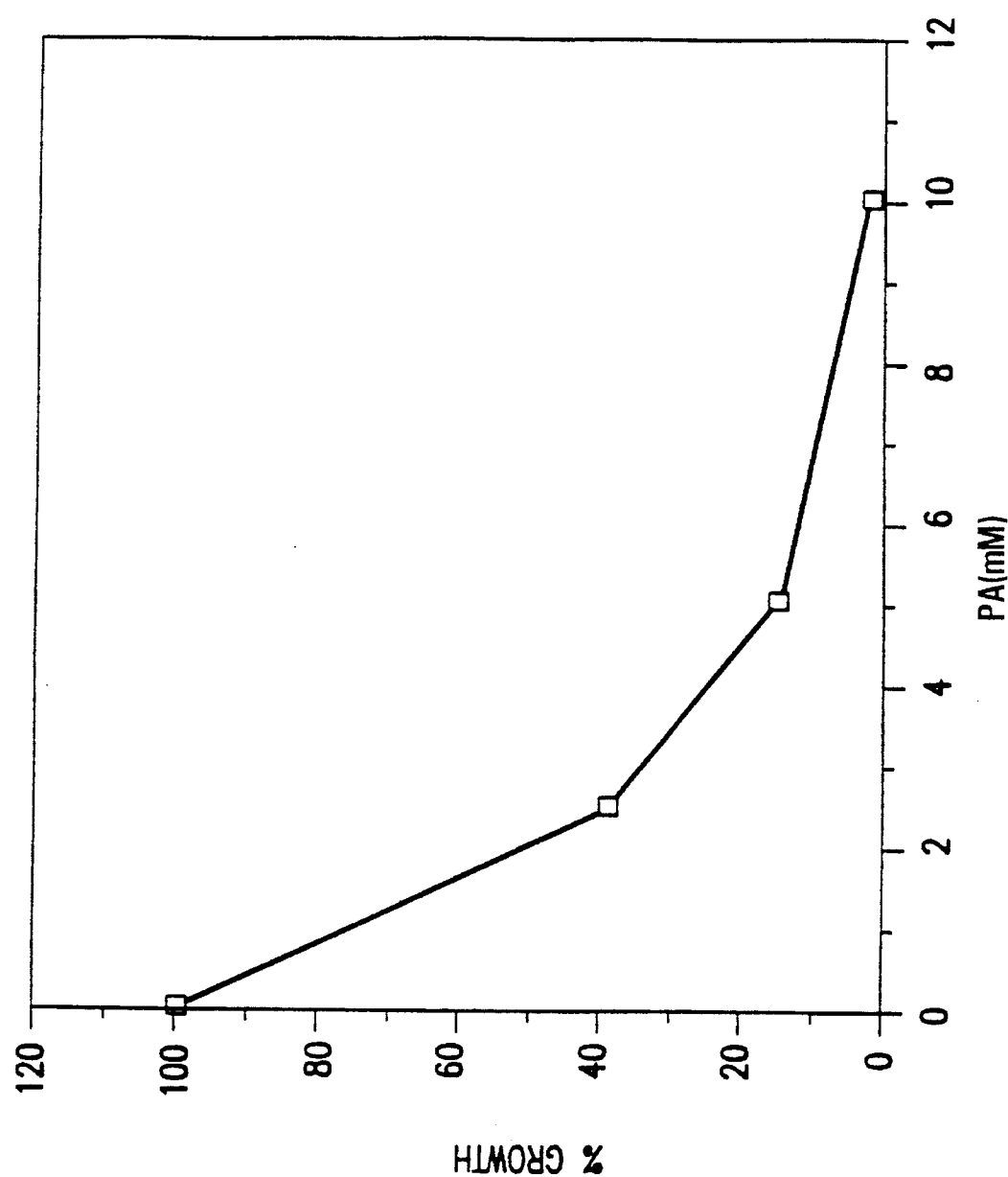


FIG 25

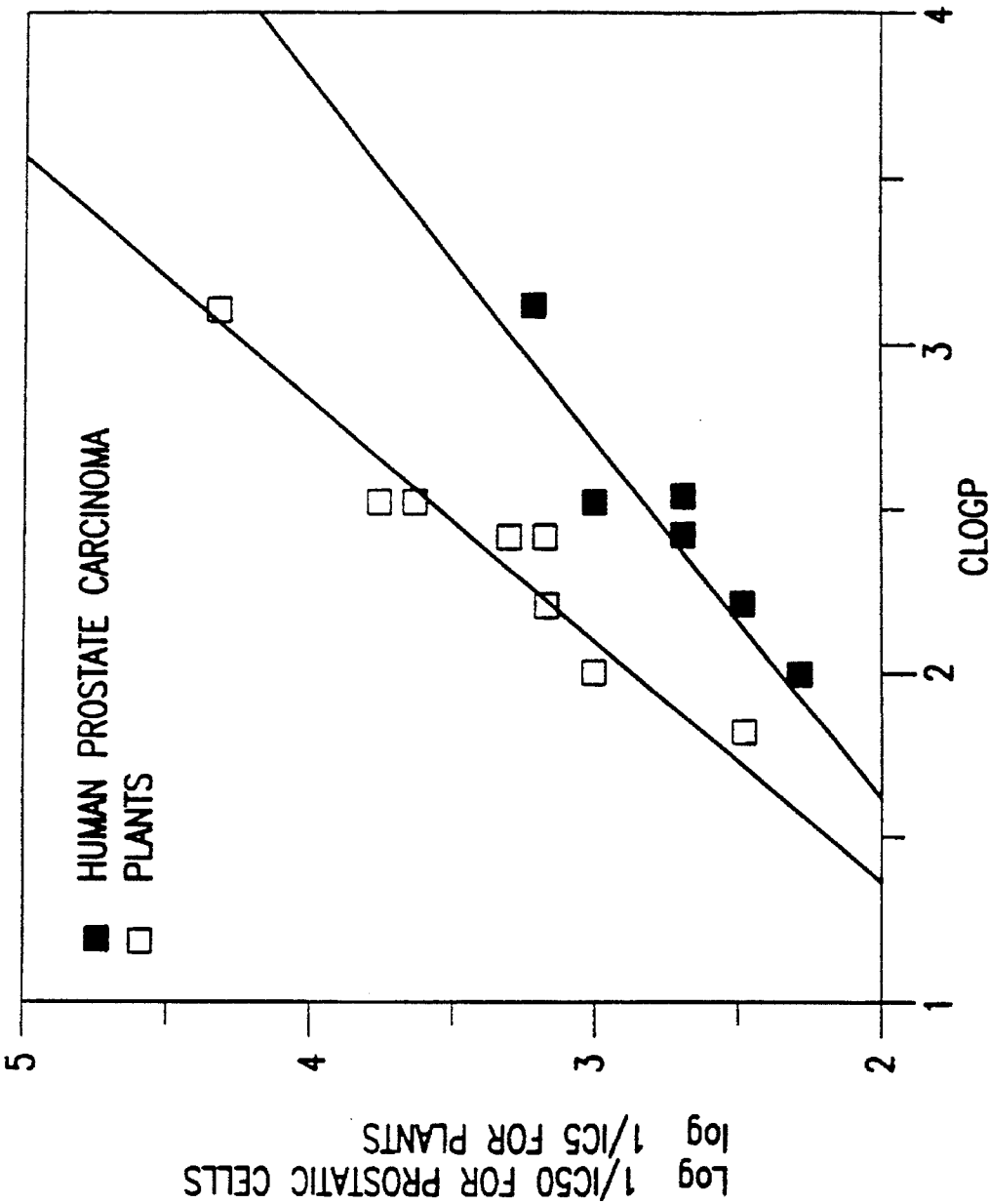
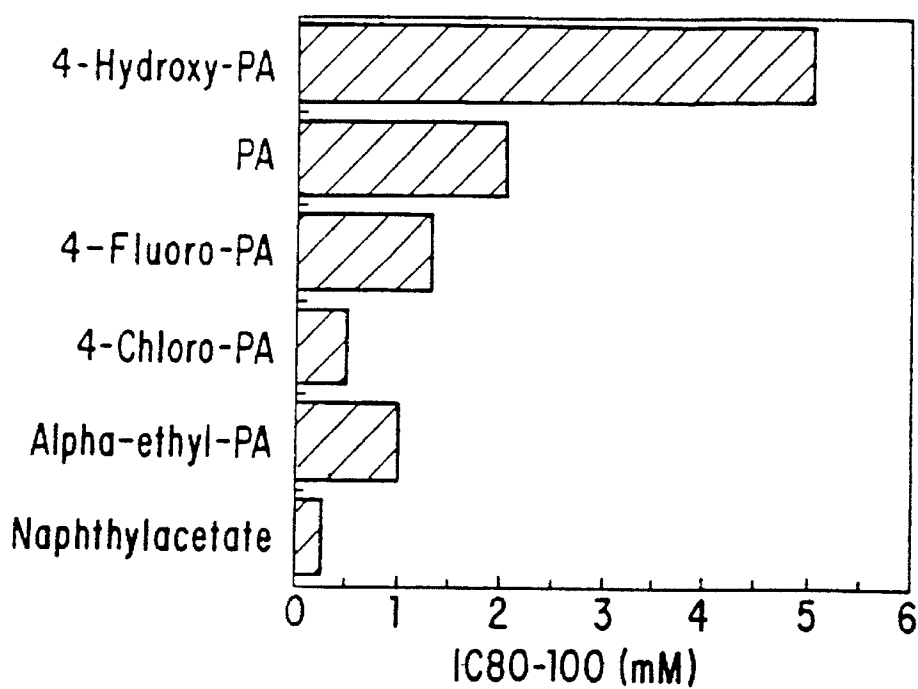
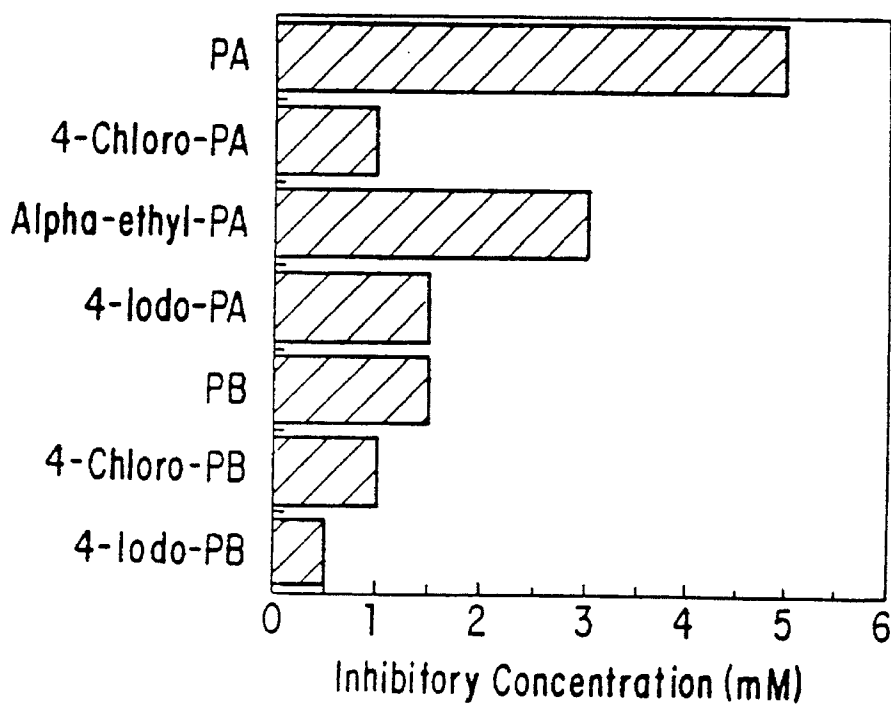
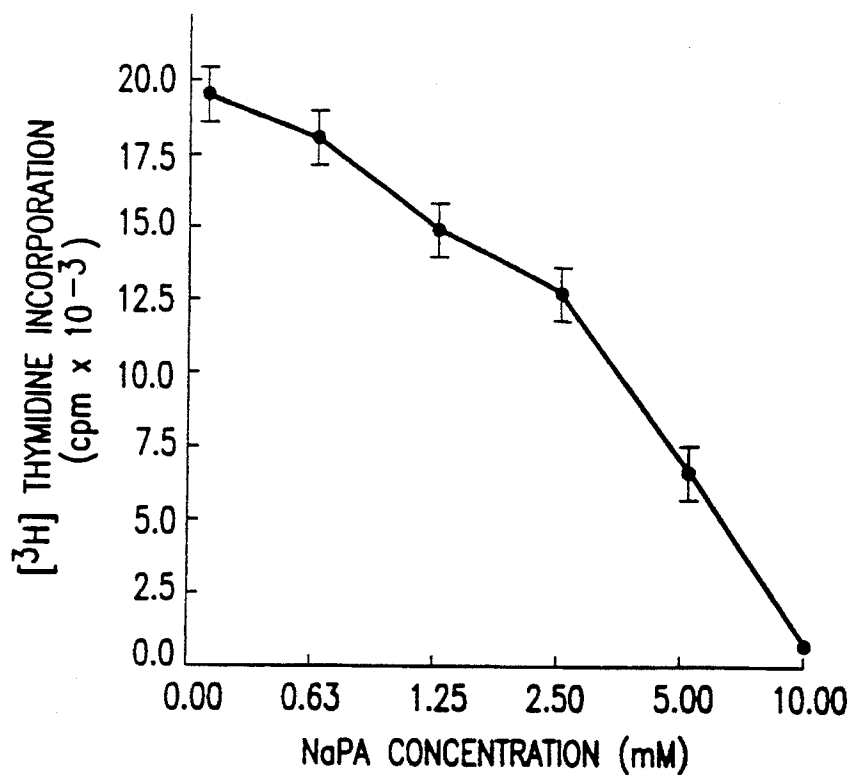
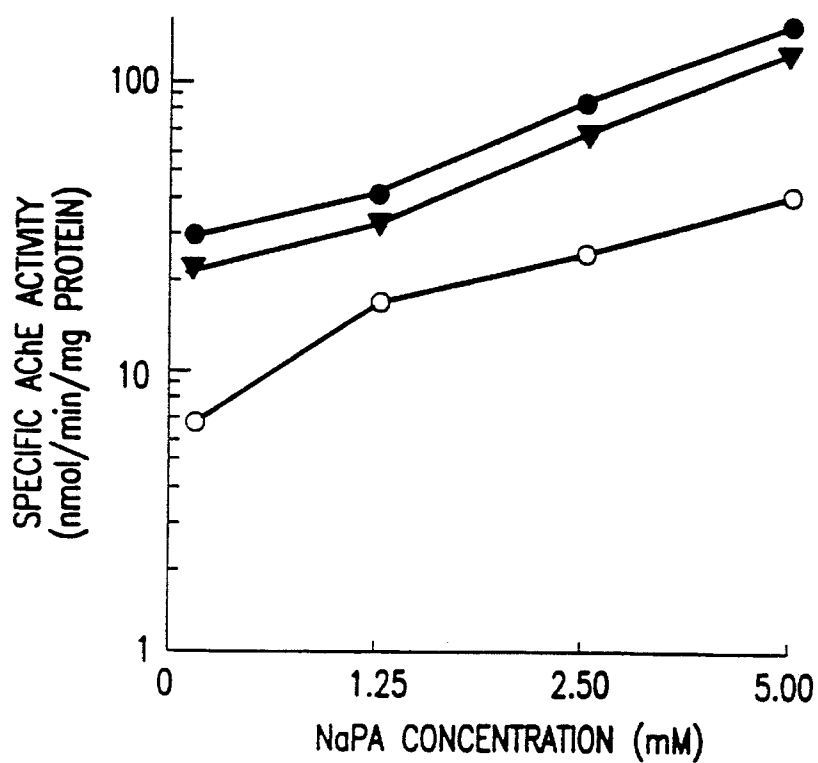


FIG 26

**FIG 27 A****FIG 27 B**

**FIG 28****FIG 30**

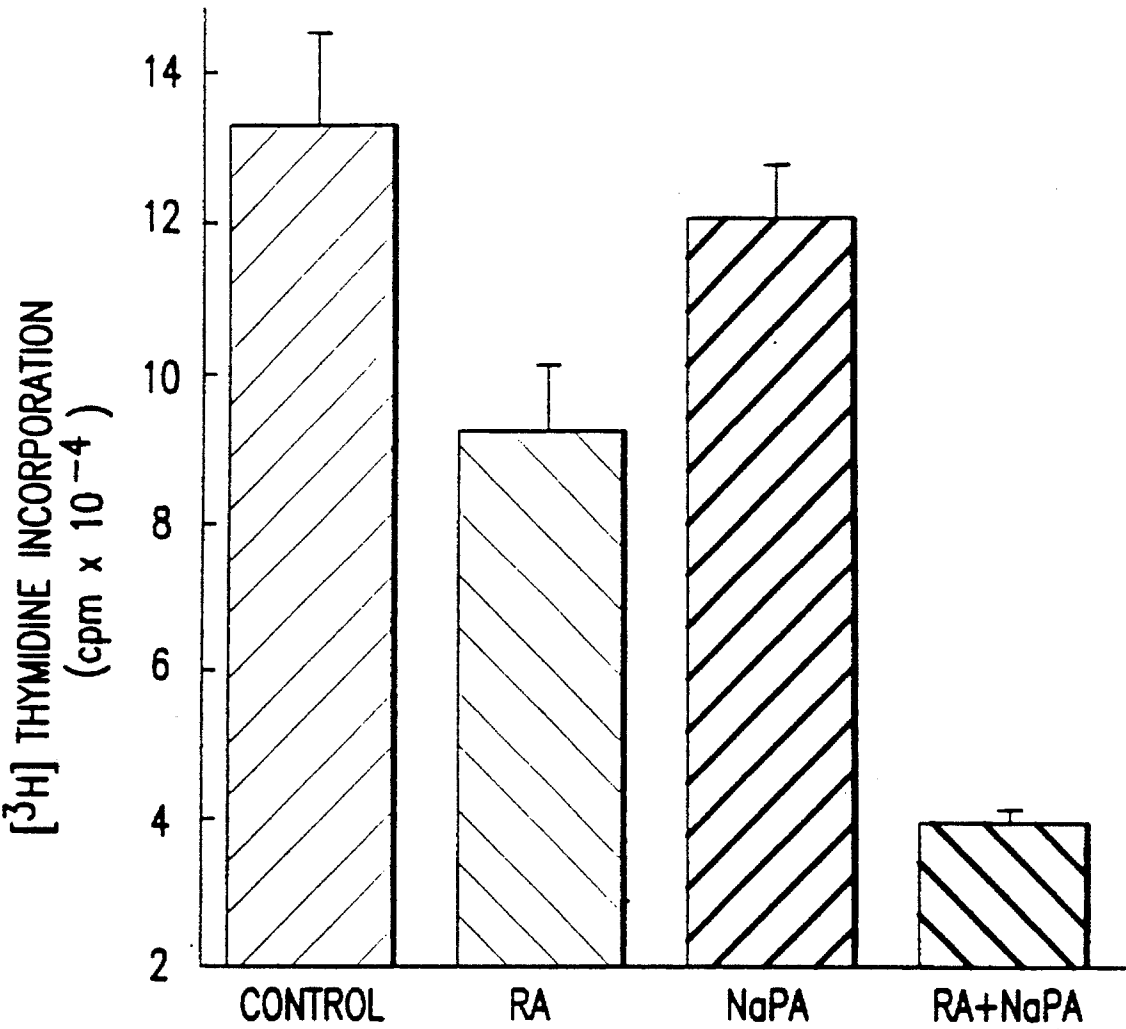


FIG 29

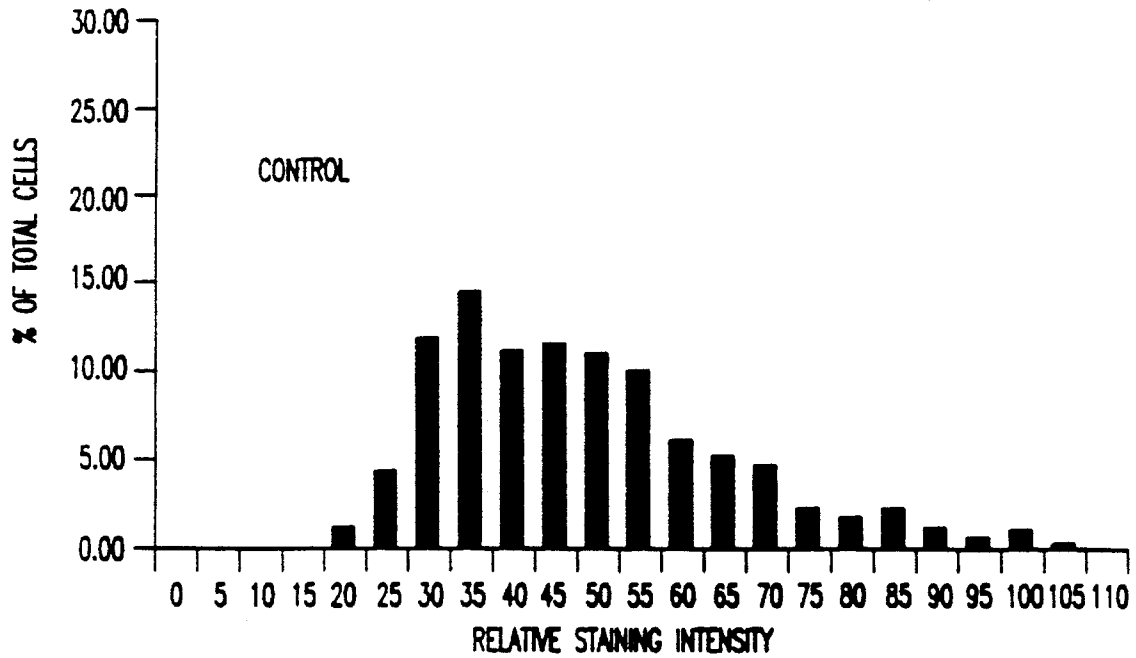


FIG 3A

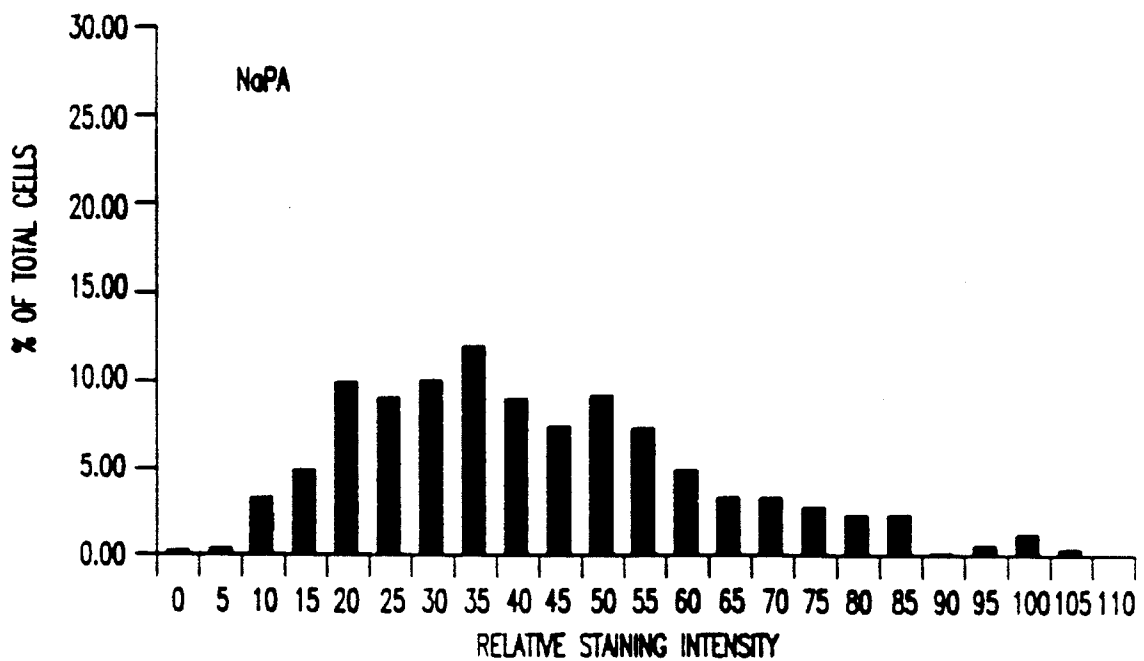


FIG 3B

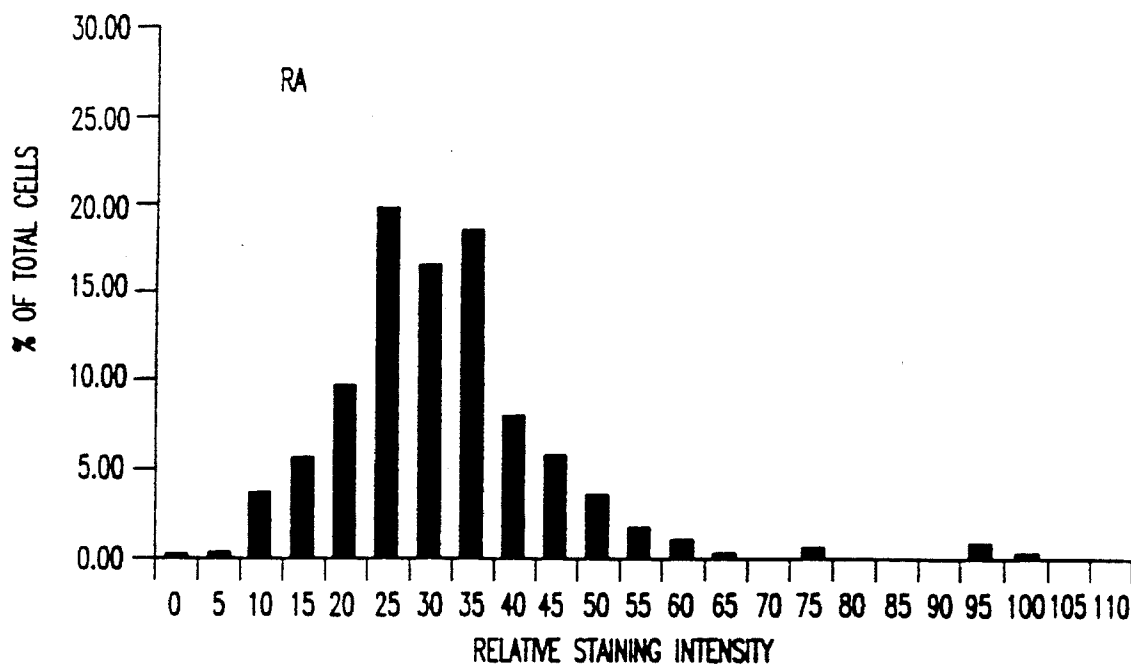


FIG 3A C

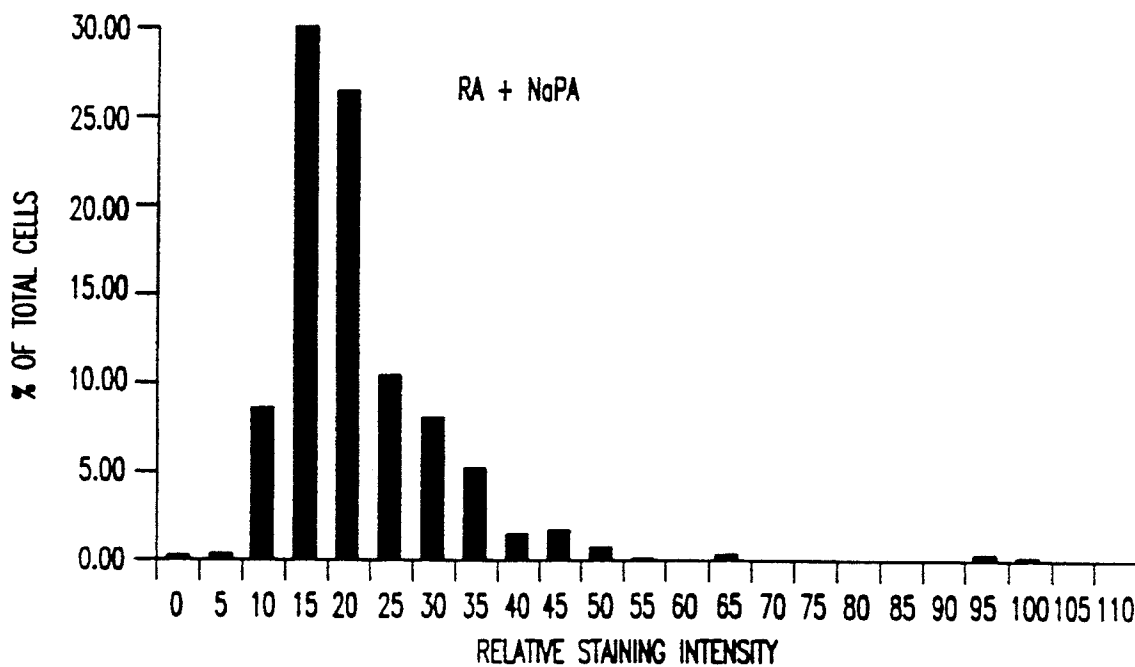
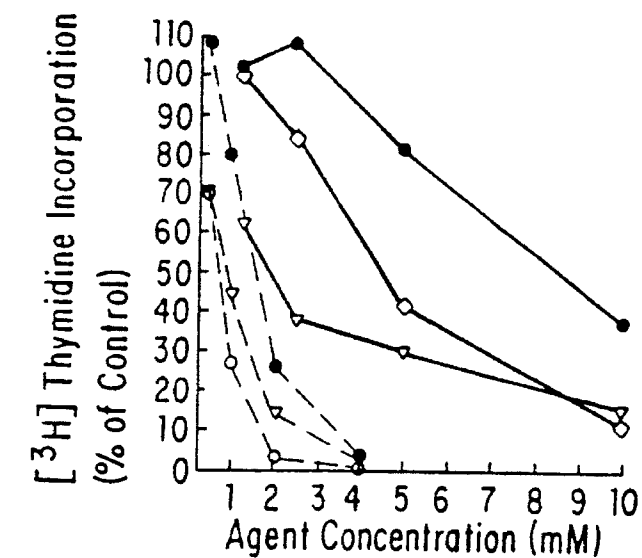
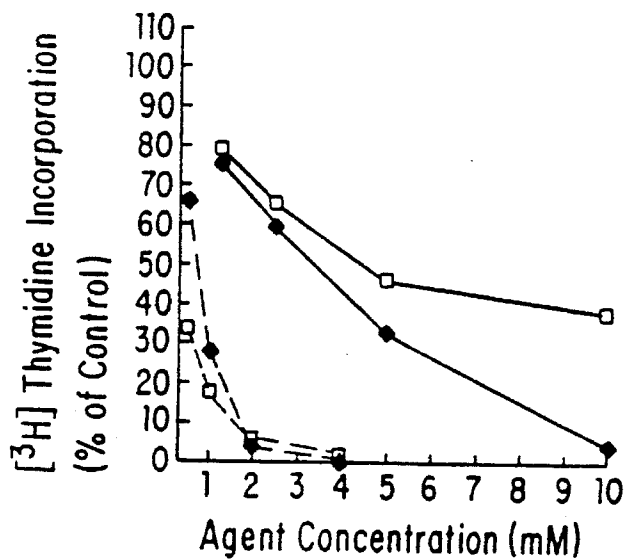


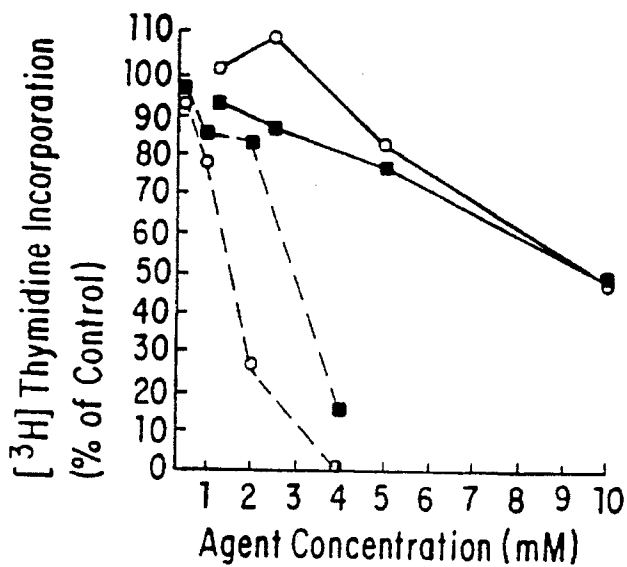
FIG 3A D



A



B



C

FIG 32

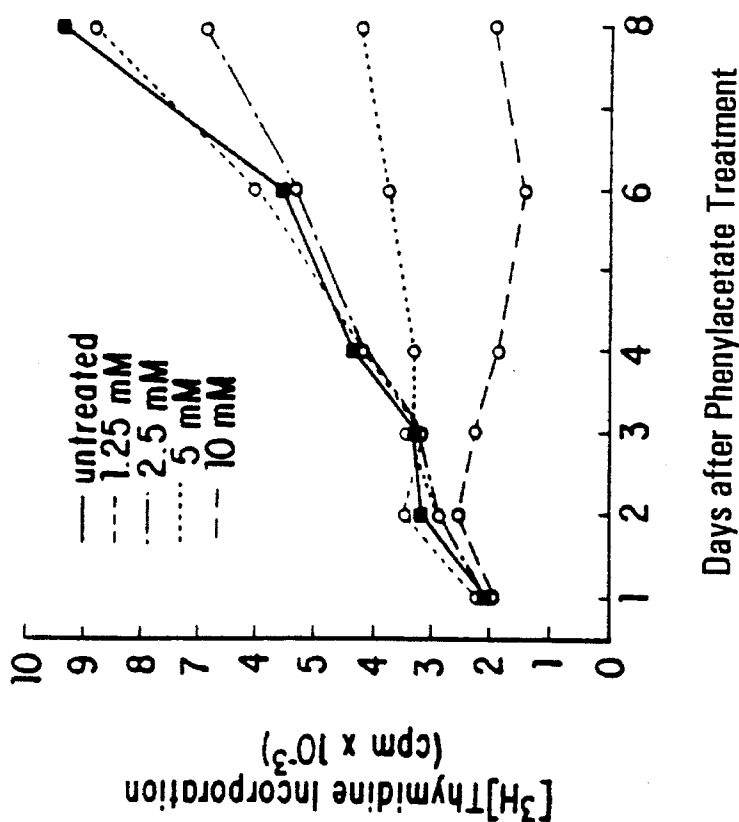


FIG 33B

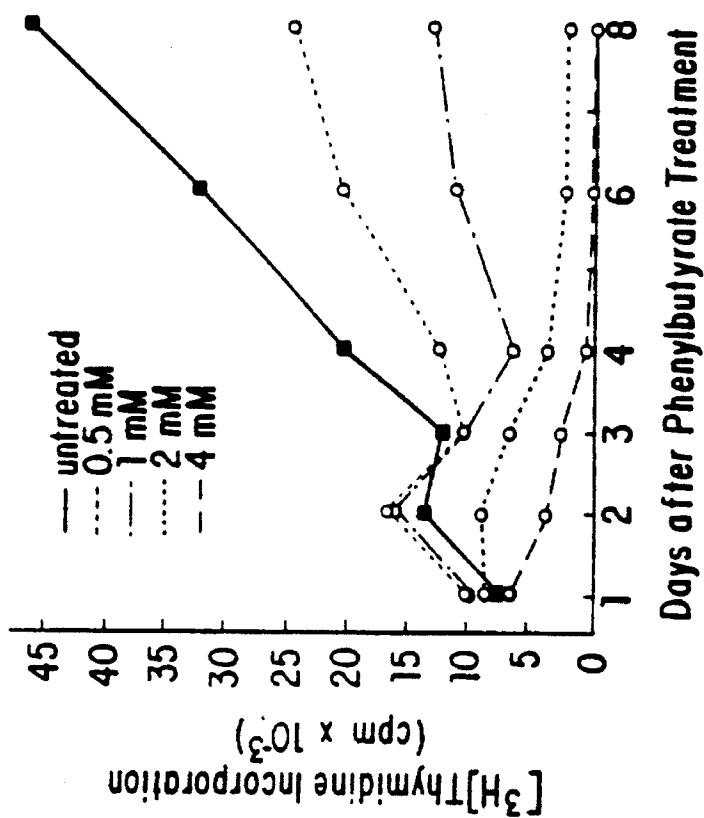
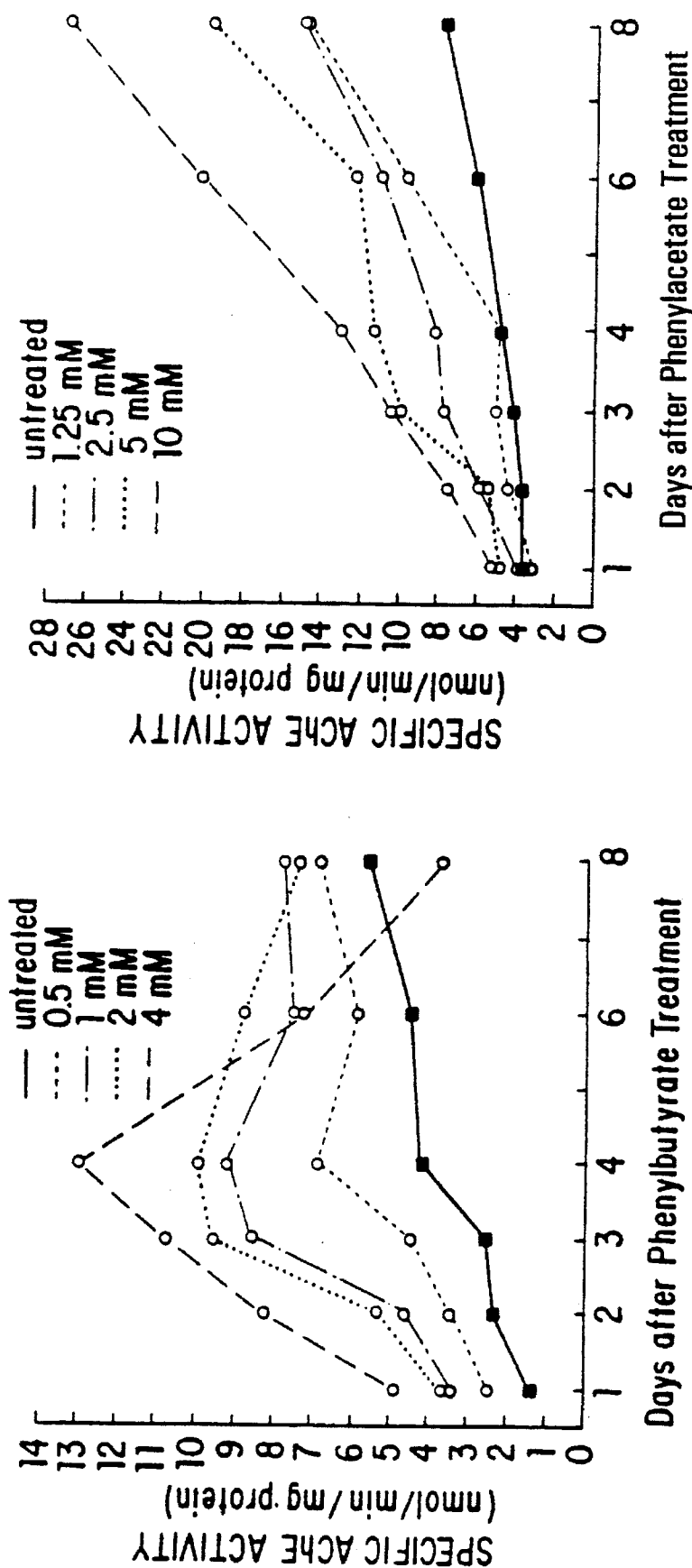


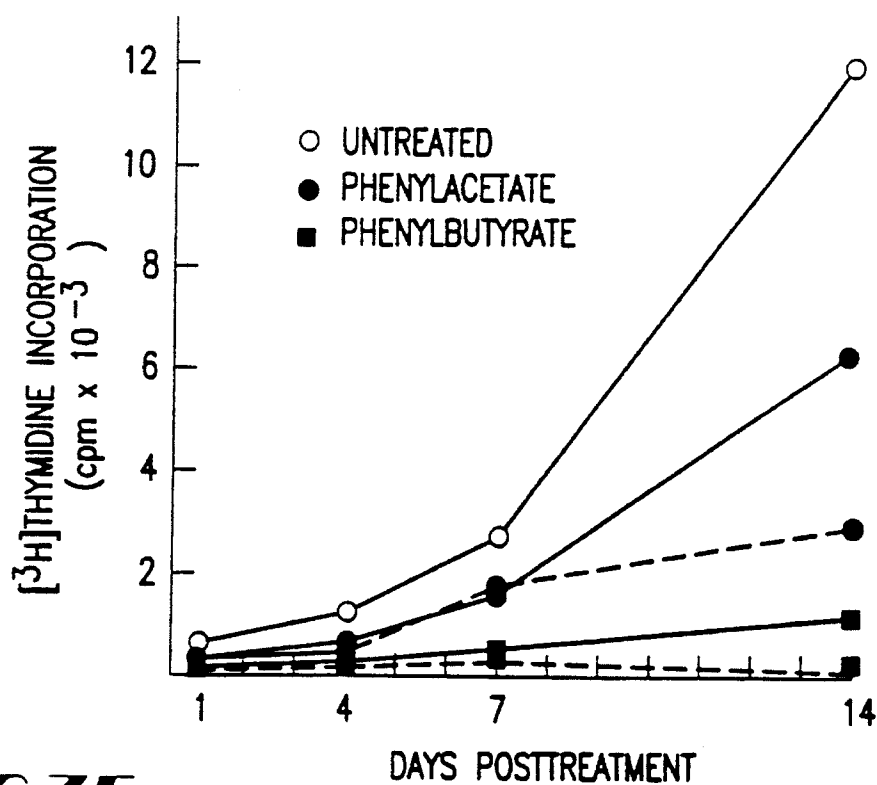
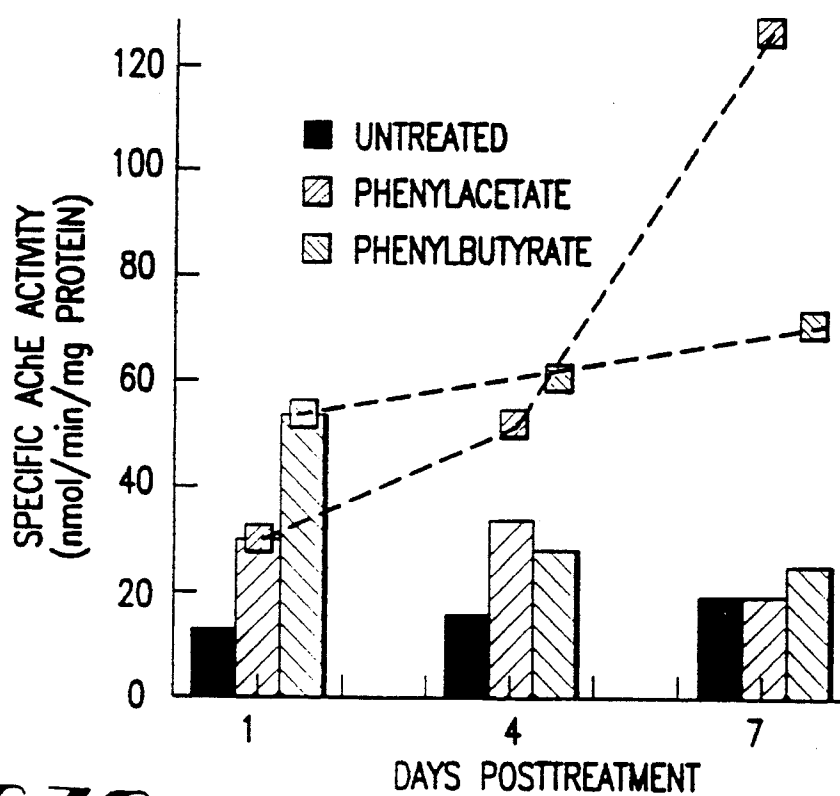
FIG 33A



A

B

FIG 34

**FIG 35****FIG 36**

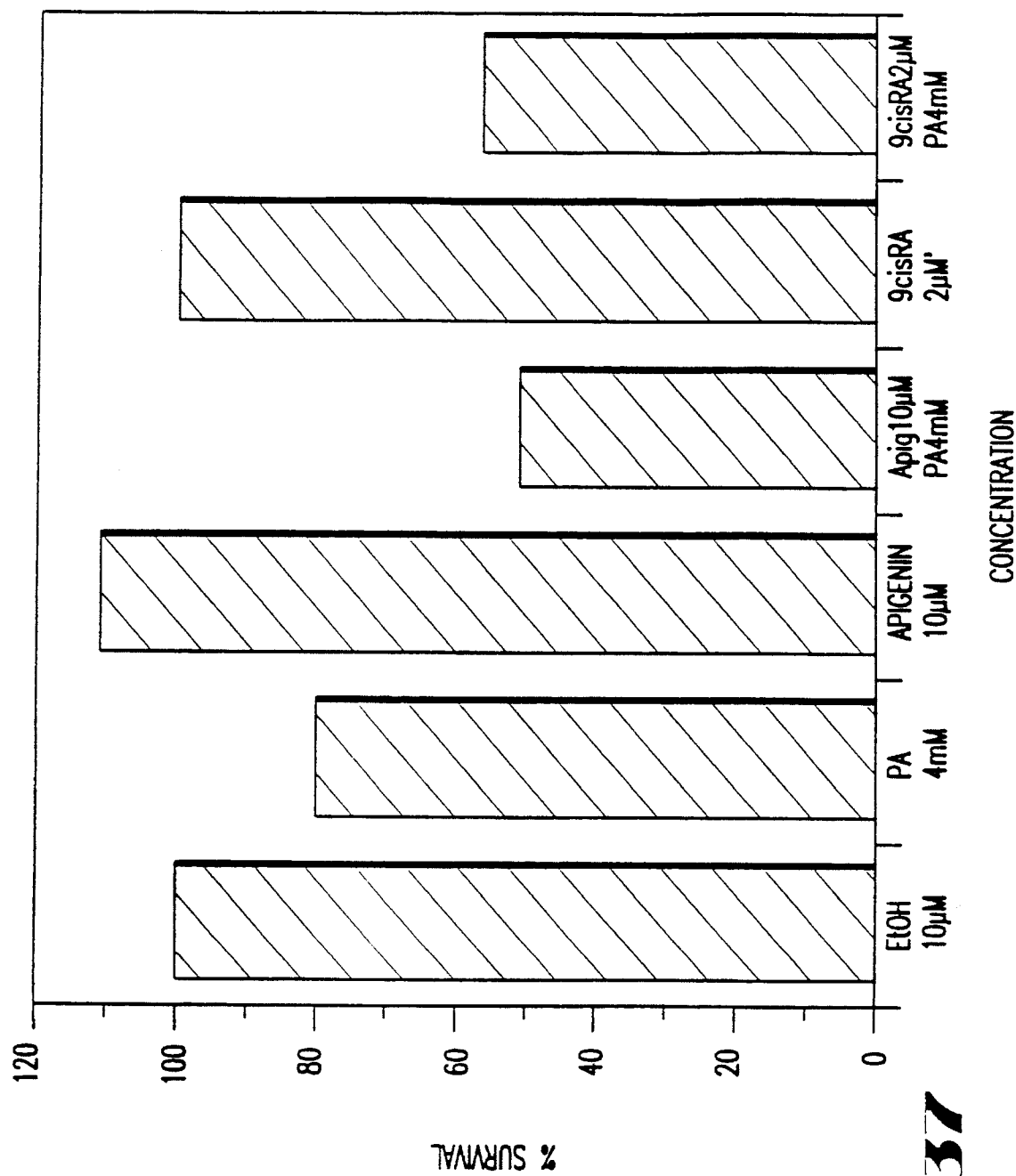


FIG 37

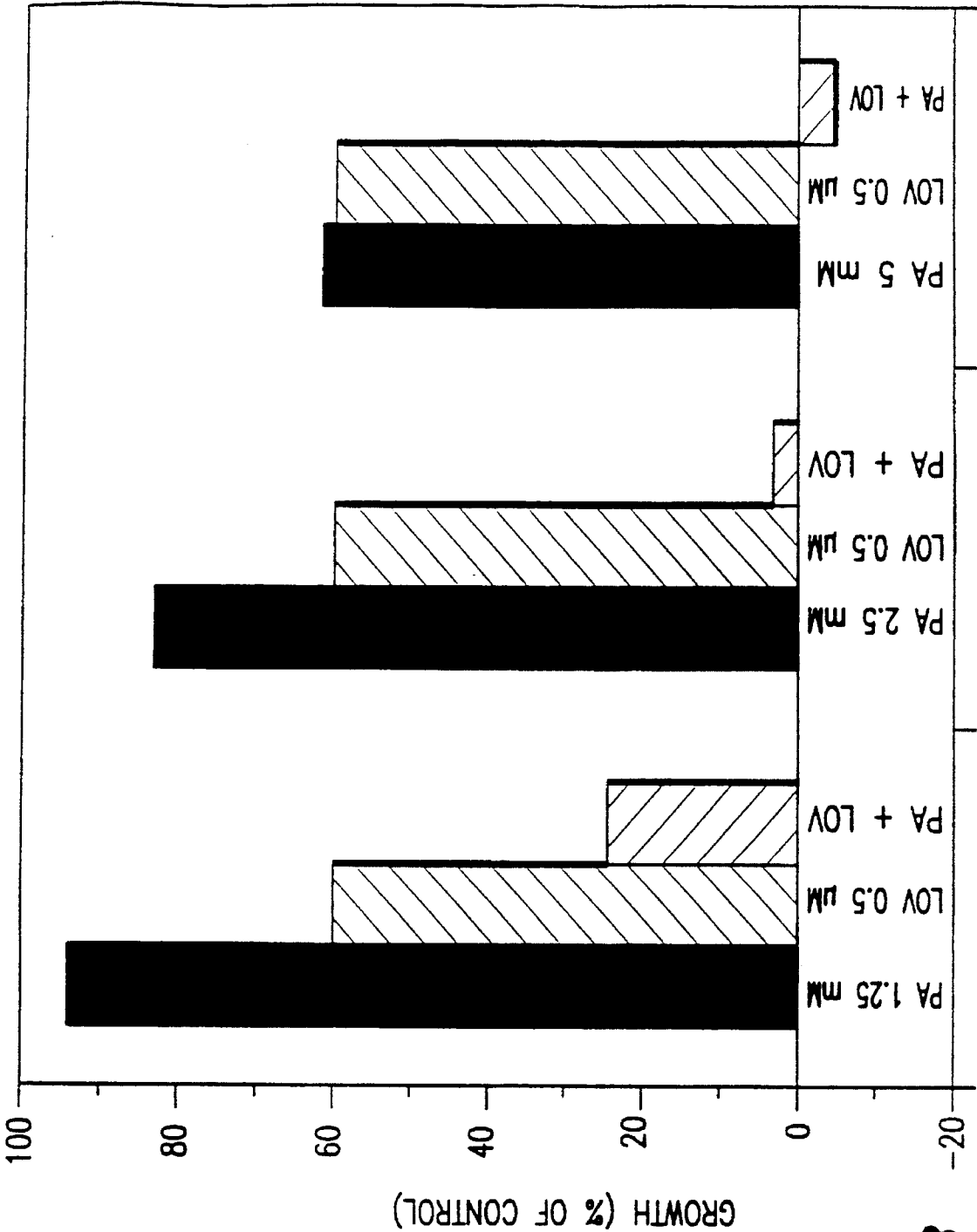


FIG 38

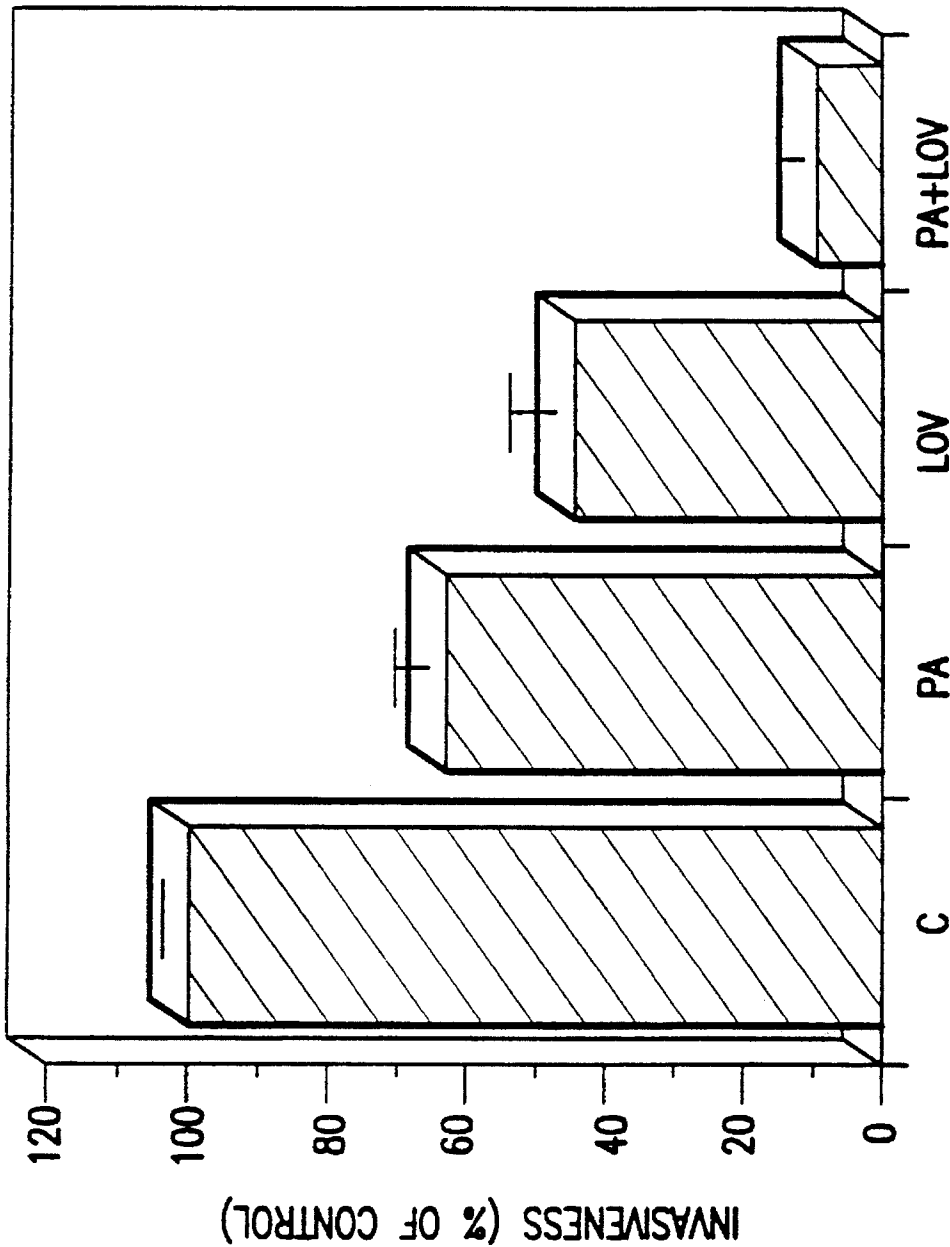
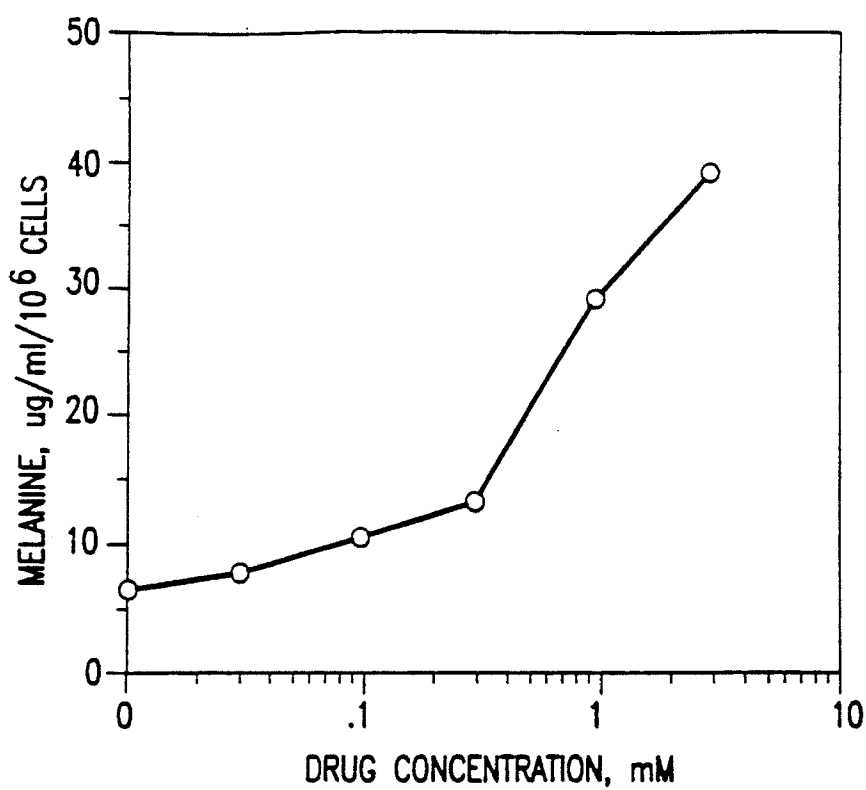
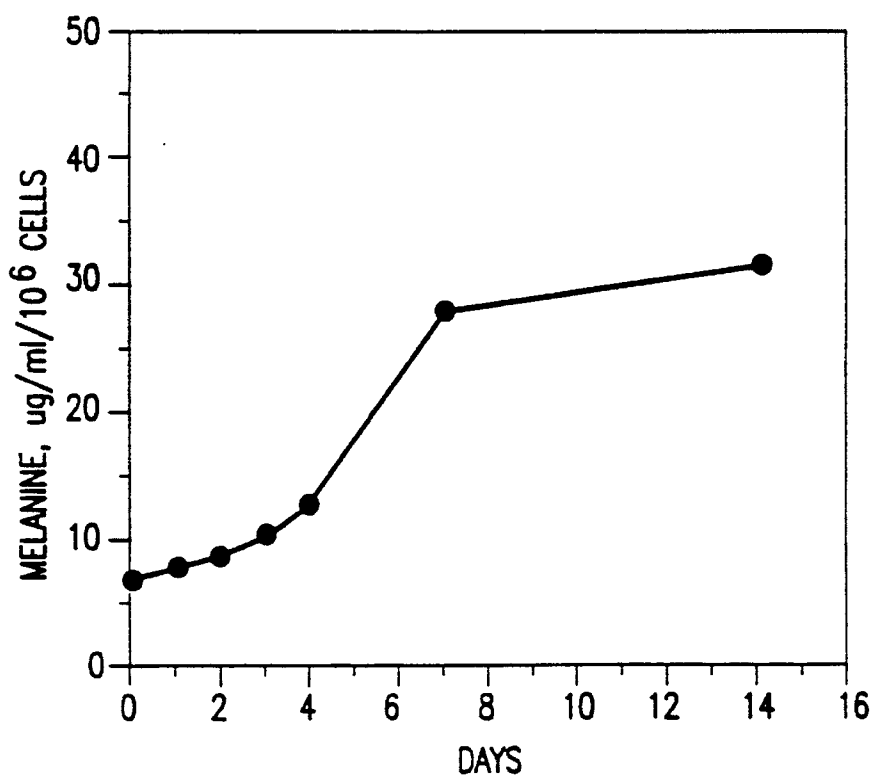


FIG 39

**FIG 40A****FIG 40B**

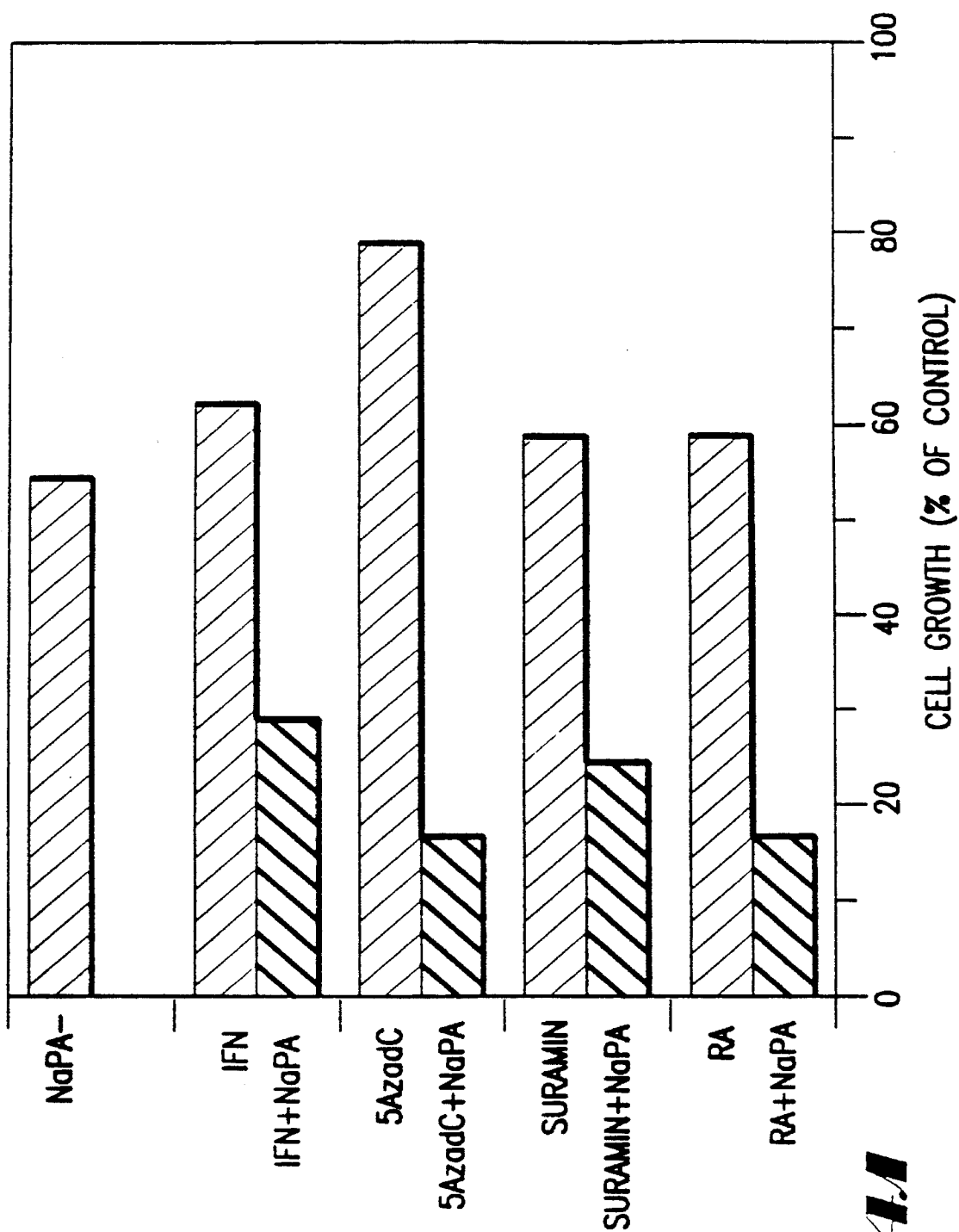


FIG 4A

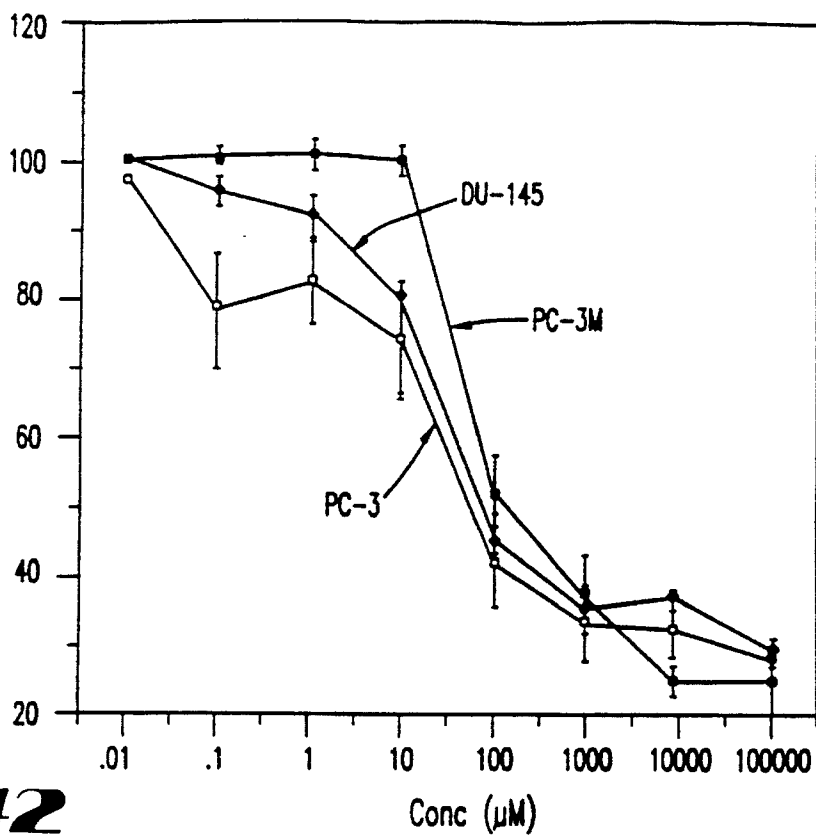


FIG 42

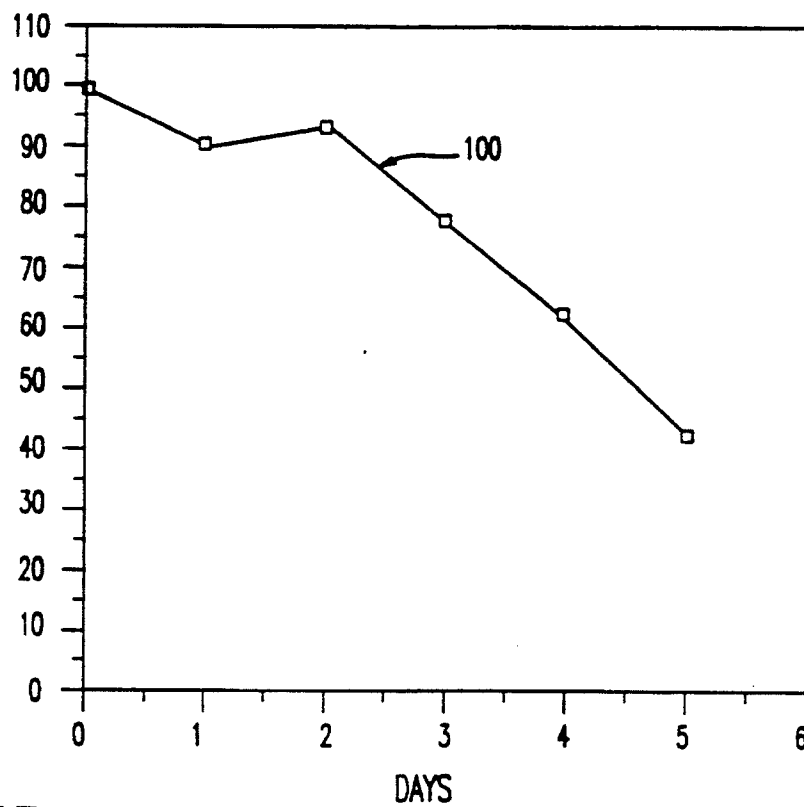


FIG 43

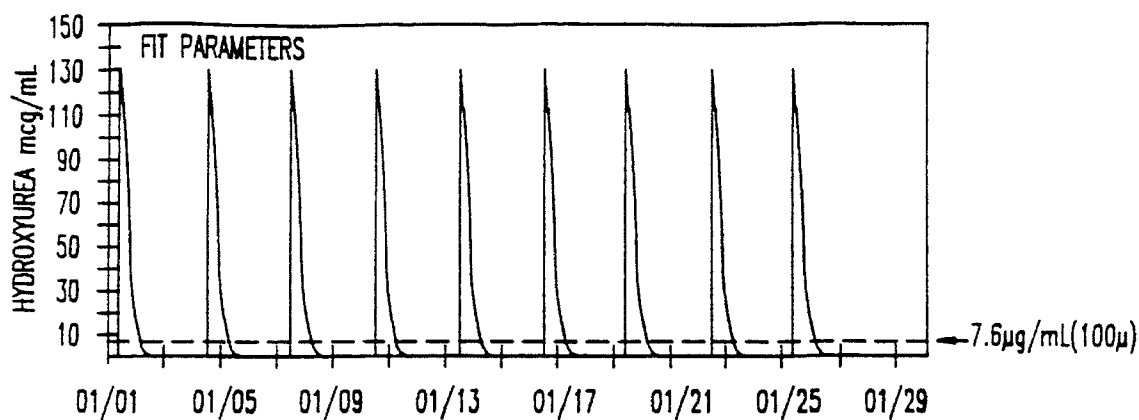


FIG 44A

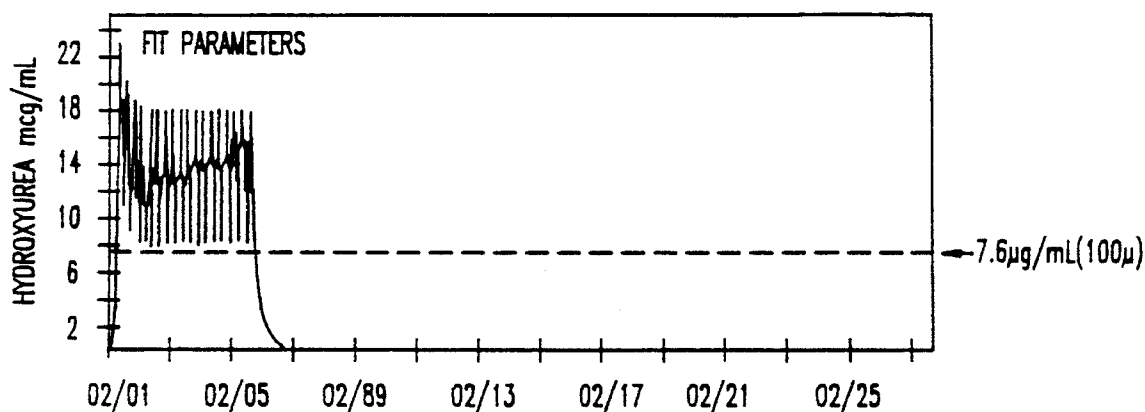


FIG 44B

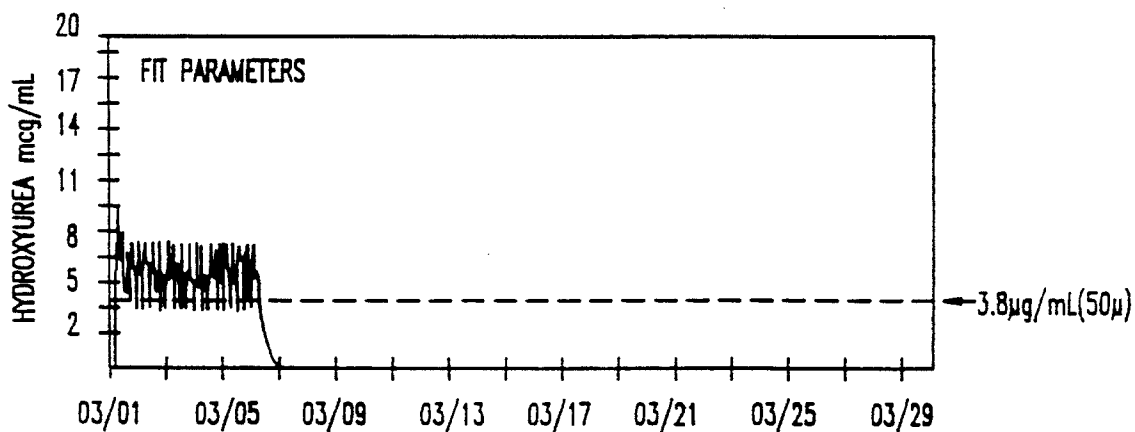


FIG 44C

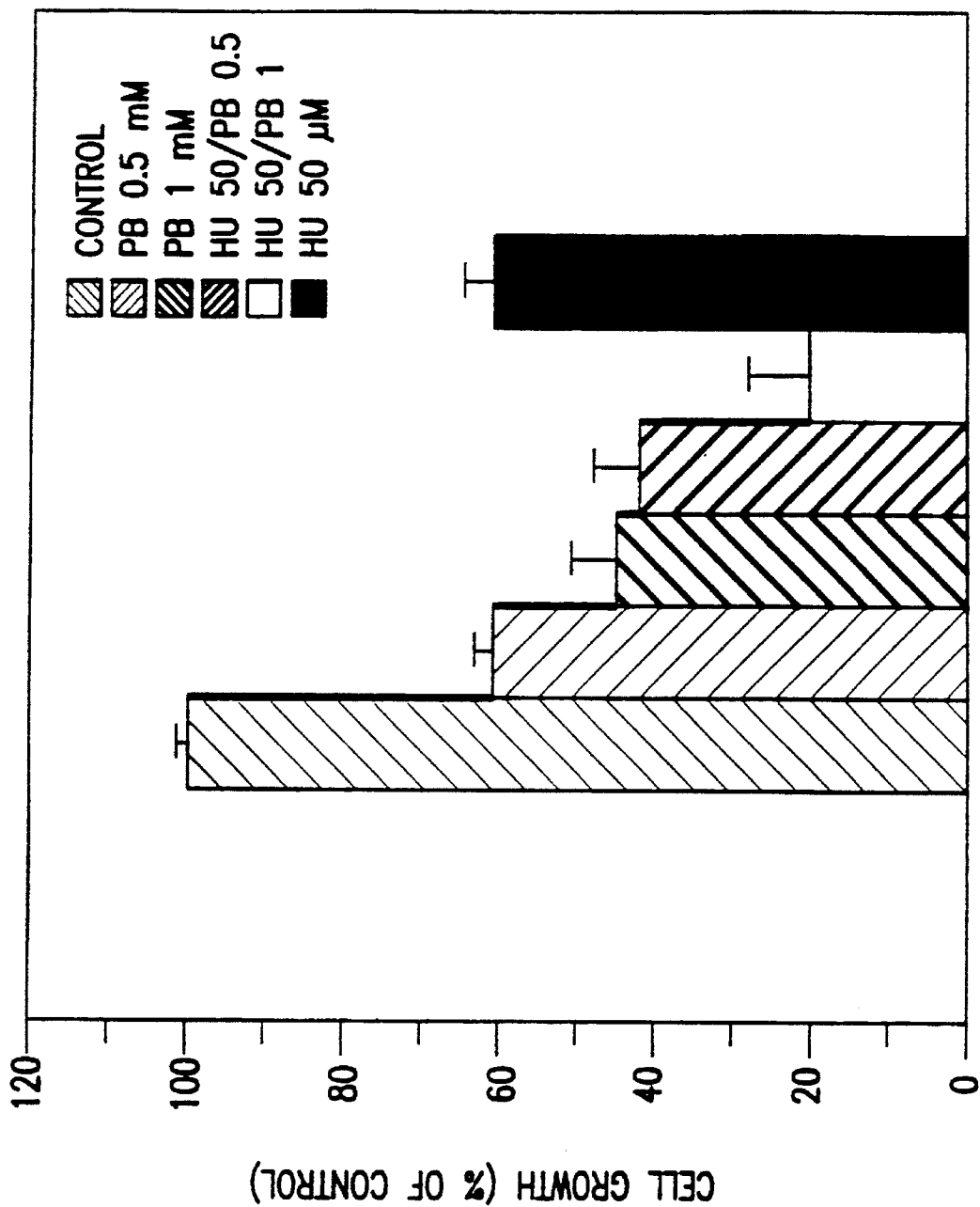


FIG 45

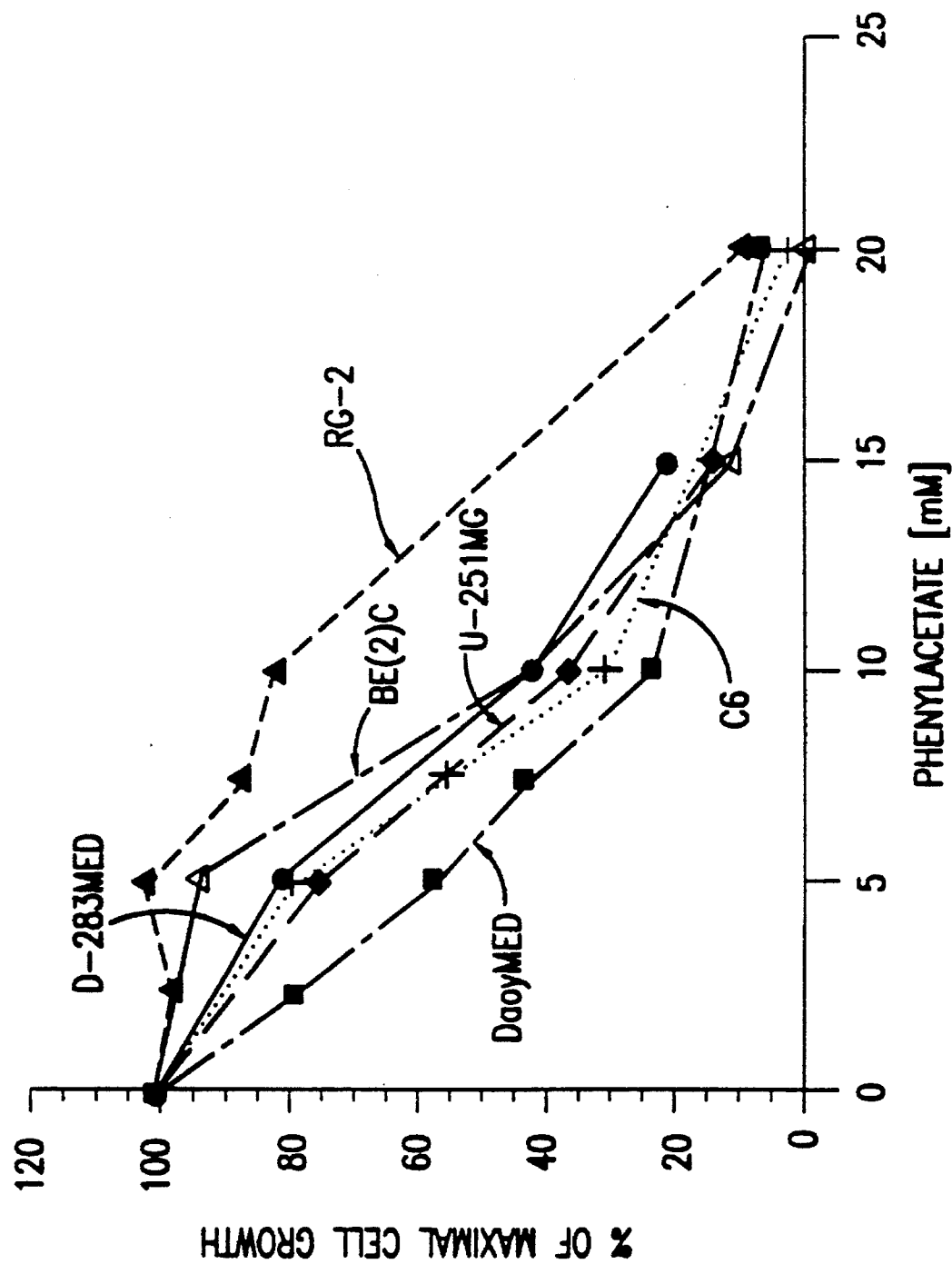
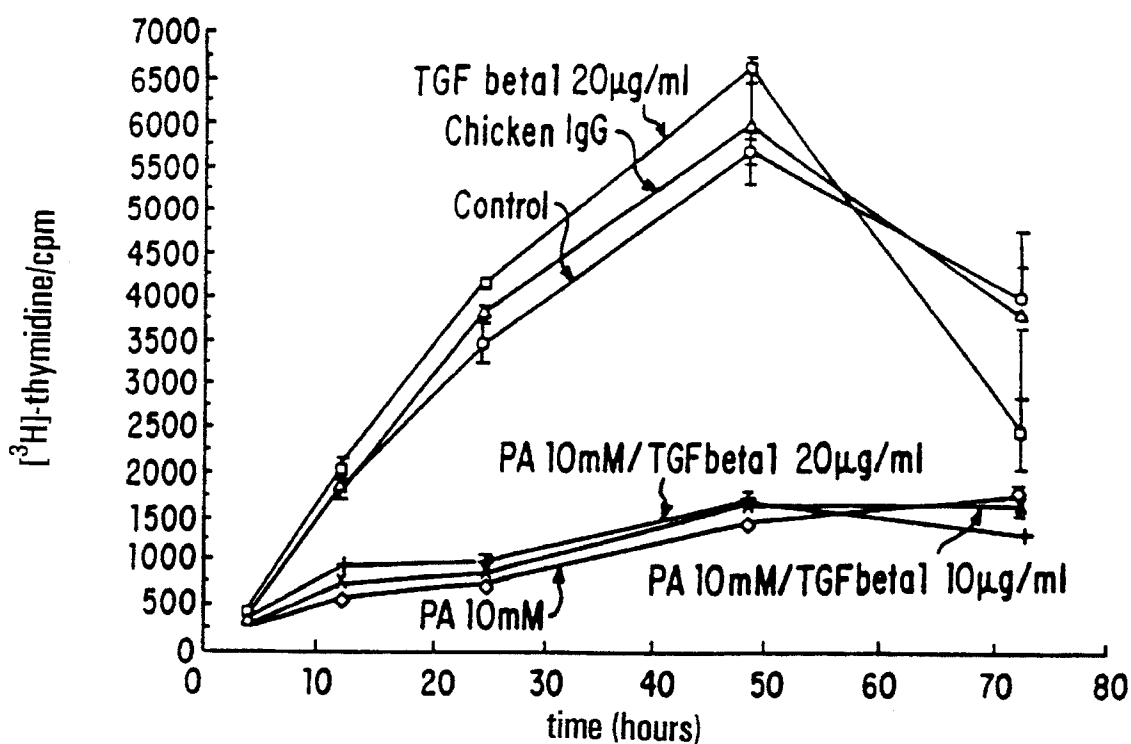
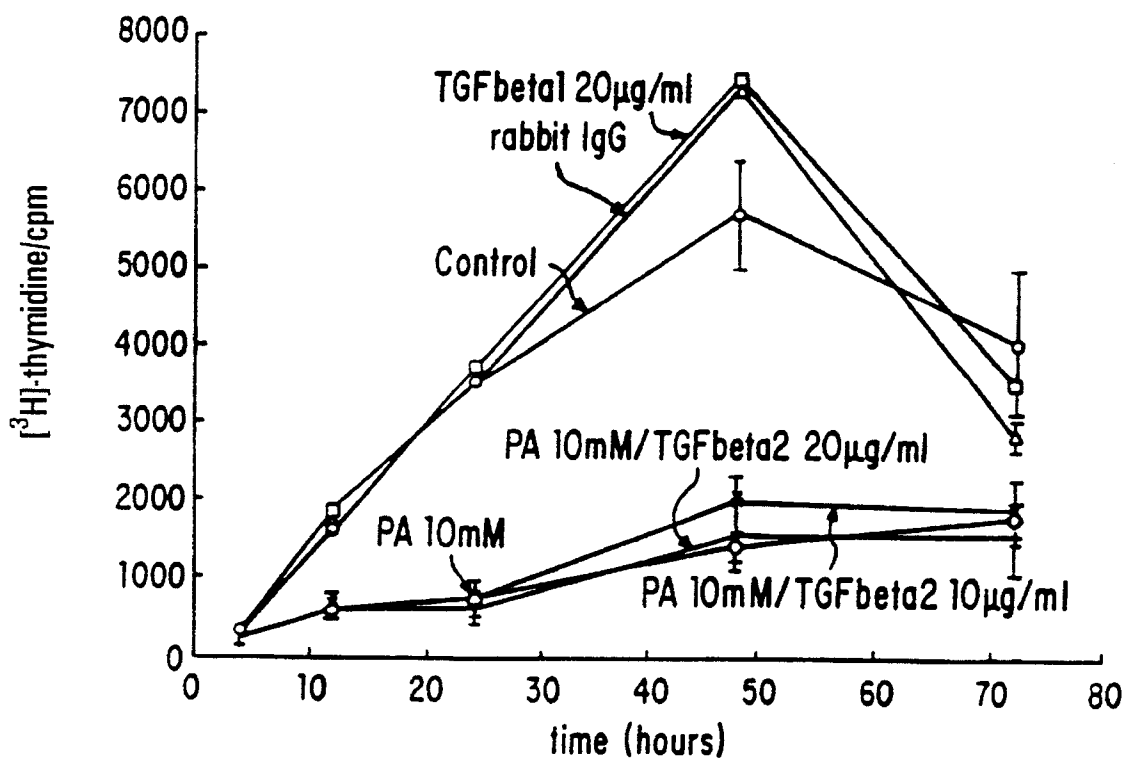


FIG 46

**FIG 47A****FIG 47 B**

COMPOSITIONS AND METHODS FOR TREATING AND PREVENTING PATHOLOGIES INCLUDING CANCER

This application is a continuation-in-part of Applicant's
copending U.S. Ser. No. 08/135,661, filed Oct. 12, 1993,
which in turn is a continuation-in-part of copending U.S.
Ser. No. 07/779,744, filed Oct. 21, 1991, the contents of
which are both hereby incorporated by this reference.

I. FIELD OF THE INVENTION

This invention relates to methods of using phenylacetic
acid and its pharmaceutically acceptable derivatives to treat
and prevent pathologies and to modulate cellular activities.
In particular, this invention relates to A) phenylacetate and
its derivatives in cancer prevention and maintenance
therapy, B) phenylacetate and its derivatives in the treatment
and prevention of AIDS, C) induction of fetal hemoglobin
synthesis in β -chain hemoglobinopathy by phenylacetate
and its derivatives, D) use of phenylacetic acid and its
derivatives in wound healing, E) use of phenylacetic acid
and its derivatives in treatment of diseases associated with
interleukin-6, F) use of phenylacetic acid and its derivatives
in the treatment of AIDS-associated CNS dysfunction, G)
use of phenylacetic acid and its derivatives to enhance
immunosurveillance, H) methods of monitoring the dosage
level of phenylacetic acid and its derivatives in a patient
and/or the patient response to these drugs, I) the activation
of the PPAR by phenylacetic acid and its derivatives, J) use
of phenylacetic acid and its derivatives in treatment of
cancers having a multiple-drug resistant phenotype, K)
phenylacetic acid and its derivatives, correlation between
potency and lipophilicity, L) phenylacetic acid and its
derivatives in synergistic combination with lovastatin for the
treatment and prevention of cancers such as malignant
gliomas or other CNS tumors, M) phenylacetic acid and its
derivatives in synergistic combination with retinoic acid for
the treatment and prevention of cancers such as those
involving neuroblastoma cells, N) phenylacetic acid and its
derivatives for the treatment and prevention of cancers and
other differentiation disorders such as those involving malig-
nant melanoma or other neuroectodermal tumors, O) phe-
nylacetic acid and its derivatives in synergistic combination
with hydroxyurea (HU) for the treatment and prevention of
cancers such as prostate cancer, P) phenylacetic acid and its
derivatives for the treatment and prevention of cancers
involving medulloblastoma and astrocytoma derived cells,
Q) phenylacetic acid and its derivatives in human studies
relating to treatments with PA and PB, R) phenylacetic acid
and its derivatives in methods of altering lipid metabolism,
including reducing serum triglycerides, and S) methods of
administering phenylacetic acid and its derivatives.

II. BACKGROUND OF THE INVENTION

Phenylacetic acid (PAA) is a protein decomposition prod-
uct found throughout the phylogenetic spectrum, ranging
from bacteria to man. Highly conserved in evolution, PAA
may play a fundamental role in growth control and differ-
entiation. In plants, PAA serves as a growth hormone (auxin)
promoting cell proliferation and enlargement at low doses
(10^{-5} – 10^{-7} M), while inhibiting growth at higher concentra-
tions. The effect on animal and human cells is less well
characterized. In humans, PAA is known to conjugate
glutamine with subsequent renal excretion of phenylacetyl-
glutamine (PAG). The latter, leading to waste nitrogen

excretion, has been the basis for using PAA or preferably its
salt sodium phenylacetate (NaPA, also referenced herein as
that active anionic moiety, phenylacetate or "PA") in the
treatment of hyperammonemia associated with inborn errors
of ureagenesis. Clinical experience indicates that acute or
long-term treatment with high NaPA doses is well tolerated,
essentially free of adverse effects, and effective in removing
excess glutamine. [Brusilow, S. W., Horwich, A. L. Urea
cycle enzymes. *Metabolic Basis of Inherited Diseases*, Vol.
6:629–633 (1989)]. These characteristics should be of value
in treatments of cancer and prevention of cancer, treatments
which inhibit virus replication and treatments of severe
beta-chain hemoglobinopathies.

Glutamine is the major nitrogen source for nucleic acid
and protein synthesis, and a substrate for energy in rapidly
dividing normal and tumor cells. Compared with normal
tissues, most tumors, due to decreased synthesis of
glutamine along with accelerated utilization and catabolism,
operate at limiting levels of glutamine availability, and
consequently are sensitive to further glutamine depletion.
Considering the imbalance in glutamine metabolism in
tumor cells and the ability of PAA to remove glutamine, PAA
has been proposed as a potential antitumor agent; however,
no data has previously been provided to substantiate this
proposal. [Neish, W. J. P. "Phenylacetic Acid as a Potential
Therapeutic Agent for the Treatment of Human Cancer",
Experientia, Vol 27, pp 860–861 (1971)].

Despite these efforts to fight cancer, many malignant
diseases that are of interest in this application continue to
present major challenges to clinical oncology. Prostate can-
cer, for example, is the second most common cause of cancer
deaths in men. Current treatment protocols rely primarily on
hormonal manipulations. However, in spite of initial high
response rates, patients often develop hormone-refractory
tumors, leading to rapid disease progression with poor
prognosis. Overall, the results of cytotoxic chemotherapy
have been disappointing, indicating a long felt need for new
approaches to treatment of advanced prostatic cancer. Other
diseases resulting from abnormal cell replication, for
example metastatic melanomas, brain tumors of glial origin
(e.g., astrocytomas), and lung adenocarcinoma, are also
highly aggressive malignancies with poor prognosis. The
incidence of melanoma and lung adenocarcinoma has been
increasing significantly in recent years. Surgical treatments
of brain tumors often fail to remove all tumor tissues,
resulting in recurrences. Systemic chemotherapy is hindered
by blood barriers. Therefore, there is an urgent need for new
approaches to the treatment of human malignancies includ-
ing advanced prostatic cancer, melanoma, brain tumors.

The development of the methods and pharmaceuticals of
the present invention was guided by the hypothesis that
metabolic traits that distinguish tumors from normal cells
could potentially serve as targets for therapeutic interven-
tion. For instance, tumor cells show unique requirements for
specific amino acids such as glutamine. Thus, glutamine
may be a desired choice because of its major contribution to
energy metabolism and to synthesis of purines, pyrimidines,
and proteins. Along this line, promising antineoplastic
activities have been demonstrated with glutamine-depleting
enzymes such as glutaminase, and various glutamine anti-
metabolites. Unfortunately, the clinical usefulness of these
drugs has been limited by unacceptable toxicities. Conse-
quently, the present invention focuses on PAA, a plasma
component known to conjugate glutamine in vivo, and the
pharmaceutically acceptable derivatives of PAA.

In addition to its ability to bind glutamine to form
glutamine phenylacetate, phenylacetic acid (PAA) can

induce tumor cells to undergo differentiation. (See examples 1-5, 7-9, 11-13, and 16 herein). Differentiation therapy is a known, desirable approach for cancer intervention. The underlying hypothesis is that neoplastic transformation results from defects in cellular differentiation. Inducing tumor cells to differentiate would prevent tumor progression and bring about reversal of malignancy. Several differentiation agents are known, but their clinical applications have been hindered by unacceptable toxicities and/or deleterious side effects.

The utility of PAA and its derivatives is more fully delineated in the above-referenced copending applications. As discussed in these applications, PAA is a nontoxic differentiation enhancer and has antitumor activity in laboratory models and in man. Preclinical studies indicate that phenylacetate and related aromatic fatty acids induce cytostasis and promote maturation of various human malignant cells, including hormone-refractory prostatic carcinoma and glioblastoma. The marked changes in tumor biology are associated with alterations in the expression of genes implicated in tumor growth, invasion, angiogenesis, and immunogenicity. PAA and its analogs appear to share several mechanisms of action, including: (a) regulation of gene expression through activation of a nuclear receptor; and (b) inhibition of the mevalonate pathway and protein isoprenylation. Thus, PAA appears particularly suited in the treatment of various neoplastic conditions.

One such neoplastic condition treatable by NaPA is neuroblastoma. As a malignant tumor of childhood, neuroblastoma has proven to be fascinating from a biological as well as clinical viewpoint. This cancer has the highest rate of spontaneous differentiation of all malignancies and several agents have been reported to induce maturation of neuroblastoma into a variety of cells sharing a neural-crest lineage (Evans, A. E., Chatten, J., D'Angio, G. J., Gerson, J. M., Robinson, J., and Schnauffer, L. A review of 17 IV-S neuroblastoma patients at the Children's Hospital of Philadelphia. *Cancer*, 45:833-839, 1980; Abemayor, E., and Sidell, N. Human neuroblastoma cell lines as models for the in vitro study of neoplastic an neuronal cell differentiation. *Environ. Health Perspect.*, 80:3-15, 1989). Among the compounds that have been explored as differentiating agents, retinoic acid (RA) (Sidell, N., Altman, A., Haussler, M. R., and Seeger, R. C. Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. *Expl. Cell Res.*, 148:21-30, 1983) was shown to be a potent compound for promoting the differentiation of a variety of human neuroblastoma cell lines (Abemayor, E., and Sidell, N. Human neuroblastoma cell lines as models for the in vitro study of neoplastic an neuronal cell differentiation. *Environ. Health Perspect.*, 80:3-15, 1989; Sidell, N., Altman, A., Haussler, M. R., and Seeger, R. C. Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. *Expl. Cell Res.*, 148:21-30, 1983; Thiele, C. T., Reynolds, C. P., and Israel, M. A. Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. *Nature*, 313:404-406, 1985); however, to date RA has demonstrated only limited clinical effectiveness in this disease (Finklestein, J. Z., Krailo, M. D., Lenarsky, C., Ladisch, S., Blair, G. K., Reynolds, C. P., Sitary, A. L., and Hammond, G. D., 13-cis-retinoic acid (NSC 122758) in the treatment of children with metastatic neuroblastoma unresponsive to conventional chemotherapy: Report from the Children's Cancer Study Group. *Med. Ped. Oncol.*, 20:307-311, 1992). In pursuit of increasing the efficacy of RA-induced differentiation of human neuroblastoma, a num-

ber of other compounds and biological response modifiers, such as cAMP-elevating agents and interferons, can potentiate the retinoid activity as well as render resistant populations sensitive to RA treatment (Lando, M., Abemayor, E., Verity, M. A., and Sidell, N. Modulation of intracellular cyclic AMP levels and the differentiation response of human neuroblastoma cells. *Cancer Res.*, 50:722-727, 1990; Wuarin, L., Verity, M. A., and Sidell, N. Effects of gamma-interferon and its interaction with retinoic acid on human neuroblastoma cells. *Int. J. Cancer*, 48:136-144, 1991). Some of these combination treatments are now being evaluated clinically or proposed for the treatment of neuroblastoma and other malignancies (Smith, M. A., Parkinson, D. R., Cheson, B. D., and Friedman, M. A. Retinoids in cancer therapy. *J. Clin. Oncol.*, 10:839-864, 1992). However, there exists a need for more effective combination treatments for the treatment of neuroblastoma and other similar cancers and pathologies.

Another neoplastic condition which heretofore has been difficult to treat is malignant glioma. Malignant gliomas are highly dependent on the mevalonate (MVA) pathway for the synthesis of sterols and isoprenoids critical to cell replication (Fumagalli, R., Grossi, E., Paoletti, P. and Paolette, R. Studies on lipids in brain tumors. I. Occurrence and significance of sterol precursors of cholesterol in human brain tumors. *J. Neurochem.* 11:561-565, 1964; Kandutsch, A. A. and Saucier, S. E. Regulation of sterol synthesis in developing brains of normal and jimpy mice. *Arch. Biochem. Biophys.* 135:201-208, 1969; Grossi, E., Paoletti, P. and Paoletti, R. An analysis of brain cholesterol and fatty acid biosynthesis. *Arch. Int. Physiol. Biochem.* 66:564-572, 1958; Azarnoff, D. L., Curran, G. L. and Williamson, W. P. Incorporation of acetate-1-¹⁴C into cholesterol by human intracranial tumors in vitro. *J. Nat. Cancer Inst.* 21:1109-1115, 1958; Rudling, M. J., Angelin, B., Peterson, C. O. and Collins, V. P. Low density lipoprotein receptor activity in human intracranial tumors and its relation to cholesterol requirement. *Cancer Res.* 50 (suppl):483-487, 1990). Targeting MVA synthesis and/or utilization would be expected to inhibit tumor growth without damaging normal brain tissues, in which the MVA pathway is minimally active. Two enzymes control the rate limiting steps of the MVA pathway of cholesterol synthesis: (a) 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyzes the synthesis of MVA from acetyl-CoA; and, (b) MVA-pyrophosphate (MVA-PP) decarboxylase controls MVA utilization and, consequently, the post-translational processing and function of intracellular signalling proteins (Goldstein, J. L. and Brown, M. S. Regulation of the mevalonate pathway. *Nature*. 343:425-430, 1990; Marshall, C. J. Protein prenylation: A mediator of protein-protein interactions. *Science*. 259:1865-1866, 1993). Therefore, it is highly desirable for the treatment of malignant gliomas or other similar cancers and pathologies to find a treatment capable of inhibiting these two steps of the MVA pathway.

A further neoplastic condition which has been difficult to treat is malignant melanoma. Disseminated malignant melanoma is characterized by a high mortality rate and resistance to conventional therapies (Ferdy Lejeune, Jean Bauer, Serge Leyvraz, Danielle Lienard (1993): Disseminated melanoma, preclinical therapeutic studies, clinical trial, and patient treatment. *Current Opinion in Oncology* 5:390-396). Differentiation therapy may provide an alternative for treatment of cancers that do not or poorly respond to cytotoxic chemotherapy (Kelloff, G. J., Boone, C. W., Malone, E. F., Steele, V. E. (1992): Chemoprevention clinical trials. *Mutation Res.* 267:291-295). Several differentiation inducers are

capable of altering the phenotype of melanoma cells in vitro. These include retinoids, butyrate, dibutyl adenosine 3':5'-cyclic monophosphate (dbc AMP), 5'-Azacytidine, interferons, hexamethylene bisacetamide (HMBA), dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), 12-tetradecanoylphorbol-13 acetate (TPA) (Mary J. Hendrix, Rebecca W. Wood, et al. (1990): Retinoic acid inhibition of human melanoma cell invasion through a reconstituted basement membrane and its relation to decreases in the expression of proteolytic enzymes and motility. *Cancer Research* 50:4121-4130; Luara Giffre, Magali Schreyer, Jean-pierre Mach, Stefan Carrel (1988): Cyclic AMP induces differentiation in vitro of human melanoma cells. *Cancer* 61:1132-1141; Jardena Nordenberg, Lina Wasserman, Einat Beery, Doron Aloni, Hagit Malik, Kurt H. Stenzel, Abraham Novogrodsky (1986): Growth inhibition of murine melanoma by butyric acid and dimethylsulfoxide. *Experimental Cell Research* 162:77-85; Eliezer Huberman, Carol Heckman, Rober Langenbach (1979): Stimulation of differentiation functions in human melanoma cells by tumor-promoting agents and dimethyl sulfoxide. *Cancer Research* 39:2618-2624; Claus Garbe, Konstantin Krasagakis (1993): Effects of interferons and cytokines on melanoma cells. *J. Invest. Dermatol.* 100:239S-244S). Unfortunately, clinical applications of these agents are limited by unacceptable toxicities, concern regarding potential carcinogenesis, or an inability to achieve and sustain effective plasma concentrations. Therefore, there exists a need for a nontoxic, clinically effective treatments for malignant melanomas or other similar cancers, pathologies or differentiation disorders.

Hydroxyurea, a ribonucleotide reductase inhibitor, is a simple chemical compound ($\text{CH}_4\text{N}_2\text{O}_2$, MW 76.05) that was initially synthesized in the late 1800's (Calabresi P, Chabner B A. Antineoplastic Agents. In: Gilman A G, Rall T W, Nies A S, Taylor P, eds. *The Pharmacological Basis of Therapeutics*. New York: McGraw Hill 1990:1251-2). It was later found to produce leukopenia in laboratory animals and subsequently was tested as an antineoplastic agent (Rosenthal F, Wislicki L, Kollek L. Ueber die beziehungen von schwersten blutgiften zu abbauprodukten des ewweises. Beitrag zum entstehungsmechanismus der perniziosen. *Anamie. Klin. Wschr.* 1928;7:972). At present, the primary clinical role of hydroxyurea is in the treatment of myeloproliferative disorders. It is now considered the preferred initial therapy for chronic myelogenous leukemia (Donohewer R C. Hydroxyurea. In: Chabner B A, Collins J M, eds *Cancer Chemotherapy, Principles and Practice*, Philadelphia: J B Lippincott 1990:225-33).

Hydroxyurea has been evaluated in a number of solid tumors, including: malignant melanoma, squamous cell carcinoma of the head and neck, renal cell carcinoma, and transitional cell carcinoma of the urothelium (Bloedow C E. A phase II study of hydroxyurea in adults: miscellaneous tumors. *Cancer Chemother Rep* 1964;40-39-41; Ariel I M. Therapeutic effects of hydroxyurea: experience with 118 patients with inoperable tumors. *Cancer* 1970;25:714; Nevinny H, Hall T C. Chemotherapy with hydroxyurea in renal cell carcinoma. *J Clin Pharmacol* 1968;88:352-9; Beckloff G L, Lerner H J, Cole D R, et al. Hydroxyurea in bladder carcinoma. *Invest Urol* 1967;6:530-4). Initial studies appeared promising in several of these diseases, but further investigation has not defined a role for hydroxyurea in any of the standard therapy regimens for solid tumors.

Inasmuch as hydroxyurea is an S-phase cell cycle specific agent, it is surprising that several clinical trials of this drug in hormone-refractory metastatic prostate cancer suggested that it possessed some activity (Lerner H J, Malloy T R.

Hydroxyurea in stage D carcinoma of prostate. *Urol* 1977;10:35-8; Kvols L K, Eagan R T, Myers R P. Evaluation of melphalan, ICRF-159, and hydroxyurea in metastatic prostate cancer: a preliminary report. *Cancer Treat Rep* 1977;61:311-2; Loening S A, Scott W W, deKernion J, et al. A Comparison of hydroxyurea, methyl-chloroethyl-cyclohexy-nitrosourea and cyclophosphamide in patients with advance carcinoma of the prostate. *J Urol* 1981;125:812-6; Mundy A R. A pilot study of hydroxyurea in hormone "escaped" metastatic carcinoma of the prostate. *Br J Urol* 1982;54:20-5; Stephens R L, Vaughn C, Lane M, et al. Adriamycin and cyclophosphamide versus hydroxyurea in advanced prostatic cancer. *Cancer* 1984;53:406-10) particularly given (1) the slowly progressive nature of the disease and (2) the schedules of drug administration used in these trials, e.g., once daily to once every three days. It seemed unlikely that either the doses or schedules of hydroxyurea administration used in these trials would capture a significant proportion of tumor cells in a susceptible phase of the cell cycle. Table 30 summarizes the reported clinical data regarding hydroxyurea's activity in hormone-refractory prostate cancer. The overall objective response rate is 23% and the frequency of subjective improvement is 36% (Lerner H J, Malloy T R. Hydroxyurea in stage D carcinoma of prostate. *Urol* 1977;10:35-8; Kvols L K, Eagan R T, Myers R P. Evaluation of melphalan, ICRF-159, and hydroxyurea in metastatic prostate cancer: a preliminary report. *Cancer Treat Rep* 1977;61:311-2; Loening S A, Scott W W, deKernion J, et al. A Comparison of hydroxyurea, methyl-chloroethyl-cyclohexy-nitrosourea and cyclophosphamide in patients with advance carcinoma of the prostate. *J Urol* 1981;125:812-6; Mundy A R. A pilot study of hydroxyurea in hormone "escaped" metastatic carcinoma of the prostate. *Br J Urol* 1982;54:20-5; Stephens R L, Vaughn C, Lane M, et al. Adriamycin and cyclophosphamide versus hydroxyurea in advanced prostatic cancer. *Cancer* 1984;53:406-10). Thus, there exists a need for an improved therapy using hydroxyurea for treatment of prostatic or similar cancers.

Treatment for most primary central nervous system (CNS) tumors has been, to date, unsatisfactory. Chemotherapy, radiation therapy, and surgery are primarily cytoreductive, aiming to reduce the number of viable tumor cells in the host. While the application of these techniques has been successful in some human malignancies, the use of cytoreductive strategies for medulloblastoma and malignant astrocytoma has had limited success because of inaccessibility of the primary tumor, early dissemination of the malignant cells into the cerebrospinal fluid, lack of effective cytoreductive agents or unacceptable toxicity. Thus, there exists a need for satisfactory treatments for CNS tumors which overcome the prior drawbacks of conventional cytoreductive therapy.

In addition, the link between problems with lipid metabolism and heart disease are now well-accepted. Thus, it is desirable to find treatment capable of modulating or altering lipid metabolism in subjects with related maladies. In particular treatments and methods for reducing serum triglycerides are highly desirable.

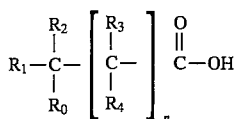
While radiation therapy has been widely used in the management of neoplastic disease, it is limited by the lack of radiosensitivity of specific regions of malignant tumors. Chemical enhancement of tumor sensitivity to radiation has largely been unsuccessful and remains a critical problem in radiotherapy. Thus, there exists a need for improved methods involving radiotherapy.

Accordingly, the present invention provides methods and compositions for treating the above-mentioned and other

pathologies with PAA and its pharmaceutically acceptable salts, derivatives, and analogs.

III. SUMMARY OF THE INVENTION

The invention provides a method of treating various pathologies in a subject. The invention also provides for the modulation of various cellular activities in a subject. The pathologies and cellular activities are treated and modulated utilizing a compound having the formula:



; wherein

R_0 =aryl, phenoxy, substituted aryl or substituted phenoxy;

R_1 and R_2 =H, lower alkoxy, lower straight and branched chain alkyl or halogen;

R_3 and R_4 =H, lower alkoxy, lower straight and branched chain alkyl or halogen; and

n =an integer from 0 to 2.

Specifically, the invention provides a method of treating or preventing various neoplastic conditions. Relatedly, a method of inducing differentiation of a cell is provided. The invention also provides a method of inducing the production of fetal hemoglobin and treating pathologies associated with abnormal hemoglobin activity or production.

The invention also provides a method of treating or preventing a viral infection in a subject. Relatedly, the invention provides a method of treating an AIDS-associated dysfunction of the central nervous system in a subject.

Also provided is a method of modulating the production of IL-6 or TGF α and TGF- β 2 both in vitro and in vivo. Typically, IL-6 and TGF- β 2 are inhibited while TGF α is induced.

The invention also provides a method of enhancing immunosurveillance and promoting wound healing in a subject.

Also provided is a method of monitoring the bioavailability of a compound for treatment of a pathology not associated with hemoglobin. The method comprises administering to a subject the compound and measuring the level of fetal hemoglobin TGF- β 2, IL-6 or TGF α .

A method of treating a neoplastic condition in cells resistant to radiation and chemotherapy is provided. Specifically, multiple drug resistant cells are particularly sensitive to the compounds of this invention.

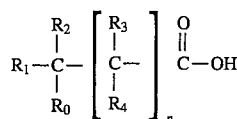
The present invention provides, in several embodiments, combinations which inhibit certain key regulatory enzymes. Thus, HMG-CoA reductase and MVA-PP decarboxylase can be blocked by lovastatin (LOV) and phenylacetate (PA), respectively.

In another embodiment, the present invention overcomes the toxicity problems with monotreatments with hydroxyurea. Because it was anticipated that the plasma concentrations of hydroxyurea required for cytotoxicity would result clinically in an unacceptable degree of myelosuppression, another objective of the present invention was to evaluate the activity in combination with phenylbutyrate, a relatively non-toxic differentiating agent.

In other embodiments, the present invention encompasses the following subject matter:

The present invention provides a method of treating a neoplastic condition in a subject comprising administering a therapeutic amount of a phenylacetic acid derivative of the formula:

General Structure A



; wherein

R_0 =aryl, phenoxy, substituted aryl or substituted phenoxy;

R_1 and R_2 =H, lower alkoxy, lower straight and branched chain alkyl or halogen;

R_3 and R_4 =H, lower alkoxy, lower straight and branched chain alkyl or halogen; and

n =an integer from 0 to 2;

salts thereof; stereoisomers thereof; and mixtures thereof. This general structure is hereinbelow referred to as General Structure A without reference to any particular method or composition. The neoplastic conditions treatable by this method include neuroblastoma, acute promyelocytic leukemia, acute myelodysplasia, acute glioma, prostate cancer, breast cancer, melanoma, non-small cell lung cancer, medulloblastoma, and Burkitt's lymphoma. The compounds for the above method (as disclosed in General Structure A), and for any of the methods or compositions disclosed elsewhere herein, specifically include sodium phenylacetate and sodium phenylbutyrate.

Further provided is a method of preventing a neoplastic condition in a subject comprising administering a prophylactic amount of a phenylacetic acid derivative of General Structure A. This method encompasses a method where the compound is administered in combination with an anti-neoplastic agent.

The present invention also provides a method of inducing the differentiation of a cell comprising administering to the cell a differentiation inducing amount of a phenylacetic acid derivative of General Structure A.

Also included is a method of inducing the production of fetal hemoglobin in a subject comprising administering to the subject a fetal hemoglobin inducing amount of a phenylacetic acid derivative of General Structure A.

The present invention includes a method of treating a pathology associated with abnormal hemoglobin activity in a subject comprising administering to the subject a therapeutic amount of a phenylacetic acid derivative of General Structure A. This method may be used to treat a pathology which is anemia. More specifically, the anemia may be selected from the group consisting of sickle cell and beta thalassemia.

Further provided is a method of treating a viral infection in a subject comprising administering to the subject a therapeutic amount of a phenylacetic acid derivative of General Structure A. The viral infection treated by the above method may be an infection by a retrovirus. Specifically, this method may be used to treat a subject infected by a Human Immunodeficiency Virus.

The present invention provides a method of preventing a viral infection in a subject comprising administering to the subject a prophylactic amount of a phenylacetic acid derivative of General Structure A.

In another embodiment, the present invention provides a method of inhibiting the production of IL-6 in a cell comprising contacting the cell with an IL-6 inhibiting amount of a phenylacetic acid derivative of General Structure A. This method of inhibiting may be used in a subject having any of the following pathologies: rheumatoid arthritis, Castleman's disease, mesangial proliferation, glomerulonephritis, uveitis, sepsis, automimmunity inflammatory bowel, type I diabetes, vasculitis and a cell differentiation associated skin disorder. The inhibition, of course, will be sufficient to treat the disorder.

The present invention also provides a method of inducing the production of TGF α in a cell comprising contacting the cell with a TGF α inducing amount of a phenylacetic acid derivative of General Structure A. This method may be used where the induction is in a wound of a subject and the induction is sufficient to promote wound healing.

The present invention also provides a method of inhibiting the production of TGF- β 2 in a cell comprising contacting the cell with a TGF- β 2 inhibiting amount of a phenylacetic acid derivative of General Structure A.

Further provided is a method of treating an AIDS-associated dysfunction of the central nervous system in a subject comprising administering to the subject a therapeutic amount of a phenylacetic acid derivative of General Structure A.

Another embodiment of the present invention is a method of enhancing immunosurveillance in a subject comprising administering to the subject an immunosurveillance enhancing amount of a phenylacetic acid derivative of General Structure A.

The present invention also provides a method of monitoring the bioavailability of a compound of General Structure A. This method is applicable for the treatment of a pathology not associated with hemoglobin and it comprises administering to a subject the compound and measuring the level of fetal hemoglobin, an increase in the amount of fetal hemoglobin indicating an increased bioavailability of the compound to treat the pathology and a decrease in the amount of fetal hemoglobin indicating a decrease in the bioavailability of the compound to treat the pathology. This method is useful for monitoring a pathology which is a neoplastic condition.

The present invention provides a method of promoting the healing of a wound in a subject comprising administering to a wound in the subject a wound healing amount of a phenylacetic acid derivative of General Structure A.

Further provided is a method of treating a neoplastic condition in a subject resistant to radiation and chemotherapy comprising administering to said subject a therapeutic amount of a phenylacetic acid derivative of General Structure A. This method is particularly useful for treatment of a neoplastic condition exhibiting the multiple drug resistant phenotype.

Thus, the present invention also includes the following embodiments:

A method of treating a neoplastic condition in a subject comprising administering a therapeutic amount of a retinoid in combination with a therapeutic amount of a phenylacetic acid derivative of General Structure A.

The present invention also provides a method of treating a neoplastic condition in a subject comprising administering a therapeutic amount of an inhibitor of the mevalonate pathway in combination with a therapeutic amount of a phenylacetic acid derivative of General Structure A. A related method uses the above steps and further includes the steps of continuously monitoring the subject for rhabdomy-

olysis-induced myopathy and in the presence of rhabdomyolysis-induced myopathy, administering ubiquinone to the subject.

A further method of the present invention is a method of inhibiting HMG-coA reductase and MVA-PP decarboxylase in a subject with a neoplastic condition, comprising administering a therapeutic amount of an inhibitor of the mevalonate pathway in combination with a therapeutic amount of a phenylacetic acid derivative of General Structure A. A related method also includes the additional steps of continuously monitoring the subject for rhabdomyolysis-induced myopathy and in the presence of rhabdomyolysis-induced myopathy, administering ubiquinone to the subject.

The present invention also provides a method of treating a neoplastic condition in a subject, comprising administering a therapeutic amount of a flavonoid in combination with a therapeutic amount of a phenylacetic acid derivative of General Structure A.

The present invention provides a further method of treating a neoplastic condition in a subject, comprising administering a therapeutic amount of hydroxyurea in combination with a therapeutic amount of a phenylacetic acid derivative of General Structure A.

In another embodiment, the present invention provides a method of modulating lipid metabolism in a subject, comprising administering a therapeutic amount of a phenylacetic acid derivative of General Structure A. In a related embodiment, the present invention provides a method of reducing serum triglycerides in a subject, comprising administering a therapeutic amount of a phenylacetic acid derivative of General Structure A.

The present invention further provides a method of locally treating a neoplastic condition of an internal tissue of a subject, comprising administering, intravesically, a therapeutic amount of a phenylacetic acid derivative of General Structure A.

The present invention provides a method of sensitizing a subject to radiation therapy, comprising administering a therapeutic amount of a phenylacetic acid derivative of General Structure A.

Also provided is a product for simultaneous, separate, or sequential use in treating a neoplastic condition in a subject, comprising, in separate preparations, a therapeutic amount of a vastatin and a therapeutic amount of a phenylacetic acid derivative of General Structure A.

A further embodiment of the present invention provides a product for simultaneous, separate, or sequential use in treating a neoplastic condition in a subject, comprising, in separate preparations, a therapeutic amount of a retinoid and a therapeutic amount of a phenylacetic acid derivative of General Structure A.

The present invention also provides a product for simultaneous, separate, or sequential use in treating a neoplastic condition in a subject, comprising, in separate preparations, a therapeutic amount of hydroxyurea and a therapeutic amount of a phenylacetic acid derivative of General Structure A.

The present invention provides a composition, comprising a therapeutic amount of a vastatin and a therapeutic amount of a phenylacetic acid derivative of General Structure A.

The present invention further provides a composition, comprising a therapeutic amount of a retinoid and a therapeutic amount of a phenylacetic acid derivative of General Structure A.

Finally, the present invention provides a composition, comprising a therapeutic amount of hydroxyurea and a therapeutic amount of a phenylacetic acid derivative of General Structure A.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the inhibition of HL-60 leukemia and premalignant 10T1/2 cell proliferation by NaPA.

FIG. 2 shows the induction of HL-60 cell differentiation. The number of NBT positive cells was determined after 4 [solid bars] or 7 days [hatched bars] of treatment. NaPA (h), 1.6 mg/ml; NaPA (l), 0.8 mg/ml. 4-hydroxyphenylacetate and PAG were used at 1.6 mg./ml. Potentiation by RA 10 nM was comparable to that by IFN gamma 300 IU/ml, and the effect of acivicin 3 µg/ml similar to DON 30 µg/ml. Glutamine Starvation (Gln, <0.06 mM) was as described. Cell viability was over 95% in all cases, except for DON and acivicin (75% and 63%, respectively).

FIG. 3 shows adipocyte conversion in 10T1/2 cultures.

FIG. 4 shows NaPA's ability to invoke growth arrest of human glioblastoma cells. Dose-dependent inhibition of human glioblastoma cell proliferation by sodium phenylacetate. Growth rates were determined, after 4-5 days of continuous treatment, by an enzymatic assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide and confirmed by cell enumeration with a hemocytometer. Reduction in cell number paralleled changes in de novo DNA synthesis (not shown).

FIG. 5 shows selective cytostasis induced by phenylacetate (5 mM) combined with glutamine starvation (0.2 mM glutamine, i.e., 2-3 fold below the normal plasma levels). The results indicate increased vulnerability of glioblastoma A172 when compared to actively replicating normal human umbilical vein endothelial cells (HUV). Cell viability was over 95% in all cases.

FIG. 6 shows that phenylacetate inhibits the mevalonate pathway of cholesterol synthesis in glioblastoma cells. FIG. 6 shows key steps of the MVA pathway discussed in text.

FIG. 7 shows the selective inhibition of cholesterol synthesis from mevalonate in phenylacetate-treated glioblastoma U87 cells, and enzymatic inhibition of mevalonate decarboxylation in cell homogenates. For analysis of steroid synthesis, logarithmically growing cells were labeled with tritiated MVA in the presence or absence of 5 mM phenylacetate, and their steroids were separated by silica thin layer chromatography. MVA decarboxylation was measured in cell homogenates. The effect of phenylacetate on cholesterol synthesis and MVA decarboxylation was selective as, under the experimental conditions used, total protein and DNA synthesis levels were unaffected.

FIG. 8 shows the effects of phenylacetate on rate of proliferation after in vitro exposure of 9 L tumor cells to various concentrations of phenylacetate for 5 days. Significant decline in DNA-synthesis was observed. Data are expressed as means \pm S.D. counts per minute (cpm).

FIG. 9 shows the treatment with phenylacetate from the day of intracerebral tumor inoculation extended survival compared with treatment with saline ($p < 0.01$; Mantel-Haenzel test).

FIG. 10 shows the treatment of established tumors with phenylacetate extended survival compared to treatment with saline ($p < 0.03$; Mantel-Haenzel test).

FIG. 11 shows the effect of NaPA on cell proliferation. PC3; DU145; LNCaP; and FS4 cultures were treated with NaPA or PAG for four days.

FIG. 12 shows a chromatogram of phenylacetate (PA) and phenylacetylglutamine (PAG). The peaks at 9.8 and 17.1 minutes represent PAG and PA, respectively. Serum concentrations of 250 µg/ml in both instances.

FIG. 13 shows serum concentrations of PA () and PAG () and plasma concentrations of glutamine () following a 150 mg/kg i.v. bolus of PA over 2 hours.

FIG. 14 shows declining phenylacetate concentrations over time during CIVI (250 mg/kg/day) in one patient, suggestive of clearance induction.

FIG. 15 shows the inhibition of tumor cell invasion by NaPA cells treated in culture for seven (7) days which were harvested and assayed for their invasive properties using a modified Boyden Chamber with a matrigel-coated filter. Results were scored six (6) to twenty-four (24) hours later.

FIG. 16 shows a simulation of a q 12 hour PA regimen (200 mg/kg/dose, 1 hour infusion) in a pharmacokinetically average patient. For simplicity, induction of clearance was not factored in.

FIG. 17 shows the effect of NaPA on cell growth and differentiation. (o) Total cell number and (●) the fraction of benzidine-positive cells were determined after 4 days of continuous treatment. Data represent means \pm SD ($n=4$). Cell viability was greater than 95%.

FIGS. 18A and 18B show the time-dependent changes in cell proliferation and Hb production. NaPA (5 mM) was added on days 2, 4, 6, and 8 of phase II cultures derived from normal donors, and the cells were analyzed on day 13. Panel A: Number of Hb-containing cells per ml ($\times 10^{-4}$), and the amounts of Hb (pg) per cell (MCH). Panel B: Total Hb (pg) per ml culture, and the proportion of HbF out of total Hb (% HbF). Data points represent the means of four determinations. The deviation of results of each determination from the mean did not exceed 10%. NaPB at 2.5 mM produced comparable effects (not shown). In all cases, cell viability was over 95%.

FIG. 19 shows the effect of NaPA on the proportions of Hb species in cultured erythroid precursors derived from a patient with sickle cell anemia. NaPA was added to 7 day phase II cultures. The cells were harvested and lysed on day 13, and the proportions of HbF, HbA₂, and HbS were determined following separation on cation exchange HPLC.

FIG. 20 shows the increased production of TGF- α by human keratinocytes upon treatment with NaPA and NaPB. Epithelial HK5 cells were treated with NaPB (3.0 mM, 1.5 mM, 0.75 mM), NaPB (10 mM, 5.0 mM, 2.5 mM) and PAG (5 mM) continuously for 4 days. Untreated cells served as a control. The amount of TGF- α (ng/ml/ 10^6 cells) was measured by using anti-TGF- α antibodies.

FIG. 21 shows the enhanced expression of the surface antigens W6/32 (MHC class I), DR (MHC class II) and ICAM-1 in melanoma cells treated with NaPB. Melanoma 1011 cells were treated with 2 mM PB for 10 days. Treatment was discontinued for 3 days to document the stability of the effect. FACS analysis revealed markedly increased expression of the antigens following treatment (shaded area); the expression of the surface antigens was similar or slightly greater on day 13 than on day 10, indicating that PB induced terminal differentiation.

FIG. 22 shows the activation of the Peroxisomal Proliferator Receptor (PPAR) by PA, PB and various phenylacetic acid analogs. The activation is measured by the increased production of the indicator gene for chloramphenicol acetyl transferase (CAT), which is controlled by the response element for acyl-CoA oxidase, relative to the control (C). The experimental details for this activation measurement method can be found in Sher et al., *Biochem.*, 32(21):5598 (1993). The concentration (in mM) of a particular drug is noted next to the following symbols for the various drugs: CF=clofibrate, PA=phenylacetate, CP=chlorophenylacetate, PB=phenylbutyrate, CPB=chlorophenylbutyrate, pAG=phenylacetylglutamine, IPB=iodophenylbutyrate, B=butyrate, IAA=indole acetic acid, NA=naphthylacetate, PP=phenoxypionic acid, 2-4D=2,4-dichlorophenoxy acetate.

FIG. 23 shows the modulation by phenylbutyrate of glutathione (GSH), gamma-glutamyl transpeptidase (GGT) and catalase activities. The antioxidant capacity (mM or units/mg protein) of the enzymes were measured for up to approximately 100 hours following treatment of prostatic PC3 cells with 2 mM NaPB.

FIG. 24 shows the radiosensitization by PA and PB of human glioblastoma U87 cells by pretreatment for 72 hours with 1, 3 and 5 mM PA and 2.5 mM PB prior to exposure to ionizing radiation (Co^{60} γ -radiation).

FIG. 25 shows the inhibition of the growth of breast MCF-7 adriamycin-resistant cancer cells by continuous exposure of up to 10 mM PA for 4 days.

FIG. 26 shows the relationship between lipophilicity and the cytostasis induced by phenylacetate derivatives in prostatic carcinoma cells and in plants. The log $1/\text{IC}_{50}$ values for prostatic cells (calculated from data presented in Table 21), were compared with the $1/\text{IC}_5$ for rapidly developing plant tissues. 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.

FIGS. 27A and 27B show the phenotypic reversion induced by phenylacetate and selected derivatives. The malignant prostatic PC3 cells were treated as described in "Material and Methods". Data indicates the relative potency of tested compounds in significantly inhibiting 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 21 and 22. The effect on anchorage-dependency was confirmed with U87 cells (not shown).

FIG. 28 shows a dose-response curve of the effect of NaPA on the incorporation of [^3H]thymidine in LA-N-5 cells after 7 days of treatment. Values represent the mean \pm S.E.M. of quadruplicate samples of a typical experiment.

FIG. 29 shows that NaPA and RA are synergistic in inhibiting growth of LA-N-5 cells. Cells were cultured in the presence of RA (10^{-7}M), NaPA (1.25 mM), RA (10^{-7}M)+ NaPA (1.25 mM), or in the absence of added compounds as indicated. After 6 days, cultures were assayed for incorporation of [^3H]thymidine. Columns represent the mean (\pm S.E.M.) of triplicate samples.

FIG. 30 shows dose response curves showing the effects of NaPA alone (o) and in the presence of 10^{-7}M (∇) and 10^{-6}M (\bullet) RA on specific AChE activity in LA-N-5 cells after 7 days of culturing. Each point represents the mean of three replicate cultures (S.E.M. <10% in all cases).

FIG. 31 shows the quantitation of N-myc nuclear staining intensities from cultures shown in FIG. 50. A total of 500 cells per treatment condition as indicated were chosen at random for analysis. Results are expressed as % of total cells counted versus relative staining intensity with median relative intensities for each treatment condition as follows: 44, control; 36, NaPA; 29, RA; 16, RA+NaPA.

FIGS. 32A through 32C show dose-response curves showing the effects of PA (solid lines) and phenylbutyrate (PB) (dashed lines) on the incorporation of [^3H]thymidine into triangles, SK-N-AS; solid diamonds, LA-N-5; hollow diamonds, Lan-1-15N; solid squares, LA-N-6; hollow squares, SK-N-SH-F; solid circles, SK-N-SH-N; and hollow circles, LA-N-2 cells after 7 days of treatment. In all cases, PB was a more potent inhibitor of cell proliferation than PA.

FIGS. 33A and 33B show the time course of PA and PB induced growth inhibition on LA-N-5 neuroblastoma. Cells

were treated with the indicated concentrations of phenylbutyrate (A) or phenylacetate (B) on day 0 and assayed for incorporation of [^3H]thymidine on a daily basis as shown.

FIGS. 34A and 34B show the time course of specific AChE activity of LA-N-5 cells in the absence or presence of various concentrations of PB (A) or PA (B) as indicated. In all cases, increased in AChE temporarily preceded induced reduction of [^3H]thymidine incorporation as shown in FIG. 56. The sharp decline in AChE activity seen with 4 mM PB after 4 days of treatment is probably due to reduced viability of the cultures.

FIG. 35 shows the reversibility of the antiproliferative effects of phenylacetate and phenylbutyrate on LA-N-5 cells. The cells were cultured in the absence (O) or presence of PA (solid circles; 5 mM) or PB (solid squares; 2 mM) for 6 days, then washed and refed with either control medium (solid lines) or medium containing the same concentration of agent as during the treatment phase (dashed lines). Following washing, the cells were assayed for incorporation of [^3H]thymidine after culturing for various days as indicated up to a posttreatment period of 2 weeks.

FIG. 36 shows the reversibility of induced increases in specific AChE activity. LA-N-5 cells were cultured in the absence (solid square) or presence of PA (vertical striped square; 5 mM) or PB (crossed striped square; 2 mM) for 6 days, then washed and refed with either control medium (solid bar graph) or medium containing the same concentration of agent as during the treatment phase (line graph with box symbols). The cells were assayed for specific AChE activity after culturing for various days as indicated up to a posttreatment period of 1 week.

FIG. 37 shows the synergistic effect of treatment of prostatic carcinoma PC3 cells with PA along with apigenin and 9-cis-retinoic acid.

FIG. 38 shows the growth arrest of A172 glioma cells upon treatment with NaPA, LOV and a combination of the two drugs (3 days continuous treatment).

FIG. 39 shows the suppression of glioma cell invasiveness (A172 cells, 3 days continuous treatment) by phenylacetate (2 mM) in combination with lovastatin (0.1 μM).

FIGS. 40A and 40B show induced melanogenesis (time and dose dependencies).

FIG. 41 shows the potentiation of other drugs.

FIG. 42 shows the dose-response curves of hydroxyurea in three prostate cancer cell lines (PC-3, PC-3M, and DU-145) compared to the control wells. The IC_{50} for all three cells is approximately 100 μM .

FIG. 43 shows the duration of drug exposure versus survival curve of hydroxyurea (100 μM) in PC-3 cells. The cells were exposed to drug for varying periods of time, but all were grown for a total of 5 days (120 hours). Drug exposure was terminated at the various intervals by replacing drug-containing medium with drug-free medium. This experiment did not detect any recovery in cell viability following brief periods of drug exposure. The IC_{50} was only achieved after 120 hours of exposure.

FIG. 44A shows a plasma concentration versus time simulation of hydroxyurea dosing regimen employed by Lener et al. (80 mg/kg every third day) depicted over 30 days. FIG. 44B shows a plasma concentration versus time simulation of the hydroxyurea dosing regimen required to produce plasma concentrations above 100 μM for 5 days in an average 70 kg man (1.0 g loading dose followed by 500 mg every 6 hours for 5 days) depicted over 30 days. FIG. 44C shows a plasma concentration versus time simulation of

the hydroxyurea dosing regimen required to produce plasma concentrations above 50 μ M for 5 days in an average 70 kg man (400 mg loading dose followed by 200 mg every 6 hours for 5 days) depicted over 30 days.

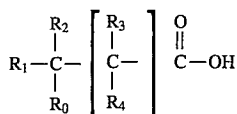
FIG. 45 shows the potentiation of antitumor activity of the combination of hydroxyurea with phenylbutyrate in PC-3 cells.

FIG. 46 shows the growth inhibitory effects of PA on various negroectodermal tumor cell lines. The cells were grown in 96 well microtiter plates in MEM+10% FCS and treated with various concentrations of PA or media alone for 96 h. Then 1 μ Ci of 3 H-thymidine was added per well. After 4 h the cells were harvested using a semiautomated cell harvester and the amount of 3 H-thymidine uptake determined by liquid scintillation counting.

FIGS. 47A and 47B show how neutralizing antibodies against human TGF β 1 (upper panel) and TGF β 2 (lower panel) failed to antagonize the antiproliferative effect of PA on U251 cells. U251 cells were cultured in 96 well microtiter plates in MEM+10% FCS in the presence of 10 mM PA or media alone. Neutralizing antibodies directed against TGF β 1 or TGF β 2 were added to the PA containing wells at the beginning of the incubation period. Normal IgG of the corresponding species served as a control. The rate of cell proliferation was determined by adding 1 μ Ci of 3 H-thymidine per well and harvesting the cells after 4 h 3 H-thymidine incorporation was determined by liquid scintillation counting.

V. DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "phenylacetic acid derivative" (or "phenylacetic acid analog") refers to a compound of the formula:



: wherein

R₀ is aryl (e.g., phenyl, naphthyl), phenoxy, substituted aryl (e.g., one or more halogen [e.g., F, Cl, Br, I], lower alkyl [e.g., methyl, ethyl, propyl, butyl] or hydroxy substituents) or substituted phenoxy (e.g., one or more halogen [e.g., F, Cl, Br, I], lower alkyl [e.g., methyl, ethyl, propyl, butyl] or hydroxy substituents);

R₁ and R₂ are each H, lower alkoxy (e.g., methoxy, ethoxy), lower straight and branched chain alkyl (e.g., methyl, ethyl, propyl, butyl) or halogen (e.g., F, Cl, Br, I);

R₃ and R₄ are each H, lower straight and branched chain alkyl (e.g., methyl, ethyl, propyl, butyl), lower alkoxy (e.g., methoxy, ethoxy) or halogen (e.g., F, Cl, Br, I); and

n is an integer from 0 to 2;

salts thereof (e.g., Na⁺, K⁺ or other pharmaceutically acceptable salts); stereoisomers thereof; and mixtures thereof.

When n is equal to 2, each of the two R₃ substituents and each of the two R₄ substituents can vary independently within the above phenylacetic acid derivative definition. It is intended that this definition includes phenylacetic acid (PAA) and phenylbutyric acid (PBA). Mixtures according to this definition are intended to include mixtures of carboxylic

acid salts, for instance, a mixture of sodium phenylacetate and potassium phenylacetate. Because the carboxylic portion of these compounds is the primarily active portion, references herein to a carboxylate, such as phenylacetate (PA) or phenylbutyrate (PB), are intended to refer also to an appropriate counter cation, such as Na⁺, K⁺ or another pharmaceutically acceptable cation such as an organic cation (e.g., arginine). Thus, as used herein, a PA or PB derivative or analog refers to the phenylacetic acid derivatives of this definition. Some of these derivatives can be interconverted when present in a biological system. For instance, PA can be enzymatically converted to PB within an animal and, similarly, PB can be converted to PA.

Thus, phenylacetic acid derivatives include, without limitation, phenylacetic acid, phenylpropionic acid, phenylbutyric acid, 1-naphthylacetic acid, phenoxyacetic acid, phenoxypropionic acid, phenoxybutyric acid, 4-chlorophenylacetic acid, 4-chlorophenylbutyric acid, 4-iodophenylacetic acid, 4-iodophenylbutyric acid, α -methylphenylacetic acid, α -methoxyphenylacetic acid, α -ethylphenylacetic acid, α -hydroxyphenylacetic acid, 4-fluorophenylacetic acid, 4-fluorophenylbutyric acid, 2-methylphenylacetic acid, 3-methylphenylacetic acid, 4-methylphenylacetic acid, 3-chlorophenylacetic acid, 3-chlorophenylbutyric acid, 2-chlorophenylacetic acid, 2-chlorophenylbutyric acid and 2,6-dichlorophenylacetic acid, and the sodium salts of the these compounds.

The compounds of the present invention can be administered intravenously, enterally, parenterally, intramuscularly, intranasally, subcutaneously, topically, intravesically or orally. The dosage amounts are based on the effective inhibitory concentrations observed in vitro and in vivo in antitumorigenicity studies. The varied and efficacious utility of the compounds of the present invention is further illustrated by the findings that they may also be administered concomitantly or in combination with other antitumor agents (such as hydroxyurea, 5-azacytidine, 5-aza-2'-deoxycytidine, and suramin); retinoids; hormones; biological response modifiers (such as interferon and hematopoietic growth factors); and conventional chemo- and radiation therapy or various combinations thereof.

The present invention also provides methods of inducing tumor cell differentiation in a host comprising administering to the host a therapeutically effective amount of PAA or a pharmaceutically acceptable derivative thereof.

The present invention also provides methods of preventing the formation of malignancies by administering to a host a prophylactically effective amount of PAA or a pharmaceutically acceptable derivative thereof.

The present invention also provides methods of treating malignant conditions, such as prostatic cancer, melanoma, adult and pediatric tumors, e.g., brain tumors of glial origin, astrocytoma, Kaposi's sarcoma, lung adenocarcinoma and leukemias, as well as hyperplastic lesions, e.g., benign hyperplastic prostate and papillomas by administering a therapeutically effective amount of PAA or a pharmaceutically acceptable derivative thereof.

In addition, the present invention provides methods of treating conditions such as neuroblastoma, promyelocytic leukemia, myelodysplasia, glioma, prostate cancer, breast cancer, melanoma, and non-small cell lung cancer.

It is understood that the methods and compositions of this invention can be used to treat animal subjects, including human subjects.

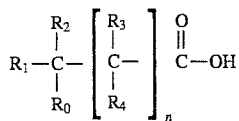
According to the present invention, phenylacetic acid derivatives, and in particular NaPA and NaPB, have been found to be excellent inhibitors of the growth of specific

tumor cells, affecting the proliferation of the malignant cells while sparing normal tissues. Also, according to the present invention, NaPA and its analogs have been found to induce tumor cell differentiation, thus offering a very desirable approach to cancer prevention and therapy. Additionally, NaPA and its analogs have been found to be of value for the treatment of viral indications such as AIDS. NaPA is also implicated in the treatment of severe beta-chain hemoglobinopathies. The exact mechanisms by which the compounds used in the methods of this invention exert their effects are uncertain. One potential mechanism may involve depletion of plasma glutamine. Based on the data reported herein, it is believed that glutamine depletion alone cannot explain the molecular and phenotypic changes observed in vitro following exposure to NaPA. It will be understood, however, that the present invention is not to be limited by any theoretical basis for the observed results.

In specific embodiments, the present invention encompasses the following subject matter:

The present invention provides a method of treating a neoplastic condition in a subject comprising administering a therapeutic amount of a phenylacetic acid derivative of the formula:

General Structure A



; wherein

R_0 =aryl, phenoxy, substituted aryl or substituted phenoxy;

R_1 and R_2 =H, lower alkoxy, lower straight and branched chain alkyl or halogen;

R_3 and R_4 =H, lower alkoxy, lower straight and branched chain alkyl or halogen; and

n =an integer from 0 to 2;

salts thereof; stereoisomers thereof; and mixtures thereof. This general structure is hereinbelow referred to as General Structure A without reference to any particular method or composition. The neoplastic conditions treatable by this method include neuroblastoma, acute promyelocytic leukemia, acute myelodysplasia, acute glioma, prostate cancer, breast cancer, melanoma, non-small cell lung cancer, medulloblastoma, and Burkitt's lymphoma. The compounds for the above method (as disclosed in General Structure A), and for any of the methods and compositions disclosed herein, specifically include sodium phenylacetate and sodium phenylbutyrate.

As used throughout this application, the phrase "in combination with" refers to treatment with the constituent drugs of the combination either simultaneously or at such intervals that the drugs are simultaneously active in the body. Furthermore, as used herein, the term "therapeutic amount" refers to an amount of an agent, drug or other compound of the generic class which is suitable for the claimed use. Therefore, "therapeutic amount" excludes members of the class which do not have the recited activity.

Further provided is a method of preventing a neoplastic condition in a subject comprising administering a prophylactic amount of a phenylacetic acid derivative of General Structure A. This method encompasses a method where the compound is administered in combination with an anti-neoplastic agent.

The present invention also provides a method of inducing the differentiation of a cell comprising administering to the cell a differentiation inducing amount of a phenylacetic acid derivative of General Structure A.

Also included is a method of inducing the production of fetal hemoglobin in a subject comprising administering to the subject a fetal hemoglobin inducing amount of a phenylacetic acid derivative of General Structure A.

The present invention includes a method of treating a pathology associated with abnormal hemoglobin activity in a subject comprising administering to the subject a therapeutic amount of a phenylacetic acid derivative of General Structure A. This method may be used to treat a pathology which is anemia. More specifically, the anemia may be selected from the group consisting of sickle cell and beta thalassemia.

Further provided is a method of treating a viral infection in a subject comprising administering to the subject a therapeutic amount of a phenylacetic acid derivative of General Structure A. The viral infection treated by the above method may be an infection by a retrovirus. Specifically, this method may be used to treat a subject infected by a Human Immunodeficiency Virus.

The present invention provides a method of preventing a viral infection in a subject comprising administering to the subject a prophylactic amount of a phenylacetic acid derivative of General Structure A.

In another embodiment, the present invention provides a method of inhibiting the production of IL-6 in a cell comprising contacting the cell with an IL-6 inhibiting amount of a phenylacetic acid derivative of General Structure A. This method of inhibiting may be used in a subject having any of the following pathologies: rheumatoid arthritis, Castleman's disease, mesangial proliferation, glomerulonephritis, uveitis, sepsis, autoimmune inflammatory bowel, type I diabetes, vasculitis and a cell differentiation associated skin disorder. The inhibition, of course, will be sufficient to treat the disorder.

The present invention also provides a method of inducing the production of TGF α in a cell comprising contacting the cell with a TGF α inducing amount of a phenylacetic acid derivative of General Structure A. This method may be used where the induction is in a wound of a subject and the induction is sufficient to promote wound healing.

The present invention also provides a method of inhibiting the production of TGF- β 2 in a cell comprising contacting the cell with a TGF- β 2 inhibiting amount of a phenylacetic acid derivative of General Structure A.

Further provided is a method of treating an AIDS-associated dysfunction of the central nervous system in a subject comprising administering to the subject a therapeutic amount of a phenylacetic acid derivative of General Structure A.

Another embodiment of the present invention is a method of enhancing immunosurveillance in a subject comprising administering to the subject an immunosurveillance enhancing amount of a phenylacetic acid derivative of General Structure A.

The present invention also provides a method of monitoring the bioavailability of a compound of General Structure A. This method is applicable for the treatment of a pathology not associated with hemoglobin and it comprises administering to a subject the compound and measuring the level of fetal hemoglobin, an increase in the amount of fetal hemoglobin indicating an increased bioavailability of the compound to treat the pathology and a decrease in the amount of fetal hemoglobin indicating a decrease in the

bioavailability of the compound to treat the pathology. This method is useful for monitoring a pathology which is a neoplastic condition.

The present invention provides a method of promoting the healing of a wound in a subject comprising administering to a wound in the subject a wound healing amount of a phenylacetic acid derivative of General Structure A.

Further provided is a method of treating a neoplastic condition in a subject resistant to radiation and chemotherapy comprising administering to said subject a therapeutic amount of a phenylacetic acid derivative of General Structure A. This method is particularly useful for treatment of a neoplastic condition exhibiting the multiple drug resistant phenotype.

As used herein, the terms "retinoid" or "retinoids" includes any suitable members of the generic class including, but not limited to: 9-cis-retinoic acid and all-trans-retinoic acid. Retinoid combination therapy is suitable for the treatment of cancers including breast cancer, leukemia and malignant melanoma.

As used herein, inhibitors of the mevalonate pathway include compounds such as terpenes and statins. Suitable statins include lovastatin. Suitable terpenes include limonene and its analogs.

The bioflavonoids are a class of compounds also known as the vitamin P complex and are known for their influence on capillary fragility (hemostatic). They are generally known to decrease capillary permeability and fragility. As used herein, flavonoids include apigenin and quercetin. However, other flavonoids would be expected to elicit the similar utility.

The particular activity of each of the compounds can be screened using the assays and models described in the Examples.

As used herein, "modulating lipid metabolism" describes the ability of a therapy to alter lipid production and degradation in vivo. For example, one modulation of lipid metabolism is the reduction of serum triglycerides (the level of low and high density lipoproteins in a subject's serum), i.e. the lowering of a subject's cholesterol level.

Thus, the present invention also includes the following embodiments:

A method of treating a neoplastic condition in a subject comprising administering a therapeutic amount of a retinoid in combination with a therapeutic amount of a phenylacetic acid derivative of General Structure A. The retinoid may be all-trans-retinoic acid or 9-cis-retinoic acid. Furthermore, this method may be used to treat neoplastic conditions such as neuroblastoma.

The present invention also provides a method of treating a neoplastic condition in a subject comprising administering a therapeutic amount of an inhibitor of the mevalonate pathway in combination with a therapeutic amount of a phenylacetic acid derivative of General Structure A. This method may be practised using inhibitors which are statins or an analogs thereof. One particularly suitable statin useful for this method is lovastatin. Another class of inhibitors is the terpenes, and, in particular, limonene. Other inhibitors can be screened using the methods set forth in the examples. This method may be used to treat neoplastic conditions including malignant glioma, adenocarcinoma and melanoma. In addition, the neoplastic condition may be of a non-malignant nature, including, but not limited to, such conditions as non-malignant glioma, benign prostatic hyperplasia, and papillomavirus infection. A related method uses the above steps and further includes the steps of continuously monitoring the subject for rhabdomyolysis-induced myopa-

thy and in the presence of rhabdomyolysis-induced myopathy, administering ubiquinone to the subject.

A further method of the present invention is a method of inhibiting HMG-coA reductase and MVA-PP decarboxylase in a subject with a neoplastic condition, comprising administering a therapeutic amount of an inhibitor of the mevalonate pathway in combination with a therapeutic amount of a phenylacetic acid derivative of General Structure A. Suitable inhibitors include the class of statins and their analogs, and, in particular lovastatin. Other suitable inhibitors are the terpenes and their analogs, and, in particular, limonene. This method may be used to treat neoplastic conditions, if necessary, including malignant glioma, adenocarcinoma and melanoma. A related method also includes the additional steps of continuously monitoring the subject for rhabdomyolysis-induced myopathy and in the presence of rhabdomyolysis-induced myopathy, administering ubiquinone to the subject.

The present invention also provides a method of treating a neoplastic condition in a subject, comprising administering a therapeutic amount of a flavonoid in combination with a therapeutic amount of a phenylacetic acid derivative of General Structure A. Suitable flavonoids include apigenin and quercetin. This method may be used to treat neoplastic conditions including prostatic carcinoma.

The present invention provides a further method of treating a neoplastic condition in a subject, comprising administering a therapeutic amount of hydroxyurea in combination with a therapeutic amount of a phenylacetic acid derivative of General Structure A. This combination therapy method may be used to treat neoplastic conditions including prostatic carcinoma.

In another embodiment, the present invention provides a method of modulating lipid metabolism in a subject, comprising administering a therapeutic amount of a phenylacetic acid derivative of General Structure A. In a related embodiment, the present invention provides a method of reducing serum triglycerides in a subject, comprising administering a therapeutic amount of a phenylacetic acid derivative of General Structure A.

The present invention provides a method of locally treating a neoplastic condition of an internal tissue of a subject, comprising administering, intravesically, a therapeutic amount of a phenylacetic acid derivative of General Structure A. This method may be used to locally treat neoplastic conditions of externally accessible internal orifices and bladders. Thus, the intravesicle method may be used to treat neoplastic conditions such as bladder carcinoma and kidney cancer.

The present invention provides a method of sensitizing a subject to radiation therapy, comprising administering a therapeutic amount of a phenylacetic acid derivative of General Structure A.

Also provided is a product for simultaneous, separate, or sequential use in treating a neoplastic condition in a subject, comprising, in separate preparations, a therapeutic amount of a statin and a therapeutic amount of a phenylacetic acid derivative of General Structure A. To this composition may be added a therapeutic amount of ubiquinone. A therapeutic amount of ubiquinone is an amount sufficient to permit tolerance of increased dosage of statin (or, specifically, lovastatin) without substantial, concomitant side effects.

A further product for simultaneous, separate, or sequential use in treating a neoplastic condition in a subject, comprises, in separate preparations, a therapeutic amount of a retinoid and a therapeutic amount of a phenylacetic acid derivative of General Structure A. This composition can be made with

retinoids including all-trans-retinoic acid and 9-cis-retinoic acid (or both).

Another novel product for simultaneous, separate, or sequential use in treating a neoplastic condition in a subject is provided. This product comprises, in separate preparations, a therapeutic amount of hydroxyurea and a therapeutic amount of a phenylacetic acid derivative of General Structure A.

The present invention provides a composition, comprising a therapeutic amount of a vastatin and a therapeutic amount of a phenylacetic acid derivative of General Structure A.

The present invention further provides a composition, comprising a therapeutic amount of a retinoid and a therapeutic amount of a phenylacetic acid derivative of General Structure A.

Finally, the present invention provides a composition, comprising a therapeutic amount of hydroxyurea and a therapeutic amount of a phenylacetic acid derivative of General Structure A.

VI. EXAMPLES

The herein offered examples, including experiments, provide methods for illustrating, without any implied limitation, the practice of this invention focusing on phenylacetic acid and its derivatives directed to A. Cancer therapy and prevention; B. Treatment and prevention of AIDS; C. Induction of fetal hemoglobin synthesis in β -chain hemoglobinopathies; D. Use of phenylacetic acid and its derivatives in wound healing; E. Use of phenylacetic acid and its derivatives in treatment of diseases associated with interleukin-6; F. Use of phenylacetic acid and its derivatives in the treatment of AIDS-associated CNS dysfunction; G. Use of phenylacetic acid and its derivatives to enhance immunosurveillance; H. Method of monitoring the dosage level of phenylacetic acid and its derivatives in a patient and/or the patient's response to these drugs; I. The activation of the PPAR by phenylacetic acid and its derivatives; J. Use of phenylacetic acid and its derivatives in treatment of cancers having a multiple-drug resistant phenotype; K. phenylacetic acid and its derivatives, correlation between potency and lipophilicity, L. phenylacetic acid and its derivatives in synergistic combination with lovastatin for the treatment and prevention of cancers such as malignant gliomas or other CNS tumors, M. phenylacetic acid and its derivatives in synergistic combination with retinoic acid for the treatment and prevention of cancers such as those involving neuroblastoma cells, N. phenylacetic acid and its derivatives for the treatment and prevention of cancers and other differentiation disorders such as those involving malignant melanoma or other neuroectodermal tumors, O. phenylacetic acid and its derivatives in synergistic combination with hydroxyurea (HU) for the treatment and prevention of cancers such as prostate cancer, P. phenylacetic acid and its derivatives for the treatment and prevention of cancers involving medulloblastoma and astrocytoma derived cells, Q. phenylacetic acid and its derivatives in human studies relating to treatments with PA and PB, R. phenylacetic acid and its derivatives in methods of altering lipid metabolism, including reducing serum triglycerides, and S. methods of administering phenylacetic acid and its derivatives.

Section A: Phenylacetate in Cancer Prevention and Maintenance Therapy

Recent advances in molecular techniques enable the detection of genetic disorders associated with a predisposition to cancer. Consequently, it is now possible to identify high-risk individuals as well as patients in a state of remission but afflicted with a residual disease. Despite such remarkable capabilities, there is still no acceptable preven-

tive treatment. Chemopreventive drugs are also needed for adjuvant therapy, to minimize the carcinogenic effects of the prevailing anticancer agents and yet maintain tumor responses.

To qualify for use in chemoprevention, a potential drug should have antitumor activities, be non-toxic and well tolerated by humans, easy to administer (e.g., orally or intravenously), and inexpensive. We suggest that NaPA possesses all of the above characteristics.

1. Prevention of Neoplastic Transformation—Oncogene Transfer Studies

NIH 3T3 cells carrying activated Ejras oncogene (originally isolated from human bladder carcinoma) were used as a model to study the potential benefit of NaPA treatment to high risk individuals, in whom predisposition is associated with oncogene activation. Cell treatment with NaPA was initiated 24–48 hours after oncogene transfer. Results, scored 14–21 days later, show dose-dependent reduction in the formation of ras-transformed foci in cultures treated with NaPA. Molecular analyses indicated that the drug did not interfere with oncogene uptake and transcription, but rather prevented the process of neoplastic transformation. The effect was reversible upon cessation of treatment. In treated humans, however, the fate of the premalignant cells may be substantially different due to involvement of humoral and cellular immunity (see discussion below).

2. Prevention of Tumor Progression by Genotoxic Chemotherapy

Current approaches to combat cancer rely primarily on the use of chemicals and radiation, which are themselves carcinogenic and may promote recurrences and the development of metastatic disease. One example is the chemotherapeutic drug 5-aza-2'-deoxycytidine (5AzaC). While this drug shows promise in treatment of some leukemias and severe inborn anemias, the clinical applications have been hindered by concerns regarding toxicity and carcinogenic effects. However, for the first time the data indicate that NaPA can prevent tumor progression induced by treatment with 5AzaC.

The experimental model involved nonmalignant 4C8a10 cells (revertants of Ha-ras-transformed NIH 3T3 fibroblasts). Transient treatment of the premalignant cells with 5AzaC resulted in malignant conversion evident within 2 days, as determined by cell morphology, loss of contact inhibition and anchorage dependent growth in culture, and acquired invasive properties and tumorigenicity in recipient athymic mice. Remarkably, NaPA prevented the development of these malignant phenotypes in the 5AzaC treated cultures (Table 1).

TABLE 1

| Treatment | Tumor Formation ^a | | Growth on matrigel ^b |
|--|------------------------------|-------------|---------------------------------|
| | Incidence | Size (mm) | |
| None | 3/8 | 1 (0.5–2) | – |
| 5AzaC (0.1 μ M) | 8/8 | 11.5 (4–19) | + |
| NaPA (1.5 mg/ml) | 0/8 | – | – |
| 5AzaC + NaPA (0.1 μ M) (1.5 mg/ml) | 0/8 | 0 | – |

^aCells pretreated in culture were injected s.c. (5×10^5 cells per site) into 3 month old female athymic nude mice (Division of Cancer Treatment, NCI Animal Program, Frederick Cancer Research Facility). Results indicate the incidence (tumor bearing/injected animals), as well as tumor size as mean (range), determined after 3 weeks.

^bCells were plated on top of matrigel (reconstituted basement membrane) and observed for malignant growth pattern, i.e., active replication, development of characteristic processes, and invasion.

3. Activity in Humans

In terms of cancer prevention, the beneficial effect of NaPA to humans may be even more dramatic than that

observed with the experimental models. In humans, NaPA is known to deplete circulating glutamine, an amino acid critical for the development and progression of cancer. The enzymatic reaction leading to glutamine depletion takes place in the liver and kidney. It is not clear whether or not glutamine depletion occurs in the cultured tumor cells. Moreover, molecular analysis revealed that NaPA induced the expression of histocompatibility class I antigens, which are localized on the surface of tumor cells and affect the immune responses of the host. While the therapeutic benefit of NaPA observed in cultures is in some cases reversible upon cessation of treatment, in patients the residual tumor cells would eventually be eliminated by the immune system. Even if chemoprevention will require continuous treatment with NaPA, such treatment would be acceptable considering the lack of toxicity.

Pharmaceutical compositions containing phenylacetate have been shown to cause reversal of malignancy and to induce differentiation of tumor cells. To demonstrate the capacity of drugs to induce differentiation of tumor cells, three in vitro differentiation model systems and one in vivo phase I clinical trial were used (further described herein). The first system used a human promyelocytic leukemia cell line HL-60. This cell line represents uncommitted precursor cells that can be induced to terminally differentiate along the myeloid or monocytic lineage. In the second system, immortalized embryonic mesenchymal C3H 10T1/2 cells were used which have the capability of differentiating into myocytes, adipocytes, or chondrocytes. In the third system, human erythroleukemia K562 cells were used because they can be induced to produce hemoglobin. Finally, the in vivo experiments demonstrated the efficacy of NaPA in inducing terminal differentiation in humans and animals.

NaPA and NaPB have also been shown to affect tumor growth in vitro and in animal models at pharmacological, non-toxic concentrations. These aromatic fatty acids induced cytostasis and promoted maturation of various human malignant cells, including hormone-refractory prostatic carcinoma, glioblastoma, malignant melanoma, and lung carcinoma. The marked changes in tumor biology were associated with alterations in the expression of genes implicated in tumor growth, invasion, angiogenesis, and immunogenicity. Multiple mechanisms of drug action appear to be involved. These mechanisms include (a) modification of lipid metabolism, (b) regulation of gene expression through DNA hypomethylation and transcriptional activation, and (c) inhibition of protein isoprenylation. Phase I clinical trials confirmed the efficacy of these novel, nontoxic differentiation inducers (see Example 15).

Example 1

HL-60 and 10T1/2 Cells—PAG and NaPA Treatment

Referring now to the data obtained using the first system (results illustrated in FIG. 1), logarithmically growing HL-60 [—●—] and 10T1/2 [—○—] cells were treated for four days with NaPA [solid line] or phenylacetylglutamate (PAG) [dashed line]. The adherent cells were detached with trypsin/EDTA and the cell number determined using a hemocytometer. Data points indicate the mean \pm S.D. of duplicates from two independent experiments. The cell lines were obtained from the American Type Culture Collection and maintained in RPMI 1640 (HL-60) or Dulbecco's Modified Eagle's Medium (10T1/2) supplemented with 10% heat inactivated fetal calf serum (Gibco Laboratories), 2 mM

L-Glutamine, and antibiotics. PAA (Sigma, St. Louis, Mo.) and PAG were each dissolved in distilled water, brought to pH 7.0 by the addition of NaOH, and stored in -20° C. until used. As demonstrated in FIG. 1, NaPA treatment of the HL-60 and 10T1/2 cultures was associated with dose dependent inhibition of cell proliferation.

EXAMPLE 2

HL-60 Cells—Induction of Granulocyte Differentiation

To further evaluate the effectiveness of NaPA as an inducer of tumor cell differentiation, the ability of NaPA to induce granulocyte differentiation in HL-60 was investigated. The ability of cells to reduce nitroblue tetrazolium (NBT) is indicative of oxidase activity characteristic of the more mature forms of human bone marrow granulocytes. NBT reduction thus serves as an indicator of granulocyte differentiation. In FIG. 2, the number of NBT positive cells was determined after 4 days [solid bars] or 7 days [hatched bar] of treatment. NaPA (h), 1.6 mg/ml; NaPA (1), 0.8 mg/ml. 4-hydroxyphenylacetate (4HPA) and PAG were used at 1.6 mg/ml. Potentiation by retinoic acid (RA) 10 nM was comparable to that by interferon gamma 300 IU/ml. The direction of differentiation towards granulocytes in cultures treated with NaPA, whether used alone or in combination with RA, was confirmed by microscopic evaluation of cells stained with Wright Stain and the lack of nonspecific esterase activity. The effect of acivicin (ACV) 1 μ g/ml was similar to 6-diazo-5-oxo-L-norleucine (DON) 25 μ g/ml. Glutamine starvation (Gln, <0.06 mM) was as described. Cell viability determined by trypan blue exclusion was over 95% in all cases, except for DON and ACV which were 75% and 63%, respectively. DON, ACV and HPA are glutamine antagonists. As illustrated in FIG. 2, it is clear that NaPA is capable of inducing granulocyte differentiation in HL-60. As further illustrated in FIG. 2, differentiation of HL-60, assessed morphologically and functionally, was sequential and could be further enhanced by the addition of low doses of retinoic acid [RA, 10 nM] or interferon gamma (300 IU/ml). After seven days of NaPA treatment, or four days, when combined with RA, the HL-60 cultures were composed of early stage myelocytes and metamyelocytes (30–50%), as well as banded and segmented neutrophils (30–40%) capable of NBT.

Pharmacokinetics studies in children with urea cycle disorders indicate that infusion of NaPA 300–500 mg/kg/day, a well tolerated treatment, results in plasma levels of approximately 800 μ g/ml. [Brusilow, S. W. et al. Treatment of episodic hyperammonemia in children with inborn errors of urea synthesis. *The New England Journal of Medicine*. 310:1630–1634 (1984)]. This same concentration was shown to effectively induce tumor cell differentiation in the present experimental system.

EXAMPLE 3

10T1/2 Cells—NaPA Induction of Adipocyte Conversion

FIG. 3 illustrates that NaPA is capable of inducing adipocyte conversion in 10T1/2 cultures. FIG. 3 shows quantitation adipocytosis. Cells were fixed with 37% formaldehyde and stained with Oil-Red O. The stained intracellular lipid was extracted with butanol, and the optical density was determined using a Titertek Multiskan MC, manufactured by Flow Laboratories, at a wavelength 510 nm. Increased lipid

accumulation was evident in cells treated with as little as 0.024 mg/ml of NaPA. The results in FIG. 3 show that differentiation was dose- and time-dependent, and apparently irreversible upon cessation of treatment. NaPA at 800 µg/ml was efficient and totally free of cytotoxic effect. In the 10T1/2 model, adipocyte conversion involved over 80% of the cell population. It was noted that higher drug concentrations further increased the efficiency of differentiation as well as the size of lipid droplets in each cell.

It is known that glutamine conjugation by NaPA is limited to humans and higher primates and that in rodents NaPA instead binds glycine. [James, M. O. et al. The conjugation of phenylacetic acid in man, sub-human primates and some non-primate species. *Proc. R. Soc. Lond. B.* 182:25-35 (1972).] Consequently, the effect of NaPA on the mouse 10T1/2 cell line could not be explained by an effect on glutamine. In agreement, neither glutamine starvation nor treatment with glutamine antagonists such as DON and ACV resulted in adipocyte conversion.

EXAMPLE 4

Induction of Lipid Accumulation and Adipocyte Differentiation

4. Clinical Use of Phenylacetate and Derivatives

TABLE 2

| Phenylacetate and Derivatives: Induction of cellular differentiation in premalignant 10T1/2 cells | | |
|---|-----------------------------|-------------------------|
| Compounds (sodium salts) | Differentiation at 1 mM (%) | DC ₅₀ * (mM) |
| Phenylacetate | 65 | 0.7 |
| 1-naphthylacetate | >95 | <0.1 |
| 3-chlorophenylacetate | 80 | 0.5 |
| 4-chlorophenylacetate | 50 | 1.0 |
| 2,6-dichlorophenylacetate | 75 | 0.5 |
| 4-fluorophenylacetate | 65 | 0.7 |

*DC₅₀, concentration of compound causing 50% differentiation

As shown in Table 2, phenylacetate and its derivatives efficiently induced lipid accumulation and adipocyte differentiation in premalignant cells. These and other results indicate that the tested compounds might be of value in:

- A. Cancer prevention. Non-replicating, differentiated tumor cells are not likely to progress to malignancy.
- B. Differentiation therapy of malignant and pathological nonmalignant conditions.
- C. Treatment of lipid disorders, in which patients would benefit from increased lipid accumulation.
- D. Wound healing. This is indicated by the ability of phenylacetate to induce collagen synthesis in fibroblasts (see Section D herein).

Studies in plants have revealed that NaPA can interact with intracellular regulatory proteins and modulate cellular RNA levels. In an attempt to explore the possible mechanism of action, Northern blot analysis of HL-60 and 10T1/2 cells was performed according to conventional methods. Cytoplasmic RNA was extracted, separated and analyzed (20 µg/lane) from confluent cultures treated for 72 hours with NaPA or PAG (mg/ml); C is the untreated control. The aP2 cDNA probe was labeled with [³²P]dCTP (New England Nuclear) using a commercially available random primed DNA labeling kit. Ethidium bromide-stained 28S rRNA indicates the relative amounts of total RNA in each lane.

The results of the Northern blot analysis of HL-60 and 10T1/2 cells, showed marked changes in gene expression shortly after NaPA treatment. Expression of the adipocyte-specific aP2 gene was induced within 24 hours in treated 10T1/2 confluent cultures reaching maximal mRNA levels by 72 hours.

EXAMPLE 5

HL-60 Cells—myc Down Regulation

In HL-60, cell transformation has been linked to myc amplification and over-expression, and differentiation would typically require down regulation of myc expression. [Collins, S. J. The HL-60 promyelocytic leukemia cell line: Proliferation, differentiation, and cellular oncogene expression. *Blood.* 70:1233-1244 (1987)]. To demonstrate the kinetics of myc inhibition and HLA-A induction, Northern blot analysis of cytoplasmic RNA (20 µg/lane) was carried out on cells treated with NaPA and PAG for specified durations of time and untreated controls (-). The dose-dependency and specificity of the effect of NaPA was observed. Two concentrations of NaPA, 1.6 mg/ml (++) and 0.8 mg/ml (+), and PAG at 1.6 mg/ml were investigated. The ³²P-labeled probes used were myc 3rd exon (Oncor) and HLA-A3 Hind III/EcoRI fragment. NaPA caused a rapid decline in the amounts of myc mRNA. This occurred within 4 hours of treatment, preceding the phenotypic changes detectable by 48 hours, approximately two cell cycles, after treatment. Similar kinetics of myc inhibition have been reported for other differentiation agents such as dimethyl sulfoxide, sodium butyrate, bromodeoxyuridine, retinoids, and 1, 25-dihydroxyvitamin D₃. The results observed suggest that down regulation of oncogene expression by NaPA may be responsible in part for the growth arrest and induction of terminal differentiation. In addition, it is evident that NaPA treatment of the leukemic cells was associated with time- and dose-dependent accumulation of HLA-A mRNA coding for class I major histocompatibility antigens. This enhances the immunogenicity of tumors in vivo.

EXAMPLE 6

K562 Cells—NaPA Promotes Hemoglobin Biosynthesis

Further support for the use of NaPA as a non-toxic inducer of tumor cell differentiation is found in the ability of NaPA to promote hemoglobin biosynthesis in erythroleukemia cells. K562 leukemic cells have a nonfunctional beta-globin gene and, therefore, do not normally produce significant amounts hemoglobin. When K562 human erythroleukemia cells were grown in the presence of NaPA at 0.8 and 1.6 mg/ml concentrations, hemoglobin accumulation, a marker of differentiation, was found to increase 4 to 9 fold over that of control cells grown in the absence of NaPA. Hemoglobin accumulation was determined by Benzidine staining of cells for hemoglobin and direct quantitation of the protein. The results of this study are reported in Table 16.

It has been shown that high concentrations of NaPA inhibit DNA methylation in plants. [Vanjusin, B. J. et al. *Biochemia* 1, 46:47-53 (1981)]. Alterations in DNA methylation can promote oncogenesis in the evolution of cells with metastatic capabilities. [Rimoldi, D. et al. *Cancer Research.* 51:1-7 (1991)]. These observations prompted some concerns regarding potential long-term adverse effects with the use of NaPA. To determine the potential tumorigenicity of NaPA, a comparative analysis was performed using NaPA

and the known hypomethylating agent 5-aza-2'-deoxycytidine (5AzadC).

Premalignant cells ($3-4 \times 10^5$) were plated in 75 cm² dishes and 5AzadC 0.1 μ M was added to the growth medium at 20 and 48 hrs after plating. The cells were then subcultured in the absence of the nucleoside analog for an additional seven weeks. Cells treated with NaPA at 1.6 mg/ml were subcultured in the continuous presence of the drug. For the tumorigenicity assay, 4-5 week-old female athymic nude mice were inoculated s.c. with 1×10^6 cells and observed for tumor growth at the site of injection.

The results set forth in Table 3 show that NaPA, unlike the cytosine analog, did not cause tumor progression.

TABLE 3

| Tumorigenicity of C3H 10T1/2 Cells in Athymic Mice | | | |
|---|---|-----------------------------|-----------------|
| Tumors | | | |
| Treatment | Incidence (positive/ injected mice) | Diameter (mm \pm S.D.) | Time (weeks) |
| None | 0/8 | 0 | 13 |
| 5AzadC | 8/8 | 5.5 \pm 2.5 | 8 |
| NaPA | 0/8 | 0 | 13 |

The transient treatment of actively growing 10T1/2 cells with 5AzadC resulted in the development of foci of neoplastically transformed cells with a frequency of about 7×10^{-4} . These foci eventually became capable of tumor formation in athymic mice. By contrast, actively replicating 10T1/2 cultures treated for seven weeks with NaPA, 800-1600 μ g/ml, differentiated solely into adipocytes, forming neither neoplastic foci in vitro nor tumors in vivo in recipient mice.

Furthermore, experiments have demonstrated that NaPA can prevent spontaneous or 5AzadC-induced neoplastic transformation, thus demonstrating its novel role in cancer prevention. It is known that the treatment of premalignant 4C8 and 10T1/2 cells with carcinogens such as 5AzadC produces malignant conversion of the respective cells. When 4C8 [Remold: et al., *Cancer Research*, 51:1-7 (1990)] and 10T1/2 cells were exposed to 5AzadC, malignant conversion became evident in two days and two weeks, respectively. NaPA (0.8-1.6 mg/ml) prevented the appearance of the malignant phenotype, as determined by cell morphology, contact inhibition and anchorage dependent growth in culture.

EXAMPLE 7

Growth Arrest in Malignant Gliomas

In addition, Phenylacetate has been implicated in damage to immature brain in phenylketonuria. Because of similarities in growth pattern and metabolism between the developing normal brain and malignant central nervous system tumors, phenylacetate may be detrimental to some brain cancers. Phenylacetate can induce cytostasis and reversal of malignant properties of cultured human glioblastoma cells, when used at pharmacological concentrations that are well tolerated by children and adults. Interestingly, treated tumor cells exhibited biochemical alterations similar to those observed in phenylketonuria-like conditions, including selective decline in de novo cholesterol synthesis from mevalonate. Since gliomas, but not mature normal brain cells, are highly dependent on mevalonate for production of sterols and isoprenoids vital for cell growth, phenylacetate

would be expected to affect tumor growth in vivo, while sparing normal tissues. Systemic treatment of rats bearing intracranial gliomas resulted in significant tumor suppression with no apparent toxicity to the host. The experimental data, which are consistent with clinical evidence for selective activity against undifferentiated brain, suggest that phenylacetate may offer a safe and effective novel approach to treatment of malignant gliomas.

Clinical experience, obtained during phenylacetate treatment of children with urea cycle disorders, indicates that millimolar levels can be achieved without significant adverse effects. The lack of neurotoxicity in these patients is, however, in marked contrast to the severe brain damage documented in phenylketonuria (PKU), an inborn error of phenylalanine metabolism associated with excessive production of phenylacetate, microcephaly, and mental retardation. [Scriver, C. R., and C. L. Clow. 1980. Phenylketonuria: epitome of human biochemical genetics. *New Engl. J. Med.* 303: 1394-1400]. The differences in clinical outcome can be explained by the fact that, although phenylacetate readily crosses the blood-brain barrier in both prenatal and postnatal life, neurotoxicity is limited to the immature brain. Compelling evidence for a developmentally restricted window of susceptibility is provided by the phenomenon of "maternal PKU syndrome": PKU females who are diagnosed early and maintained on a phenylalanine-restricted diet, develop normally and subsequently tolerate a regular diet. These women often give birth to genetically normal, yet mentally retarded infants due to the untreated maternal PKU. The elevated levels of circulating phenylacetate, while sparing the mature tissues of the mother, are detrimental to the fetal brain. The primary pathological changes in PKU involved rapidly developing glial cells and are characterized by alterations in lipid metabolism and myelination with subsequent neuronal dysfunction. The vulnerable fetal glial tissues resemble neoplastic glial cells in numerous molecular and biochemical aspects, including unique dependence upon mevalonate (MVA) metabolism for synthesis of sterols and isoprenoids critical to cell replication [Kandutsch, A. A., and S. E. Saucier. 1969. Regulation of sterol synthesis in developing brains of normal and jimpy mice. *Arch. Biochem. Biophys.* 135: 201-208; Fumagalli, R., E. Grossi, P. Paoletti, and R. Paoletti. 1964. Studies on lipids in brain tumors. I. Occurrence and significance of sterol precursors of cholesterol in human brain tumors. *J. Neurochem.* 11: 561-565; Grossi, E., P. Paoletti, and R. Paoletti. 1958. An analysis of brain cholesterol and fatty acid biosynthesis. *Arch. Int. Physiol. Biochem.* 66: 564-572], and on circulating glutamine as the nitrogen donor for DNA, RNA and protein synthesis [Perry, T. L., S. Hasen, B. Tischler, R. Bunting, and S. Diamond. 1970. Glutamine depletion in phenylketonuria, a possible cause of the mental defect. *New Engl. J. Med.* 282: 761-766; Weber, G. 1983. Biochemical strategy of cancer cells and the design of chemotherapy: G. H. A. Clowes Memorial Lecture. *Cancer Res.* 43: 3466-3492]. The hypothesis underlying these studies was that phenylacetate, known to conjugate and deplete serum glutamine in humans, and to inhibit the MVA pathway in immature brain [Castillo, M., M. F. Zafra, and E. Garcia-Peregrin. 1988. Inhibition of brain and liver 3-hydroxy-3-methylglutaryl-CoA reductase and mevalonate-5-pyrophosphate decarboxylase in experimental hyperphenylalaninemia. *Neurochem. Res.* 13: 551-555; Castillo, M., J. Iglesias, M. F. Zafra, and E. Garcia-Peregrin. 1991. Inhibition of chick brain cholesterogenic enzymes by phenyl and phenolic derivatives of phenylalanine. *Neurochem. Int.* 18: 171-174; Castillo, M., M. Martinez-Cayuela, M. F. Zafra, and E. Garcia-Peregrin. 1991. Effect of phenylalanine derivatives on the main regulatory enzymes of hepatic cholestrogenesis. *Mol. Cell. Biochem.* 105: 21-25], might attack these critical control points in malignant gliomas. The efficacy of phenylacetate was demonstrated using both in vitro and in vivo tumor models.

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

Evaluation of Cell Replication and Viability. Growth rates were determined by an enzymatic assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide (Sigma, St. Louis, Mo.) [Alley, M. C., D. A. Scudiero, A. Monks, M. L. Hursey, M. J. Czerwinski, D. L. Fine, B. J. Abbott, J. G. Mayo, R. H. Schoemaker, and M. R. Boyd. 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* 48: 589-601], cell enumeration with a hemocytometer following detachment with trypsin/EDTA, and by thymidine incorporation into DNA. The different assays produced essentially the same results. Cell viability was assessed by trypan blue exclusion.

Colony Formation in Semi-Solid Agar. Tumor cells were detached with trypsin/EDTA, re-suspended in growth medium containing 0.36% agar, and placed onto a base layer of solid agar (0.9%) in the presence or absence of drugs. Colonies composed of 30 or more cells were scored after three weeks.

Immunocytochemistry. Cells were immunostained with antivimentin monoclonal antibodies using Dako PAP kit K537 (Dako Corporation, California).

Measurement of Cholesterol, Protein and DNA Synthesis. For studies of steroid synthesis, cells were labeled for 24 hours with 5×10^6 DPM [$5\text{-}^3\text{H}$]-mevalonate (35 Ci/mmol) (New England Nuclear, Boston, Mass.) in growth medium containing 3 μM lovastatin and 0.5 mM unlabeled mevalonate, in the presence or absence of 5 mM phenylacetate or 2.5 mM phenylbutyrate. Cellular steroids were extracted with hexane and separated by silica thin layer chromatography. The R_f of the hexane-soluble radiolabeled product was identical to that of a radiolabeled cholesterol standard in three different solvent systems. Similarly treated cells were tested for de novo protein and DNA synthesis by metabolic labeling with [^3H]-leucine (158 Ci/mmol) or [^3H]-deoxythymidine (6.7 Ci/mmol) (New England Nuclear). Measurements of $^{14}\text{CO}_2$ released from [$1\text{-}^{14}\text{C}$]-mevalonate (49.5 mCi/mmol) (Amersham, Chicago, Ill.) in cell homogenates incubated with phenylacetate/phenylbutyrate were performed with minor modifications to established procedures.

Analysis of Protein Isoprenylation. Cell cultures were incubated with 10 mM phenylacetate or 2.5 mM phenylbutyrate for 24 hours in complete growth medium, and labeled with RS-[2- ^{14}C]-mevalonate (16 $\mu\text{Ci}/\text{ml}$, specific activity 15 $\mu\text{Ci}/\text{mmol}$) (American Radiolabeled Chemicals, Inc. St. Louis, Mo.) during the final 15 hours of treatment. Whole cell proteins were extracted, resolved on 10% SDS-polyacrylamide gels, and stained with Commassie Brilliant Blue. Gels were then dried and exposed to Kodak X-Omat film for 4 days.

Animal Studies. To determine the effect of phenylacetate on the tumorigenic phenotype of human glioblastoma cells, cultures were pre-treated for one week and then harvested, resuspended in medium containing 30% matrigel (Collaborative Biomedical Products, Bedford, Mass.), and transplanted s.c. (2.5×10^6 cells per site) into 5-week old female athymic mice (Division of Cancer Treatment, NCI Animal Program, Frederick Cancer Research Facility). The animals were then observed for tumor growth at the site of injection.

To further evaluate drug efficacy in vivo, Fisher 344 rats received a stereotaxic inoculation of syngeneic 9 L gliosarcoma cells (4×10^4) into the deep white matter of the right cerebral hemisphere, as previously described [Weizsaecker, M., D. F., Deen, M. L. Rosenblum, T. Hoshino, P. H. Gutin, and M. Baker. 1981. The 9 L rat brain tumor: description and application of an animal model. *J. Neurol.* 224: 183-192; Culver, K. W., Z. Ram, S. Walbridge, H. Ishii, E. H. Oldfield, and R. M. Blaese. 1992. In vivo gene transfer with retroviral vector producer cells for treatment of experimental brain tumors. *Science.* 256: 1550-1552]. The animals were then subjected to two weeks of continuous treatment with sodium phenylacetate (550 mg/kg/day, s.c.), using osmotic minipumps transplanted subcutaneously. In control rats the minipumps were filled with saline. Statistical analysis of data employed the Fisher's Exact Test.

Induction of cytostasis and phenotypic reversion in cultured human glioblastoma cells. Treatment of glioblastoma cells with phenylacetate resulted in time- and dose-dependent growth arrest (FIG. 4), accompanied by similarly diminished DNA synthesis. After 4-6 days of continuous treatment with 4 mM phenylacetate, there was approximately 50% inhibition of growth in U87, A172, U373, U343, and HS683 cultures (IC_{50} 4.4 ± 0.6 mM). Reflecting on the heterogeneous nature of tumor cell responses, glioblastoma U251 and U138 cells were less sensitive with IC_{50} values of 8-10 mM. Further studies, mimicking pharmacological conditions that are expected in patients, involved exposure of cells to phenylacetate in glutamine-depleted medium. These conditions completely blocked glioblastoma cell growth, but had little effect on the replication of normal endothelial cells (FIG. 5). Phenylbutyrate, an intermediate metabolite of phenylacetate formed in the brain by fatty acid elongation, also inhibited tumor cell replication (IC_{50} 2.2 ± 0.2 mM in A172, U87 and U373), while the end metabolite, phenylacetylglutamine, was inactive. In addition to inducing selective tumor cytostasis, both phenylacetate and phenylbutyrate promoted cell maturation and reversion to a nonmalignant phenotype, manifested by an altered pattern of cytoskeletal intermediate filaments, loss of anchorage-independence, and reduced tumorigenicity in athymic mice (Table 4). These profound changes in tumor behavior were accompanied by alterations in the expression of genes implicated in growth control, angiogenesis, and immunosuppression (e.g., TGF α , HbF, and TGF- β 2).

TABLE 4

| Treatment | Reversal of Malignancy of Human Glioblastoma Cells | |
|----------------|--|--|
| | Clonogenicity in Soft Agar ¹ (%) | Tumor Incidence ² Positive/Injected Sites |
| None | 8.1 | 9/10 |
| Phenylacetate | | |
| 2.5 mM | 0.5 | ND |
| 5 mM | >0.01 | 2/10 |
| Phenylbutyrate | | |
| 1.25 mM | 0.15 | ND |
| 2.5 mM | >0.01 | 1/10 |

¹U87 cells were detached with trypsin/EDTA, resuspended in growth medium containing 0.36% agar, and placed onto a base layer of solid agar (0.9%) in the presence or absence of drugs. Colonies composed of 30 or more cells were scored after three weeks.

²U87 cells pre-treated in culture for one week, were harvested, resuspended in medium containing 30% matrigel, and transplanted s.c. into 5-week old female athymic mice (2.5×10^6 cells per mouse). Data were recorded 5 weeks after cell inoculation.

ND = not determined.

Phenylacetate inhibits the mevalonate pathway and protein isoprenylation. The most consistent biochemical change

observed in glial cells exposed to phenylacetate involved alterations in lipid metabolism and inhibition of the MVA pathway (FIG. 6). Active de novo synthesis of cholesterol and isoprenoids from precursors such as acetyl-CoA and MVA is an important feature of the developing brain (but not the mature brain), coinciding with myelination. It is also a hallmark of malignant gliomas [Azarnoff, D. L., G. L. Curran, and W. P. Williamson. 1958. Incorporation of acetate- ^{14}C into cholesterol by human intracranial tumors in vitro. *J. Nat. Cancer Inst.* 21: 1109–1115; Rudling, M. J., B. Angelin, C. O. Peterson, and V. P. Collins. 1990. Low density lipoprotein receptor activity in human intracranial tumors and its relation to cholesterol requirement. *Cancer Res.* 50 (suppl): 483–487]. Cholesterol production and protein isoprenylation diminished within 24 hours of glioblastoma treatment with either phenylacetate or phenylbutyrate (FIG. 7), preceding changes in DNA and total protein synthesis, which were detectable after 48 hours. The reduction in isoprenylation was paralleled by a decrease in MVA decarboxylation (to less than 50% of control), an effect previously observed in embryonic brain in PKU-like conditions. MVA-5-pyrophosphate decarboxylase, a key enzyme regulating cholesterol synthesis in brain, is inhibited by phenylacetate under conditions in which MVA kinase and MVA-5-phosphate kinase are only minimally affected. Phenylacetate might also interfere with MVA synthesis from acetyl-CoA. Glioblastoma cells could not, however, be rescued by exogenous MVA (0.3–3 mM), suggesting that MVA utilization, rather than its synthesis, is the prime target.

Mevalonate is a precursor of several isopentenyl moieties required for progression through the cell cycle such as sterols, dolichol, the side chains of ubiquinone and isopentenyladenine, and prenyl groups that modify a small set of critical proteins [Goldstein, J. L., and M. S. Brown. 1990. Regulation of the mevalonate pathway. *Nature.* 343: 425–430; Marshall, C. J. 1993. Protein prenylation: A mediator of protein-protein interactions. *Science.* 259: 1865–1866; Braun, P. E., D. De Angelis, W. W. Shtybel, and L. Bernier. 1991. Isoprenoid modification permits 2',3'-cyclic nucleotide 3'-phosphodiesterase to bind to membranes. *J. Neurosci. Res.* 30: 540–544]. The latter include plasma membrane G and G-like proteins (e.g., ras) involved in mitogenic signal transduction (molecular weight 20–26 kDa), the myelination-related enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase, and nuclear envelope lamins that play a key role in mitosis (44–74 kDa). Inhibition of sterol and isoprenoid synthesis during rapid development of the brain could lead to the microcephaly and impaired myelination seen in untreated PKU. Targeting MVA in dedifferentiated malignant gliomas, on the other hand, would be expected to inhibit tumor growth in vivo without damaging the surrounding normal tissues, as the MVA pathway is significantly less active in mature brain.

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

TABLE 5

| Treatment ¹ | Phenylacetate Activity in Experimental Brain Cancer | | | |
|------------------------|---|-------------|-------------|------------|
| | Brain Tumors ² | | | |
| | No. of animals | Macroscopic | Microscopic | Tumor Free |
| Saline | 10 | 8 | 1 | 1 |
| Phenylacetate | 15 | 3 | 4 | 8 |

¹Fisher 344 rats received a stereotaxic inoculation of syngeneic 9L gliosarcoma cells into the deep white matter of the right cerebral hemisphere, as described in Material and Methods. Animals were then subjected to two weeks of continuous treatment with either sodium phenylacetate (550 mg/kg/day, s.c.) or saline, using osmotic minipumps transplanted subcutaneously.

²Animals were sacrificed 23 days after tumor inoculation to determine antitumor effects. Findings were confirmed by histological evaluation of the inoculated site.

Summary and Prospective. Phenylacetate has long been implicated in damage to the developing fetal brain. As primary CNS tumors are highly reminiscent of immature fetal brain, malignant gliomas should be equally vulnerable. Moreover, viewing maternal PKU syndrome as a natural human model, phenylacetate would be expected to suppress the growth of brain neoplasms without harming normal tissues. Experimental data supports this hypothesis. Phenylacetate induced selective cytostasis and promoted maturation of glioma cells in vitro and in vivo. Premature growth arrest and differentiation could also underlie the damage to fetal brain in PKU. Multiple mechanisms of action are involved, including inhibition of protein isoprenylation and depletion of plasma glutamine in humans. The demonstrable antitumor activity, lack of toxicity, and ease of administration (oral or intravenous), demonstrate the clinical efficacy of phenylacetate in management of malignant gliomas, and perhaps of other neoplasms as well. Previously, phenylacetate showed activity in prostate cancer in vitro. Phase I clinical studies with phenylacetate in the treatment of adults with cancer confirmed that therapeutic levels can be achieved in the plasma and cerebrospinal fluid with no significant toxicities, and provide preliminary evidence for benefit to prostatic carcinoma and glioblastoma patients (see Example 18).

Phenylacetate was used to treat human solid tumors, including prostatic carcinoma, glioblastomas, and malignant melanoma. Treatment resulted in selective cytostasis and phenotypic reversion, as indicated by the restored anchorage-dependence, reduced invasiveness and loss of tumorigenicity in athymic mice. Molecular analysis of brain and hormone-refractory prostate cancer cells revealed marked decline in the production and secretion of TGF β , a protein implicated in growth control, angiogenesis, and immunosuppression. Treated prostatic cells exhibited decreased proteolytic activity mediated by urokinase-plasminogen activator, a molecular marker of disease progression in man.

EXAMPLE 8

Growth Arrest, Tumor Maturation, and Extended Survival in Brain Tumors Treated with NaPA

In Vitro Studies

Cell proliferation. The effect of NaPA on cell proliferation was evaluated using tritiatedthymidine incorporation assay on cultured 9 L gliosarcoma cells and cell enumeration using a hemocytometer following detachment with trypsin/EDTA. 9 L is a syngeneic malignant glial tumor derived from

Fischer 344 rats and is associated with 100% mortality within three to four weeks after intracerebral inoculation [Weizsaecker M, Deen D F, Rosenblum M L, et al. The 9 L rat brain tumor: description and application of an animal model. *J Neurol*. 1981; 224: 183-192]. Tumor cells were plated at 5×10^4 tumor cells/well in 24-well plates (Costar, Cambridge, Mass.) in Dulbecco Modified Eagle's medium (DMEM) with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah), 2 mM L-glutamine (GIBCO BRL, Gaithersburg, Md.), 50 U/ml penicillin (GIBCO) and 50 μ g/ml streptomycin (GIBCO) and 2.5 μ g/ml Fungizone (ICN Biomedicals Inc., Costa Mesa, Calif.). After 24 hours, the medium was changed and NaPA (Elan Pharmaceutical Research Corp., Gainesville, Ga.) added to the medium at 0, 2.5, 5, and 10 mM concentration for 5 days. Six hours before harvest, 0.5 mCi tritiatedthymidine (ICN Radiochemicals, Irvine, Calif.) was added to each well. Thymidine incorporation was determined by scintillation counting in triplicates. Colony formation in semi-solid agar. Anchorage independent growth (the ability of cells to form colonies in semi-solid agar) is characteristic of malignant glial cells. 9 L cells were harvested with trypsin/EDTA and resuspended at 1.0×10^4 cells/ml in growth medium containing 0.36% agar (Difco). Two ml of the cell suspension was added to 60 mm plates (Costar, Cambridge, Mass.) which were precoated with 4 ml of solid agar (0.9%). Phenylacetate was added to the agar at different concentrations (0, 1.25, 2.5, and 5 mM). In a second experiment, 9 L cells were grown for 7 days in tissue culture containing 5 mM NaPA. The cells were then transferred, as described, to agar plates without NaPA. Colonies composed of 30 or more cells were counted after 3 weeks.

9 L brain tumor inoculation and phenylacetate administration. Fisher 344 rats (n=50) weighing 230-350 grams were anesthetized using intraperitoneal (i.p.) Ketamine (90 mg/Kg, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) and Xylazine (10 mg/Kg, Mobay Corporation, Shawnee, Kans.) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, Calif.). 4×10^4 syngeneic 9 L gliosarcoma cells in 5 μ L (Hank's) balanced salt solution were injected into the deep white matter (depth of inoculation-3.5 mm) of the right cerebral hemisphere using a 10 μ L Hamilton syringe connected to the manipulating arm of the stereotaxic apparatus. In 10 rats, phenylacetate was administered by continuous subcutaneous (s.c.) release of the drug using two 2ML2 osmotic pumps release rate of 5 μ L/hr for 14 days (Alza Corporation, Palo Alto, Calif.). On the day of tumor inoculation the pumps were implanted in the subcutaneous tissue of both flanks. The concentration of the drug in the pumps was 650 mg/ml (total of 2600 mg for both pumps) for a daily dose of 550 mg/kg per rat. The minipumps were replaced after 14 days for a total treatment of 28 days. Fifteen additional rats received NaPA, as described, starting 7 days after intracerebral inoculation of the tumor. In these rats, an additional daily injection of NaPA (300 mg/kg, i.p.) was given for 28 days. Control rats (n=25) received continuous saline from two s.c. 2ML2 osmotic pumps. Perioperative penicillin (100,000 u/kg, i.m.) was given to all rats before implantation of the minipumps. Survival was recorded in each group. Three rats treated for established tumors and two control rats were sacrificed 7 days after initiation of NaPA (14 days after tumor inoculation). These were used for electron microscopic studies of treated tumors, in vivo proliferation assays, and measurement of NaPA levels in the serum and CSF. Peripheral organs (heart, lung, spleen, liver, kidney, bowel, adrenal, and gonads) were harvested and subjected for a routine histological examina-

tion. Brain specimens were sectioned and stained for routine hematoxylin and eosin (H&E) and myelin stains (Luxol-fast blue) for evidence of drug-related toxicity.

Electron microscopy. Animals were sacrificed by intracardiac perfusion with 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.4. Two hours later the fixed brains were washed in buffer and sliced into 1 mm thick coronal sections. The areas containing tumors were further dissected into 1 mm³ cubes, post-fixed with 2% osmium tetroxide in 0.1M sodium cacodylate buffer for 2 hours, washed in buffer, mordanted en block with 1% uranyl acetate at pH 5 overnight, then washed, dehydrated and embedded in Epon. Thin sections were cut at several levels into each block to ensure greater sampling. Electron micrographs of tumor cells were taken at random for morphology.

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

Measurement of NaPA levels in serum and CSF. Three NaPA-treated and 2 saline-treated rats were sacrificed after 7 days of combined s.c. and i.p. NaPA or saline administration. Blood was drawn from the heart and CSF was aspirated from the cisterna magna. Due to volume limitations of CSF, pooled serum and CSF samples were assessed in a similar fashion. Protein extraction of a 200 μ L aliquot of biological fluid was carried out with 100 μ L of a 10% perchloric acid solution. 150 μ L of supernate was neutralized with 25 μ L of 20% potassium bicarbonate and centrifuged. 125 μ L of supernate was then pipetted into sampling tubes. Chromatography was performed on a Gilson 715 HPLC system using a 30 cm Waters C18 column (i.d. 3.9 mm) at 60° C. A 75 μ L injectate was eluted with an acetonitrile/water gradient ranging from 5 to 30% over 20 minutes and flowing at 1 ml/min. UV-monitoring was performed at a wavelength of 20 nm. Elution time for phenylacetate was 14.8 minutes. Statistical analysis. The Chi-square test was used to compare proportions of BrdU-positive cells. The Mantel-Haenzel test was used to compare survival between NaPA-treated and saline-treated rats in the survival experiments.

In Vitro Results

In vitro Effect of NaPA on cell proliferation and anchorage dependency. Treatment of 9 L cells with NaPA for 5 days resulted in dose-dependent decrease in cell number with IC₅₀ at 6.0 ± 0.5 mM. This was accompanied with a decrease in tritiated-thymidine incorporation (FIG. 8). In addition, phenylacetate induced a dose-dependent restoration of anchorage dependency, indicating a reversion of the malignant phenotype (Table 19). 9 L cells that were exposed to NaPA for 7 days before plating in agar (not containing NaPA) still showed >40% inhibition in colony formation (Table 19).

TABLE 19

| Phenylacetate Inhibits Anchorage-Independent Growth of 9L Gliosarcoma Cells | | | |
|---|-----------|------------------|--------------|
| Treatment | PA in | Colony Formation | |
| in Culture | Agar (mM) | # Colonies | % Inhibition |
| none | 0 | 628 ± 50 | — |
| none | 5 | 8 ± 4 | 98.7 |
| | 2.5 | 111 ± 13 | 82.4 |
| | 1.25 | 326 ± 20 | 48.0 |
| ^a Phenylacetate | 0 | 375 ± 25 | 40.3 |

^a9L cells were treated with 5 mM phenylacetate in culture for 7 days before being plated on soft agar.

In Vivo Studies

In vivo proliferation assay and electron microscopy findings. Treatment of established brain tumors with NaPA resulted in a significant decrease in the rate of proliferation. 285 of 1283 treated tumor cells stained for BrdU compared to 429 of 1347 saline-treated tumor cells (mitotic index of 0.22 in NaPA-treated vs. 0.33 in saline-treated tumors; $p < 0.0001$).

Electron microscopy of these tumors showed a striking abundance of well-organized rough endoplasmic reticulum in the NaPA-treated tumor cells, indicating a higher degree of cell differentiation [Ghadially F N. Endoplasmic reticulum and ribosomes in cell differentiation and neoplasia. In: eds. *Ultrastructural Pathology of the Cell and Matrix*. Third, London:Butterworths; 1992:450-454]. By contrast, untreated tumors generally had Scant rough endoplasmic reticulum and numerous polyribosomes, which are characteristics of highly malignant cells. In addition, mitotic cells were more frequently found in untreated tumors.

Serum and CSF levels of NaPA. Assays of pooled serum and CSF from 3 treated and 2 control rats, obtained after 7 days of combined s.c. and i.p. NaPA (total daily dose of 850 mg/kg) or saline administration, revealed a mean phenylacetate level of 2.45 mM in the serum and 3.1 mM in the CSF. No phenylacetate was detected in the serum of CSF samples from saline-treated rats.

Survival Experiments

Simultaneous tumor inoculation and administration of NaPA. Seven of 10 NaPA-treated rats survived for >90 days after tumor inoculation when NaPA was administered for 4 weeks starting on the day of tumor inoculation. Nine of 10 control rats died within 34 days after tumor inoculation ($p < 0.01$, Mantel-Haenzel test) (FIG. 9).

Treatment of established tumors with NaPA. Five of 12 rats treated with s.c. and i.p. NaPA for 4 weeks (starting 7 days after tumor inoculation) are still alive 50 days after tumor inoculation, while 12 of 13 saline-treated rats died by day 36 ($p < 0.03$, Mantel-Haenzel test) (FIG. 10).

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

Discussion. Phenylacetate induced a potent cytostatic and antitumor effect in the in vitro and in vivo brain tumor models used in these studies. This effect extended beyond the duration of drug administration, indicated by the long-term survival and apparent cure of rats which received NaPA either simultaneously with tumor inoculation or after tumors were established. This extended effect of NaPA shows that the malignant phenotype of treated tumor cells reverted, perhaps irreversibly in some animals, to one that was more benign and differentiated. Anchorage independence, i.e., the ability of cells to form colonies in semi-solid agar, is characteristic of malignant glioma cells. Phenylacetate

caused a dose-dependent restoration of anchorage dependency, indicating reversion of the glioma cells to a non-malignant phenotype. More than 80% inhibition of colony formation was achieved at NaPA concentration in the agar plate of 2.5 mM, similar to the serum and CSF levels measured in treated rats. In addition, after one week of exposure to NaPA, more than 40% of tumor cells maintained a benign growth pattern despite the absence of NaPA in the agar plates (Table 19). A significant in vivo indicator of cell differentiation was observed in our study in the subcellular organelles of treated brain tumor cells. The disorganized cytoplasmic polyribosomes in the saline-treated tumor cells were transformed by NaPA to a hyperplastic, well organized, rough endoplasmic reticulum. The endoplasmic reticulum is a highly specialized structure that performs many distinct functions. Hence a well-developed endoplasmic reticulum represents cell differentiation and functional activity. An inverse relationship has been noted between the amount of rough endoplasmic reticulum and the growth rate and degree of malignancy of tumors [Ghadially F N. Diagnostic Electron Microscopy of Tumours. eds. 2. London:Butterworth; 1985]. The numerous polyribosomes in the untreated tumor cells correlated well with the number of mitoses seen by light microscopy and were confirmed by the BrdU proliferation assay. These changes underscore the differentiating effect of NaPA on the malignant glial cells and correlate with the in vivo decrease in cell proliferation and extended survival that occurred in treated animals with brain tumors.

Therapeutic blood and CSF NaPA levels were reached in the treated rats. The high CSF levels indicate good penetration of NaPA into the central nervous system and into the developing tumor. The doses used are well below the known toxic levels of NaPA in children with inborn errors of urea synthesis (2.5 g/kg/d) or rats (1.6 g/kg/d) and indicate that NaPA can be given safely at a higher doses, possibly with enhancement of antitumor efficacy. These data indicate that phenylacetate, given to rats at a non-toxic dose, has a profound effect on tumor growth regulation and cell maturation.

EXAMPLE 9

Suppression of 5-Aza-2'-Deoxycytidine Induced Carcinogenesis

Differentiation inducers selected for their low cytotoxic and genotoxic potential could be of major value in chemoprevention and maintenance therapy. Specifically, the ability of phenylacetate to prevent carcinogenesis by the chemotherapeutic hypomethylating drug, 5-aza-2'-deoxycytidine (5AzadC), was tested in vitro and in mice. Transient exposure of immortalized, but non-tumorigenic ras-transformed 4C8 fibroblasts to 5AzadC resulted in neoplastic transformation manifested by loss of contact inhibition of growth, acquired invasiveness, and tumorigenicity in athymic mice. The latter was associated with increased ras expression and a decline in collagen biosynthesis. These profound phenotypic and molecular changes were prevented by a simultaneous treatment with phenylacetate. Protection from 5AzadC carcinogenesis by phenylacetate was: (a) highly efficient despite DNA hypomethylation by both drugs; (b) free of cytotoxic and genotoxic effects; (c) stable after treatment was discontinued, and; (d) reproducible in vivo. Whereas athymic mice bearing 4C8 cells developed fibrosarcomas following a single i.p. injection with 5AzadC, tumor development was significantly inhibited by systemic treatment with nontoxic doses of phenylacetate. Phenylac-

etate and its precursor suitable for oral administration, phenylbutyrate, may thus represent a new class of chemopreventive agents, the efficacy and safety of which should be further evaluated.

The multi-step nature of neoplastic transformation makes this disease process amenable to chemopreventive intervention. Several agents have been shown to inhibit carcinogenesis and thereby prevent the development of primary or secondary cancers [Kelloff, G. J., C. W. Boone, W. F. Malone, and V. E. Steele. 1992. Chemoprevention clinical trials. *Mutation Res.*, 267: 291-295; Weinstein, B. I. 1991. Cancer prevention: Recent progress and future opportunities. *Cancer Res.*, 51:5080s-5085s; Wattenberg, L. W. Inhibition of carcinogenesis by naturally occurring and synthetic compounds. In: Y. Kuroda, D. M. Shankel and M. D. Waters (eds), *Antimutagenesis and Anticarcinogenesis, Mechanisms II*, pp.155-166. New York: Plenum Publishing Corp., 1990; Sporn, M. B., and D. L. Newton. 1979. Chemoprevention of cancer and retinoids. *Fed. Proc.* 38:2528-2534]. Of major interest are natural products and their analogs, including vitamins (A, B12, C, D3, and E), retinoids, and terpenes. These agents can suppress neoplastic transformation subsequent to a carcinogenic insult by regulating cell growth and differentiation. One such growth regulator is phenylacetate.

The efficacy of phenylacetate as a chemopreventive agent was tested using in vitro and in vivo models of 5AzadC-induced carcinogenesis. Despite the promise of 5AzadC in the treatment of cancer and of beta-chain hemoglobinopathies, its clinical applications have been hindered by concerns regarding carcinogenic potential. The model used in the present studies involved premalignant murine fibroblasts (cell lines 4C8 and PR4), which express a transcriptionally activated c-Ha-ras protooncogene. These non-tumorigenic cells are highly susceptible to malignant conversion by pharmacological doses of 5AzadC. However, Phenylacetate can protect such vulnerable cells from 5AzadC-induced carcinogenesis both in culture and in mice.

Cell Cultures and Reagents. The subclones of mouse NIH 3T3 fibroblasts, PR4N and 4C8-A10 (designated here PR4 and 4C8) have been previously described [Wilson, V. L., R. A. Smith, H. Autrup, H. Krokan, D. E. Musci, N-N-T. Le, J. Longoria, D. Ziska, and C. C. Harris. 1986. Genomic 5-methylcytosine determination by ³²P-postlabeling analysis. *Anal. Biochem.*, 152:275-284; Dugaiczky, A., J. J. Haron, E. M. Ston, O. E. Dennison, K. N. Rothblum, and R. J. Schwartz. 1983. Cloning and sequencing of a deoxyribonucleic acid copy of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid isolated from chicken muscle. *Biochem.* 22:1605-1613]. Both cell lines are phenotypic revertants isolated from LTR/c-Ha-ras1-transformed 3T3 cells after long-term treatment with murine interferon α/β . Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (Gibco) and antibiotics. The sodium salts of phenylacetic and phenylbutyric acids (Elan Pharmaceutical Corporation) were dissolved in distilled water. 5AzadC (Sigma St. Louis, Mo.) was dissolved in phosphate buffered saline (PBS) and stored in aliquots at -20° C. until use. Exposure of 5AzadC to direct light was avoided at all times to prevent drug hydrolysis.

Treatments with 5AzadC. For treatment in culture, cells were plated at 1-2x10⁵ cells in 100 mm dishes and the drugs added to the growth medium at 20 and 48 hrs later. The cells were subsequently subcultured in the absence of the nucleoside analogs and observed for phenotypic alterations. For in vivo treatment with 5AzadC, 6-9 week-old

female athymic nude mice (Division of Cancer Treatment, NCI Animal Program, Frederick Cancer Research Facility) were inoculated subcutaneously (s.c.) with 0.5x10⁶ cells. Twenty four hours later 400 μ g of freshly prepared 5AzadC in 200 μ l of PBS was administered intraperitoneally (i.p.) into each animal (approximately 20 mg/kg). Systemic treatment with NaPA is described in the text.

Growth on Matrigel. The ability of cells to degrade and cross tissue barriers was assessed by a qualitative in vitro invasion assay that utilize matrigel, a reconstituted basement membrane (Collaborative Research). Cells were exposed for 48 hrs in T.C. plastic dishes with 5AzadC alone or in combination with NaPA. NaPA treatment continued for additional 1-2 weeks. Cells were then replated (at 5x10⁴ per point) onto 16 mm dishes (Costar, Cambridge, Mass.), which were previously coated with 250 μ l of matrigel (10 mg/ml). NaPA was 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 hours; invasion into the matrigel was evident after 6-9 days.

Tumor Formation in Athymic Mice. Cells were injected s.c. (5x10⁵ cells per site) into 4-6 week old female athymic nude mice (Division of Cancer Treatment, NCI animal Program, Frederick Cancer Research Facility). The number, size, and weight of tumors were recorded after 3-4 weeks. For histological examination, tumors were excised, fixed in Bouin's solution (picric acid: 37% formaldehyde: glacial acetic acid, 15:5:1 vol/vol), and stained with H&E.

Measurement of DNA Methylation. To determine the 5-methylcytosine content, samples of cultures were taken 24 hours after the second 5AzadC treatment. The cell pellets were lysed in 0.5% SDS, 0.1M NaCl, 10 mM EDTA pH 8.0, added with 400 μ g/ml of proteinase K (Boehringer Mannheim), and stored at -70° C. until DNA isolation and analysis. The content of methylated/unmethylated cytosine residues in the cellular DNA was measured by a ³²P-postlabeling technique as previously described.

Northern Blot Analysis and DNA Probes. Cytoplasmic RNA was extracted from exponentially growing cells and separated by electrophoresis in 1.2% agarose-formaldehyde gels. RNA preparation, blotting onto nylon membranes (Schleicher and Schuell), hybridization with radiolabeled DNA probes, and autoradiography were performed as described [Rimoldi, D., V. Srikantan, V. L. Wilson, R. H. Bassin, and D. Samid. 1991. Increased sensitivity of nontumorigenic fibroblasts expressing ras or myc oncogenes to malignant transformation induced by 5-aza-2'-deoxycytidine. *Cancer Res.*, 51:324-330]. The DNA probes included: 6.2 kb EcoRI fragment of v-Ki-ras, 2.9 kb SacI fragment of the human c-Ha-ras1 gene, and a BamHI 4.5 kb fragment of the c-myc gene. Glyceraldehyde phosphate dehydrogenase cDNA [Dugaiczky, A., J. J. Haron, E. M. Ston, O. E. Dennison, K. N. Rothblum, and R. J. Schwartz. 1983. Cloning and sequencing of a deoxyribonucleic acid copy of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid isolated from chicken muscle. *Biochem.* 22:1605-1613] was provided by M. A. Tainsky (University of Texas, Houston), and a mouse transin cDNA by G. T. Bowden (University of Arizona, Tucson). The cDNA probe for mouse histocompatibility class I antigens was a gift from G. Jay (NIH, Bethesda). Radiolabeled probes were prepared with [³²P]dCTP (NEN) using a random primed DNA labeling kit (Boehringer Mannheim, Germany).

In Vitro Carcinogenesis Induced by 5AzadC and Its Prevention by Phenylacetate. Untreated 4C8 and PR4 formed contact-inhibited monolayers composed of epithelial-like cells. In agreement with previous observations, transient

exposure of these cultures to 0.1 μ M 5AzadC during logarithmic phase of growth resulted in rapid and massive neoplastic transformation. Within one week of 5AzadC treatment, the great majority of the cell population became refractile and spindly in shape, and formed multilayered cultures with increased saturation densities (Table 7), indicative of loss of contact inhibition of growth. These phenotypic changes could be prevented by the addition of 5–10 mM NaPA (Table 7). Several different regimens of NaPA treatment were found to be similarly effective. These included: (a) pre-treatment with NaPA, starting one day prior to the addition of 5AzadC; (b) simultaneous exposure to both drugs, and; (c) addition of NaPA one day after 5AzadC. In all cases, cells were subsequently subjected to continuous treatment with NaPA for at least one week. Cells cultured under these conditions, like those treated with NaPA alone, formed contact-inhibited monolayers resembling untreated controls. These cells maintained the benign growth pattern for at least three weeks after NaPA treatment was discontinued.

That NaPA prevents neoplastic transformation was further indicated by the inability of cells to invade reconstituted basement membranes (matrigel), and form tumors in athymic mice. When plated onto matrigel, 5AzadC-transformed 4C8 and PR4 cells developed net-like structures characteristic of highly malignant cells, and eventually degraded the extracellular matrix components. In marked contrast, NaPA-treated cultures formed small, non-invasive colonies on top of the matrigel, as previously observed with normal fibroblasts. Untreated parental cells exhibited an intermediate phenotype, as their colonies were slow growing and non-invasive, yet irregular in shape possibly due to increased cell motility. The chemopreventive effect of phenylacetate could be mimicked by its precursor, phenylbutyrate. Cells exposed to 5AzadC in the presence of sodium phenylbutyrate (NaPB, 1.5–3 mM) maintained contact inhibited growth and exhibited a benign phenotype when placed onto matrigel (Table 7).

TABLE 7

| Effect of 5AzadC and NaPA on DNA Methylation | | | |
|--|------------------------|--------------------|--------------|
| Cells | Treatment ^a | DNA Methylation | |
| | | % 5mC ^b | % of Control |
| 4C8 | none | 3.49 \pm 0.06 | 100 |
| | 5AzadC | 1.52 \pm 0.27 | 43 |
| | NaPA | 2.22 \pm 0.10 | 63 |
| | 5AzadC + NaPA | 1.62 \pm 0.18 | 46 |
| PR4 | none | 2.72 \pm 0.16 | 100 |
| | 5AzadC | 1.11 \pm 0.22 | 41 |
| | NaPA | 1.25 \pm 0.08 | 46 |
| | 5AzadC + NaPA | 1.06 \pm 0.11 | 39 |

^aCells were treated with 0.1 μ M 5AzadC and/or 10 mM NaPA and the percentage of 5 mC was determined as described in "Materials and Methods".
^bData indicate the mean \pm S.D. (n = 4) of two experiments.

The in vitro growth characteristics of cells correlated with their behavior in athymic mice. 5AzadC-treated 4C8 cells developed rapidly growing fibrosarcomas within 2 weeks of s.c. transplantation into mice. Consistent with their behavior in vitro, the parental cells were far less aggressive, forming small lesions after 3–4 weeks in three of eight recipient animals. However, no tumors developed in animals injected with 4C8 cells that had been pre-treated for one week in culture with the combination of 5AzadC and NaPA (Table 7). There was also no tumor formation in mice injected with 4C8 treated with NaPA alone. Therefore it follows that NaPA induced phenotypic reversion of the premalignant fibroblasts and prevented their malignant conversion by the cytosine analog.

Modulation of Gene Expression by NaPA. The NIH 3T3-derived cells lines, 4C8 and PR4, carry an LTR-activated c-Ha-ras protooncogene. Northern blot analysis of 5AzadC-treated 4C8 revealed a significant increase in ras mRNA levels and a decline in the differentiation marker, collagen α (type I) transcripts. No such changes in gene expression occurred in cultures to which NaPA was added. Withdrawal of NaPA after one week of continuous treatment did not cause restoration of ras expression, confirming that the therapeutic benefit of NaPA is stable in the absence of further treatment.

Effect of Phenylacetate and 5AzadC on DNA Methylation. 5AzadC is a potent inhibitor of DNA methylation, an epigenetic mechanism implicated in the control of gene expression and cell phenotype. Hypomethylation may underlay the therapeutic effect of 5AzadC in cancer and in severe inborn anemias [Mompalmer, R. L., G. E. Rivard, and M. Gyger. 1985. Clinical trial on 5-aza-2'-deoxycytidine in patients with acute leukemia. *Pharmac. Ther.*, 30:277–286; Stamatoyannopoulos, J. A., and A. W. Nienhuis. 1992. Therapeutic approaches to hemoglobin switching in treatment of hemoglobinopathies. *Annu. Rev. Med.*, 43:497–521; Ley, T. J., J. DeSimone, N. P. Anagnou, G. H. Keller, R. K. Humphries, P. H. Turner, P. H., N. S. Young, P. Heller, and A. W. Nienhuis. 1982. 5-Azacytidine selectively increases gamma-globin synthesis in a patient with beta⁺ thalassemia. *N. Engl. J. Med.* 307:1469–1475]. However, changes in DNA methylation could also be responsible for its carcinogenic potential. It was of interest therefore to determine the degree of DNA methylation in cells protected by phenylacetate. As would be expected, 5AzadC caused a significant decrease in the content of 5-methylcytosine (5 mC) (Table 6). There was, however, a comparable decline in 5 mC in cells treated with 5AzadC in combination with NaPA, as well as in those treated with NaPA alone (Table 6).

TABLE 6

In vitro Prevention by Phenylacetate of 5AzadC-Induced Carcinogenesis

| Cell | Treatment | Saturation Density ^a (cells/cm ² \times 10 ⁻³) | Invasiveness ^b | Tumorigenicity in Mice ^c | |
|------|---------------|---|---------------------------|-------------------------------------|-----------------|
| | | | | Incidence | Tumor Size (mm) |
| 4C8 | None | 3.9 | – | 3/8 | 1.0 (0.5–2) |
| | 5AzadC | 7.0 | + | 8/8 | 11.5 (4–19) |
| | 5AzadC + NaPA | 1.6 | – | 0/8 | 0 |
| | 5AzadC + NaPB | 1.1 | – | ND | |
| PR4 | NaPA | ND | – | 0/8 | 0 |
| | NaPB | 1.3 | – | ND | |

^aCell were treated simultaneously with the indicated drugs and kept in culture for 5 days post confluency at which time they were detached and counted. Exposure to 5AzadC was transient as described in Materials and Methods, while treatment with NaPA and NaPB continued throughout the experiment. Similar results were obtained when NaPA treatment was initiated one day prior or after cell exposure to 5AzadC (data not shown).

^bCells were plated on top of a matrigel layer and observed for malignant growth pattern, i.e., development of characteristic processes and degradation of the reconstituted basement membrane and invasion towards the plastic surface below.

^cCells pretreated in culture were injected s.c. (5 \times 10⁵ cells per site) into 2 month old female athymic nude mice. Results determined after 3 weeks indicate tumor incidence (tumor bearing, injected animals) and size. The values of tumor size are mean (range).
 ND = not determined.

In Vivo Chemoprevention by NaPA. To determine the efficacy of NaPA in vivo, studies were extended to include an animal model involving athymic mice bearing the non-tumorigenic 4C8 cells transplanted subcutaneously. A single i.p. injection of mice with 5AzadC (20 mg/kg) resulted in

tumor development at the site of 4C8 cell inoculation. However, when mice were pre-treated with NaPA 1.5 hr prior to 5AzadC injection, and NaPA treatment continued for 22 days thereafter, the incidence of tumor formation was significantly decreased (Table 8). There were no adverse effects associated with NaPA treatment as indicated by animal weight and behavior. Further more, despite causing DNA hypomethylation NaPA did not induce neoplastic transformation of transplanted 4C8 cells. Animals protected by NaPA either failed to develop tumors or formed slow-growing lesions at the site of 4C8 inoculation. The animal data is consistent with the in vitro findings, indicating that NaPA can prevent 5AzadC-induced neoplastic transformation without producing significant toxicities.

TABLE 8

| In vivo Chemoprevention by Phenylacetate | | | |
|---|----------------------------------|--|---|
| Group | Animal Treatment ^a | Tumor Incidence ^b positive/total | Tumor Size ^c mean (range) |
| A | PBS | 0/4 | 0 |
| B | NaPA | 0/4 | 0 |
| C | 5AzadC + PBS | 9/9 | 12 (2-29) |
| D | 5AzadC + NaPA | 4/10 | 3 (0-10) |

^a4C8 cells (5×10^5 per site) were transplanted s.c. into athymic mice. The next day, the animals in were treated i.p. with 400 mg/kg NaPA, and 1.5 hr later with 20 mg/kg 5AzadC. NaPA treatment was repeated at 4.5 hours following 5AzadC injection. Subsequent treatments involved NaPA injections twice daily for 8 days, and once a day for additional 2 weeks. PBS was used as a control.

^bData indicates tumor growth at 4 weeks after 5AzadC treatment. Spontaneous tumors developed thereafter in control animals receiving PBS, and subsequently in those treated with NaPA.

^cTumor diameter in millimeters.

There is considerable interest in the use of nontoxic differentiation inducers in cancer chemoprevention. Drug toxicity is particularly important considering the overall health condition and variable life-span of candidate populations, i.e., high-risk individuals and patients in remission. The differentiation inducer phenylacetate can prevent 5AzadC-induced carcinogenesis both in vitro and in vivo when used at nontoxic doses.

Chemoprevention can be accomplished by either blocking the "initiation" step of carcinogenesis (i.e., mutagenesis), or by suppressing "promotion" and progression to malignancy. The current studies, using premalignant cells with an activated ras oncogene as a model, examined the efficacy of phenylacetate as an anti-promotional drug. Other well characterized chemopreventive agents that block promotion include vitamin A and its synthetic retinoids; like phenylacetate, these compounds are also regulators of cell growth and differentiation.

The current studies exploited in vitro and in vivo models involving fibroblasts (designated 4C8 and PR4) that are highly vulnerable to malignant conversion by the DNA hypomethylating agents 5AzadC and 5AzaC (16,17). Transient exposure of these cells to 5AzadC, either in culture or in recipient athymic mice, caused rapid neoplastic transformation. Malignant conversion was associated with an increase in ras mRNA levels and down-regulation of collagen type I expression, indicating loss of cell differentiation. These profound biological and molecular changes brought about by 5AzadC are prevented by a simultaneous treatment with non-cytotoxic concentrations of phenylacetate and its precursor, phenylbutyrate. Phenylacetate's anti-tumor activity and lack of toxicity were confirmed in athymic mice. In the in vivo model, mice bearing the susceptible 4C8 cells transplanted s.c. were injected i.p. with 5AzadC.

All mice so treated developed rapidly growing fibrosarcomas; however, the incidence of tumor formation was markedly reduced by systemic treatment with NaPA.

The mechanism by which NaPA prevented the 5AzadC induced malignant conversion is unclear. Like other chemopreventive agents that block promotion, phenylacetate may act by inducing cytostasis and tumor maturation. There is a growing body of evidence indicating that phenylacetate can cause selective growth arrest and tumor differentiation in vitro and in rodent models. In some cases, e.g., promyelocytic leukemia, differentiation induced by phenylacetate was linked to a decline in myc oncogene expression. In NaPA-treated 4C8, protection from de-differentiation (evidenced by growth characteristics and collagen expression), was associated with inhibition of ras overexpression. Down-regulation of oncogene expression may thus be responsible in part for the chemopreventive activity of NaPA. In addition to affecting ras at the mRNA levels, phenylacetate, an inhibitor of the mevalonate pathway of cholesterol synthesis [Castillo, M., J. Iglesias, M.F. Zafra, and E. Garcia-Peregrin. 1991. Inhibition of chick brain cholesterolgenic enzymes by phenyl and phenolic derivatives of phenylalanine. *Neurochem. Int.*, 18: 171-174], could also block the post-translational modification of the ras-encoded protein, p21. Limonene, an inhibitor of p21 prenylation, is a chemopreventive agent as well.

Phenylacetate blocked carcinogenesis by 5AzadC despite the decline in 5mC content. In fact, NaPA itself was found to inhibit DNA methylation; yet, in contrast to 5AzadC, NaPA was not carcinogenic. Correlations between carcinogenic potential and DNA hypomethylating activities of chemical agents have been previously documented in tissue culture models, and alterations in DNA 5mC patterns were proposed to contribute and enhance the initiation of carcinogenesis. However, the present data indicate that quantitative changes in DNA methylation alone are not sufficient to affect cell phenotype and thus, hypomethylating activity is not sufficient to induce the tumorigenic phenotype in these in vitro and animal models.

The selective induction of specific genes by intracellular factors and chemical agents subsequent to demethylation has been reported by several laboratories. For example, an increase in human gamma-globin gene expression in vitro was found to require activation by hexamethylenebisacetamide following treatment with 5AzaC [Ley J. T., Y. L. Chiang, D. Haidaris, N. P. Anagnou V. L. Wilson, and W. F. Anderson. 1984. DNA methylation and regulation of the human β -globin like genes in mouse erythroleukemia cells containing human chromosome 11. *Proc. Natl. Acad. Sci. USA.* 81: 6618-6622]; demethylation of the gene by 5AzaC was not sufficient for gene expression. By contrast, phenylacetate and phenylbutyrate induced gamma-globin gene expression with subsequent accumulation of fetal hemoglobin in cultured erythroid progenitors and in humans. In addition to affecting DNA methylation, NaPA and NaPB also activate a nuclear receptor that functions as a transcriptional factor (the peroxisome proliferator receptor is discussed herein). Thus, one possible explanation for the differences in carcinogenic opposing activities between NaPA/NaPB and 5AzadC seen here may be the ability of the aromatic fatty acids to induce the expression of genes critical to growth control. Phenylacetate and related compounds can possibly reverse the methylation-mediated state of repression of silent anti-oncogenes. The finding of DNA hypomethylation by NaPA in mammalian cells does not come as a surprise in view of previous studies demonstrating that, at millimolar concentrations, phenylacetate inhibits

DNA methylation in plant. Interestingly, at such high concentrations, phenylacetate also inhibits plant tumor cell proliferation. Therefore, the effect of phenylacetate on DNA methylation and its role in regulating growth and differentiation have been conserved in evolution.

The outcome of combining NaPA with 5AzadC (or 5AzaC) is of particular interest. The cytosine analogs have been shown to benefit patients with severe blood disorders such as leukemia, sickle cell anemia, and β -thalassemia. There is now experimental data suggesting that 5AzadC may be active also in some solid tumors, including malignant melanoma (Weber et al, submitted) and prostate carcinoma. Unfortunately, the clinical application of 5AzadC has been limited by concerns regarding carcinogenesis. The data indicate that NaPA can minimize the carcinogenic risk, while both preserving and potentiating the therapeutic effects of 5AzadC. Studies with human leukemic cells and with erythroid progenitors derived from patients with β -hemoglobinopathies revealed that NaPA can enhance the efficacy of 5AzadC, causing superinduction fetal hemoglobin production. Moreover, the addition of NaPA/NaPB to non-toxic, yet sub-optimal concentrations of 5AzadC, induced complete growth arrest and promoted apoptosis in cultured hormone-refractory prostatic carcinoma cells (unpublished data).

It appears therefore that phenylacetate, a common amino acid derivative, may be of value as an antitumor and chemopreventive agent. NaPA, which has an unpleasant odor, can be substituted by its precursor, NaPB (or a derivative or analog of NaPB), for oral administration. Upon ingestion by humans, phenylbutyrate undergoes β -oxidation to phenylacetate. Like NaPA, NaPB exhibits antitumor and chemopreventive activities in experimental models, and both drugs already proved safe for long-term oral treatment of children with urea cycle disorders. More recent clinical studies involving adults with cancer have confirmed that millimolar plasma levels of phenylacetate and phenylbutyrate can be achieved with no significant adverse effects. NaPB/NaPA will benefit high risk individuals predisposed to cancer development, be applied in combination with other anticancer therapeutics to enhance efficacy and minimize adverse effects, and perhaps be used in maintenance therapy to prevent disease relapse.

EXAMPLE 10

HbF Induction in K562 Cells by NaPA and Derivatives

The K562 erythroleukemia line serves as a model for inherited anemias that are associated with a genetic defect in the beta globin gene leading to severe β -chain hemoglobinopathies.

The results reported in Table 9 also show that there is a synergistic affect when leukemia cells are exposed NaPA in combination with interferon alpha, a known biological response modifier or with the chemotherapeutic drug hydroxyurea (HU).

TABLE 9

| Induction of Hemoglobin Synthesis in Erythroleukemia K562 cells* | | | |
|---|-----------|--------------------------|----------------------------|
| TREATMENT | BENZIDENE | POSITIVE CELLS (%) | CELL VIABIL- ITY (%) |
| Control | | 1.8 | >95 |
| NaPA | | | |
| 0.8 mg/ml | | 6.0 | |
| 1.6 mg/ml | | 17.1 | |
| Interferon 500 IU/ml | | 13.5 | |
| HU 100 uM | | 17.2 | |
| NaPA (0.8 mg/ml) + HU or IFN | | 40-42 | |

*Results at seven days of treatment.

Analysis of gene transcripts showed accumulation of mRNA coding for gamma globin, the fetal form of globin. This was confirmed at the protein level.

Using the erythroleukemia K562 cell line described above it was found that 4-hydroxyphenylacetate was as effective as NaPA in inducing fetal hemoglobin accumulation, but was less inhibitory to cell proliferation. In contrast, some other analogs such as 2,4- or 3,5-dihydroxyphenylacetate were found to be highly toxic.

EXAMPLE 11

PC3 and DU145 Cells—NaPA as an Antitumor Agent

The effectiveness of NaPA as an antitumor agent was further evaluated in a variety of experimental models. Studies in depth were performed with two androgen-independent human prostate adenocarcinoma cell lines, PC3 and DU145, established from bone and brain metastases, respectively, as well as hormone responsive LNCaP cultures. NaPA treatment of the prostatic cells resulted in concentration-dependent growth arrest, accompanied by cellular swelling and accumulation of lipid that stained positive with Oil-Red O. The results of this study are shown in FIG. 11. As illustrated therein, an IC_{50} for NaPA occurred at 600–800 μ g/ml. Significantly higher doses were needed to affect the growth of actively replicating normal human FS4 skin fibroblasts or normal endothelial cells (IC_{50} from 12–15 μ M), indicating a selective cytostatic effect of the drug.

EXAMPLE 12

PC3 Cells—Non-invasiveness after NaPA Treatment

It is known that PC3 cells are invasive in vitro and metastatic in recipient athymic mice. [Albini, A. et al. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res. 47: 3239–3245 (1987)]. The invasiveness of PC3 cells which is indicative of their malignant phenotype can be assessed by their ability to degrade and cross tissue barriers such as matrigel, a reconstituted basement membrane. Untreated PC3 cells and PC3 cells treated with NaPA for 4 days in culture were quantitatively analyzed in a modified Boyden chamber containing a matrigel-coated filter with FS4 conditioned medium as a chemoattractant. After 4 days of treatment with 800 μ g/ml of NaPA in T.C. plastic dishes, 5×10^4 cells were replated onto 16 mm dishes

(Costar, Cambridge, Mass.) coated with 250 μ l of matrigel 10 mg/ml. Controls showed the characteristic growth pattern of untreated cells, i.e., formation of net-like structures composed of actively replicating cells which eventually degraded the matrigel and formed monolayers on the plastic surface beneath. In contrast to the controls, the NaPA treated cells formed isolated small colonies which resembled normal human FS4 cells as shown in 8 days after plating. The NaPA treated cells failed to degrade the matrigel barrier. The formation of small noninvasive colonies on top of the matrigel is indicative of loss of malignant properties following treatment. Results of the in vitro invasion assays correlate highly with the biological behavior of cells in vivo.

EXAMPLE 13

PC3 Cells—PAG Treatment did not Hinder Invasiveness

PC3 cells treated with NaPA for one week in culture, in contrast to untreated cells or those treated with PAG, failed to form tumors when transplanted s.c. into athymic mice. These results are shown in Table 10.

TABLE 10

| Tumorigenicity of Prostatic PC3 Cells in Nude Mice | | | |
|--|-----------|--------------------------|------------------------|
| TREATMENT (mg/ml) | Incidence | Diameter (mm \pm S.D.) | Weight (mg \pm S.D.) |
| None | 7/7 | 9 \pm 3 | 285 \pm 60 |
| NaPA 0.8 | 1/7 | 2 | 50 |
| PAG 0.8 | 3/4 | 8 \pm 2 | 245 \pm 35 |

PC3 cells were pretreated for 1 week in culture and then injected (2×10^5 cells/animal) s.c. into 4–5 week-old female athymic nude mice. The results in Table 10 indicate the incidence of tumor bearing animals/injected animals as well as tumor size measured as mean diameter \pm S.D. 8 weeks later.

EXAMPLE 14

Phenylacetate in Combination with Suramin

To further substantiate the phenotypic changes observed in the NaPA treated prostatic PC3 cells, Northern blot analysis revealed that NaPA inhibited the expression of collagenase type IV, one of the major metalloproteases implicated in degradation of basement membrane components, tumor cell invasion, and metastasis. Furthermore, it was found that NaPA treated prostatic PC3 cells showed an increase in the level of HLA-A mRNA which codes for major histocompatibility class I antigen known to affect tumor immunogenicity in vivo.

The malignant prostatic cell lines exhibit numerous abnormalities in gene expression, including increased production of autocrine tumor growth factor- β (TGF- β) and elevated activity of urokinase plasminogen activator (uPA). Members of the TGF- β family have been implicated in tumor growth control, angiogenesis, and immunosuppression. uPA, in contrast, is a serine protease involved in degradation of extracellular stroma and basal lamina structures, with the potential to facilitate tumor invasion and metastasis. It was of interest, therefore, to examine the effect of NaPA on TGF- β and uPA expression in the prostatic tumor cells. Northern blot analysis of PC3 after 72 h treatment

revealed a decrease in TGF- β 2 mRNA levels; the effect was specific for TGF- β 2 as there was no change in the expression of TGF- β 1. The decrease in TGF- β 2 was accompanied by approximately a twofold increase in the levels of HLA-A3 mRNA, as previously observed in treated human leukemic HL-60 cells.

Preliminary analysis of uPA transcript levels showed no significant change after NaPA treatment. There was, however, a reduction cell-surface uPA activity. The hormone-refractory malignant PC3 and DU145 cells, but not the more indolent hormone-responsive LNCaP, displayed high cell-bound uPA activity. Because the parental PC3 cultures are composed of highly heterogeneous cell populations with respect to uPA production, more homogeneous subclones were established by limiting dilutions and single-cell cloning. A subclone designated PC3-1, which resembled the parental PC3 cells in its invasive capacity and surface-localized uPA activity ($2.2 \pm 0.3 \times 10^{-6}$ Plau units per cell), was chosen for further studies. After 3 d of treatment of PC3-1 with NaPA 5 mM there was over 50% reduction in cell-associated uPA activity; the effect was dose-dependent and reversible upon cessation of treatment. Similar results were obtained with DU145 cells. Assay specificity was confirmed by the fact that pretreatment of cells with neutralizing anti-human uPA monoclonal antibodies, or addition of antibodies at the time of assay, blocked over 95% of the plasminogen-dependent proteolytic activity. Plasminogen-independent proteolysis constituted 30% of the maximal fibronectin degrading activity, and was similar for both NaPA-treated cells and untreated controls.

NaPA in Combination with Suramin

TABLE 11

| Malignant Melanoma A375 | | | |
|-------------------------|-----------------|-----------------------|---------------|
| Treatment (μ g/ml) | | Growth (% of control) | Viability (%) |
| None | | 100 | >95 |
| NaPA 400 | | 63.3 | >95 |
| Suramin | 38 | 78.3 | >95 |
| | 75 | 56.8 | >95 |
| | 150 | 38.6 | 92 |
| | 300 | 26.6 | 82 |
| NaPA (400) | + Suramin (38) | 45.5 | >95 |
| | + Suramin (75) | 30.1 | 94 |
| | + Suramin (150) | 21.8 | 92 |

TABLE 12

| Prostate Adenocarcinoma PC3 | | | |
|-----------------------------|-----------------|-----------------------|---------------|
| Treatment (μ g/ml) | | Growth (% of control) | Viability (%) |
| None | | 100 | >95 |
| NaPA 800 | | 59.6 | >95 |
| Suramin | 75 | 58.5 | nd |
| | 150 | 46.5 | nd |
| | 300 | 31.0 | nd |
| NaPA (800) | + Suramin (75) | 24.2 | 90 |
| | + Suramin (150) | 10.9 | 64 |

NaPA was found to significantly potentiate the therapeutic effect of suramin, the only experimental drug known to be active against prostate cancer.

However, drug toxicities have been a major concern. In agreement with previous in vitro studies, we found that toxic doses of suramin (300 μ g/ml) were needed in order to achieve over 50% inhibition of prostatic DU145 cell growth.

This cellular model was used to examine whether NaPA could enhance the activity of suboptimal but less toxic doses of suramin. Results of this examination show that NaPA and suramin act in an additive manner to inhibit DU145 cell proliferation. Moreover, suramin was found to be significantly more active if added to glutamine-depleted medium. Despite significant differences in tumor sensitivities, there was complete growth arrest when DU145 and PC3 cells were treated for 6 d with both NaPA and suramin in glutamine-depleted medium, under conditions in which each treatment alone had only a partial effect. Similarly, Tables 11 and 12 show the effect of combined NaPA and suramin treatment of malignant melanoma A375 cells and prostate adenocarcinoma PC3 cells.

It is known that a disease state characterized by the presence of benign hyperplastic lesions of the prostate exists as a separate disease entity and has been identified in many patients that progress to a diagnosis of prostatic cancer. Based on the above, it is anticipated that NaPA, in addition to being effective in the treatment of prostatic cancer, would be effective in treating patients having benign hyperplastic prostatic lesions.

Further experiments demonstrated that NaPA appears to have broad antitumor activity affecting a wide spectrum of malignancies. The experimental data presented in Table 13 indicate that NaPA 0.4–0.8 mg/ml caused about 50% inhibition of growth in treated adenocarcinoma of the prostate cell lines PC3 and DU145, melanoma A375 and SK MEL 28, lung adenocarcinoma H596 and H661, and astrocytoma U87, U373, and 343. Somewhat higher concentrations (1.0–1.5 mg/ml) were needed to cause a similar inhibition of squamous cell carcinoma A431, breast tumor MCS-7, osteosarcoma KRIB, and fibrosarcoma V7T. Typically, NaPA treatment was associated with growth arrest, induction of differentiation markers, reduced invasiveness in vitro, and loss of tumorigenicity in nude mice.

TABLE 13

| Responses of Different Tumor Cell Lines to NaPA Treatment | | |
|--|---------------------------|--|
| # | Tumor Cell Line | % Inhibition by NaPA 0.8 mg/ml ^a |
| 1 | Melanoma | |
| | A375 | ≥70 |
| | SK MEL 28 | >50 |
| 2 | Prostatic Ca ^b | |
| | PC3 | ≥50 |
| | DU145 | ≥50 |
| | LaNCoP | >50 |
| 3 | Astrocytoma | |
| | U87 | ≥50 |
| | U343 | ≥50 |
| | U373 | ≥50 |
| | Kaposi's Sarcoma | ≥40 |
| 4 | KS | |
| | Leukemia HL-60 | ≤40 |
| | Leukemia K562 | ≤30 |
| | Breast Cancer | ≤30 |
| 8 | MCF-7 | |
| | Osteosarcoma | |
| | KRIB | ≤30 |
| | HOS | <20 |
| 9 | Fibrosarcoma | |
| | V7T | ≤30 |
| | RS485 | ≤30 |

TABLE 13-continued

| Responses of Different Tumor Cell Lines to NaPA Treatment | | |
|--|---|--|
| # | Tumor Cell Line | % Inhibition by NaPA 0.8 mg/ml ^a |
| 10 | Squamous Cancer Of Head & Neck A431 | <30 |

^aPharmacologically attainable concentration
^bCarcinoma

Of major interest in Table 13 are the following:

#1–3 Tumor cells show significant response i.e., ≥50% inhibition of proliferation within one week of treatment. Cf. FIG. 15.

#4 KS, an HIV-associated disorder, may be more dramatically affected by NaPA in humans, due to inhibition of HIV expression and of essential growth factors released by infected lymphocytes.

#5,6 The treated HL-60 promyelocytic leukemic cells undergo terminal differentiation, a desirable outcome of chemotherapy. In the K562 erythroleukemia, NaPA induced reversible erythroid differentiation with no significant growth arrest (<30%); thus the K562 data is of interest with respect to treatment of certain anemias, not cancer.

Less attractive:

#7–10 For effective responses, the tumors may require much higher drug concentrations if used alone.

Although some of the malignant cell lines seem more sensitive than others, all were significantly more affected by NaPA when compared to normal or benign cells. For example, NaPA inhibited the growth of malignant osteosarcoma (KRIB) cells more so than benign osteosarcoma-derived HOS cells. A differential effect was seen also in ras-transformed fibrosarcoma V7T, when compared to the parental non-tumorigenic NIH 3T3 cells. As to normal human cells, as much as 2–4 mg/ml of NaPA were needed to cause a significant inhibition of growth to primary human skin FS4 fibroblasts. It should be noted that the treatment was not toxic to either the malignant or the normal cells.

The concentration range found to selectively suppress malignant growth can be readily obtained in the clinical setting without causing significant side effects. Intravenous infusion of NaPA into humans at 250–500 mg/kg/day which results in plasma levels of 600–800 µg/ml has been found to be a well tolerated treatment. Cytotoxicity in tissue culture was observed when the NaPA concentration was as high as 3 mg/ml or higher.

EXAMPLE 15

Phase I Clinical Trials

Patient Population. Patients were eligible for this study if they had advanced solid tumors for which conventional therapy had been ineffective, a Karnofsky performance status greater than 60%, normal hepatic transaminases and total bilirubin, a serum creatinine less than 1.5 mg/dl, and normal leukocyte and platelet counts. All patients signed an informed consent document that had been approved by the National Cancer Institute (NCI) Clinical Research Subpanel. Seventeen patients, 16 men and 1 woman, with a median age of 57 years (range: 36–75) were enrolled between January and June 1993. Disease distribution included progressive, metastatic, hormone-refractory prostate cancer (9 patients),

anaplastic astrocytoma or glioblastoma multiform (6 patients), ganglioglioma (1 patient) and malignant pleural mesothelioma (1 patient).

Drug Preparation and Administration. Sodium phenylacetate for injection was prepared from bulk sodium phenylacetate powder supplied by Elan Pharmaceutical Research Co. (Gainesville, Ga.). The finished injectable stock solution was manufactured by the Pharmaceutical Development Service, Pharmacy Department, Clinical Center, NIH, in vials containing sodium phenylacetate at a concentration of 500 mg/ml in sterile water for injection, USP, with sodium hydroxide and/or hydrochloric acid added to adjust the pH to approximately 8.5. Doses of sodium phenylacetate to be infused over 30 minutes to 2 hours were prepared in 150 ml of sterile water for injection, USP. Doses of phenylacetate to be given over 24 hours were prepared similarly to yield a total volume of 1,000 ml and were administered using an infusion pump.

The protocol as originally designed delivered an i.v. bolus dose of phenylacetate (150 mg/kg over 2 hours) on the first day of therapy, to allow for the estimation of pharmacokinetic parameters. This was followed 24 hours later by a CIVI of the drug for the next 14 days. Cycles of two week drug infusions were repeated every 6 weeks. The rate of drug infusion was to be increased in sequential cohorts of at least three patients, and individual patients could escalate from one dose level to the next with sequential cycles of therapy provided they had experienced no drug-related toxicity and their disease was stable or improved.

The protocol underwent several modifications over the 6 month period. First, the size of the initial bolus dose was reduced from 150 to 60 mg/kg i.v. and the bolus infusion duration from 2 hours to 30 minutes, after the first three patients were treated. This change resulted in drug concentrations optimal for estimating the drug's pharmacokinetics (vide infra) within a six hour time period. Second, after the non-linear nature of phenylacetate's pharmacokinetics was recognized (vide infra), the protocol was changed from a fixed dose escalation (dose levels 1 and 2: 150 and 250 mg/kg/day, respectively) to a concentration-guided escalation trial (dose levels 3 and 4: 200 and 400 µg/ml, respectively). In the latter format each patient was given an i.v. bolus dose of phenylacetate (60 mg/kg over 30 minutes) one week prior to beginning a 14 day CIVI of the drug. The patient-specific pharmacokinetic parameters estimated from the bolus dose were used to calculate an infusion rate that would maintain the serum phenylacetate concentration at the targeted level during the 14 day infusion. Serum drug concentrations were measured weekly, prompting week-in reestimation of individual pharmacokinetics and dosage adjustment (adaptive control with feedback).

Sampling Schedule. With the initial 150 mg/kg i.v. bolus, blood samples were obtained through a central venous catheter at the following timepoints calculated from the beginning of the infusion: 0, 60, 115, 125, 135, 150, 165, 180, 240, 360, 480, and 600 minutes. For the 60 mg/kg bolus given over 30 minutes, blood sampling was performed at 0, 30, 60, 75, 90, 105, 120, 150, 180, 270 and 390 minutes from the beginning of the infusion. At dose levels 1 and 2, blood samples were obtained daily during the CIVI, while at dose levels 3 and 4, blood samples were obtained on days 1, 2, 3, 8, 9 and 10 of the infusion. Twenty-four hour urine collections for the determination of phenylacetate and phenylacetylglutamine excretion were obtained on days 1, 7 and 14 of therapy. Sampling of the CSF was performed only if clinically indicated.

Determination of sodium phenylacetate and phenylacetylglutamine in serum and urine by high performance liquid

chromatography (HPLC). Blood was drawn by venipuncture into a Vacutainer® tube free of anticoagulant and was then refrigerated. It was centrifuged at 1,200 g for 10 minutes in a Sorvall® RT 6000D centrifuge (DuPont Co., Wilmington, Del.) at 4° C. Serum was then removed and stored in Nunc Cryotubes (Nunc Co., Denmark) at -70° C. until the day of analysis.

A standard curve was generated by adding known amounts of sodium phenylacetate (Elan Pharmaceutical Research Co., Gainesville, Ga.) and phenylacetylglutamine (a gift from Dr. S. W. Brusilow, Johns Hopkins University, Baltimore) to a commercial preparation of pooled serum (Baxter Healthcare Corporation, Deerfield, Ill.). The standard values spanned the expected range of serum concentrations: 0, 5, 10, 20, 50, 100, 250, 500, 750 and 1,500 µg/ml.

Two hundred microliters of serum were pipetted into a 1.7 ml Eppendorf tube (PGC Scientifics, Gaithersburg, Md.). Protein extraction was carried out by adding 100 µl of a 10% (v/v) solution of perchloric acid (Aldrich Chemical Co., Milwaukee, Wis.). The tube was vortexed and then centrifuged at 4,500 g for 10 minutes. One hundred and fifty microliters of supernatant were transferred to a new 1.7 ml Eppendorf tube and 25 µl of 20% KHCO₃ (w/v) was added to neutralize the solution. This was centrifuged at 4,500 g for 10 minutes and 125 µl of supernatant were transferred to an autosampler vial and maintained at 10° C. until HPLC injection. Urine samples were processed in an identical manner after an initial 1:10 dilution with water.

The HPLC system (Gilson Medical Electronics, Middleton, Wis.) was composed of two pumps (305 and 306), an 805 manometric module, an 811C dynamic mixer, a 117 variable wavelength UV detector and a 231 autosampler fitted with a 20 µl injection loop and cooled with a Grey Line model 1200 cooling device. The column was a Waters® (Millipore Corporation, Milford, Mass.) C18 Nova-Pak, 3.9x300 mm, maintained at 60° C. with a Waters® temperature control module. The mobile phase solutions consisted of fifty microliter samples were auto-injected onto a 10 cm cation-ion exchange column integrated into a Beckman Model 6300 Amino Acid Analyzer (Beckman Instruments Inc., Palo Alto, Calif.). The solvent flow rate (2:1 water/ninhydrin) was maintained constant at 0.5 ml/min. Column temperature was raised by 1.5° C. per minute to elute sarcosine, the internal standard. The column was regenerated with lithium hydroxide at 70° C. following each injection. Absorbance was measured at 570 nm and 440 nm following post-column color development with ninhydrin-RX (Beckman Instruments Inc., Palo Alto, Calif.) at 131° C. Beckman System Gold software was used for data acquisition and data management.

Pharmacokinetic Methods. Initial estimates of V_{max} and K_m for phenylacetate were obtained by generating Lineweaver-Burk plots from concentration versus time curves following i.v. bolus doses. These initial parameter estimates were refined by non-linear least squares fitting, using the Nelder-Mead iterative algorithm, as implemented in the Abbottbase® Pharmacokinetic Systems software package (Abbott Laboratories, Abbott Park, Ill., version 1.0).

Statistical Methods. The Student's t-test was used to compare estimates of phenylacetate's pharmacokinetic parameters derived from the Lineweaver-Burk plots with those obtained using non-linear given set of dosing and concentration data was quantified by calculating the weighted sum of the errors squared following non-linear least-squares fitting. The standard deviation of the errors was modeled as a function of drug concentration multiplied by

the coefficient of variation of the assay. Confidence regions for the parameters were derived from the weighted sum of squares in the model incorporating the induction parameters, and approximate significance levels for testing between the two models were calculated using the F distribution [Draper, NR, Smith H. Applied Regression Analysis. New York, John Wiley and Sons, p. 282, 1966]. The significance levels of individual cycles were analyzed by the Spearman rank correlation method in an attempt to discern whether a relationship existed between time-dependent changes in drug clearance and dose.

Analytical Assay. The reverse phase HPLC assay allowed both serum phenylacetate and phenylacetylglutamine concentrations to be determined simultaneously from the same sample (see FIG. 17). The lower limit of detection for both compounds in serum and urine was 5 µg/ml, based upon a signal-to-noise ratio of 5:1. The interassay CV for serum concentrations was less than 6% within the range of 40 to 1,000 µg/ml. (Table 14). The lower limit of detection for glutamine was 0.5 µg/ml, with an interassay CV that did not exceed 7%.

Model Specification and Initial Parameter Estimation. FIG. 13 shows representative concentration versus time curves for simultaneously measured serum levels of phenylacetate and phenylacetylglutamine and plasma levels of glutamine following a 150 mg/kg bolus dose of sodium phenylacetate. The post-infusion decline in serum phenylacetate concentration over time is linear when plotted on a non-logarithmic scale, consistent with saturable elimination kinetics. While useful for demonstrating a zero-order process, the 150 mg/kg bolus was inadequate for parameter estimation insofar as most of the phenylacetate concentrations obtained over the six-hour sampling period were above K_m . In order to generate concentrations both above and below K_m , the bolus was changed to 60 mg/kg i.v. over 30 minutes. Visual inspection of the concentration versus time curves following these boluses revealed no evidence of an initial distributive phase, suggesting that a single compartment, open non-linear model should be adequate to describe the drug's pharmacokinetics. Initial estimates (mean±SD) of K_m (90±30 µg/ml), V_{max} (26.0±10 mg/kg/hr) and Vd (22.4±6.8 L) were calculated in 13 patients using the Lineweaver-Burk equation. Refinement of these initial parameter estimates by non-linear least squares fitting of the entire concentration versus time profile for each bolus dose yielded the following estimates: K_m =105.1±44.5 µg/ml, V_{max} =24.1±5.2 mg/kg/hr and Vd=19.2±3.3 L. The differences between the two methods of estimation were not statistically different, as measured by the Student's t-test (p=0.89).

Induction of Phenylacetate Clearance. In some patients treated at dose levels 1 and 2, we observed a tendency for the serum phenylacetate concentration to decrease with time. An example of this phenomenon is shown in FIG. 14. Considering the 12 cycles of therapy delivered at these levels, a comparison of the serum drug concentration measured on day 2 of CIVI to that observed on day 11 demonstrated a statistically significant decline in concentration with time (Wilcoxon signed rank test, p=0.016). At dose levels 3 and 4, attempts at maintaining targeted serum phenylacetate concentrations using adaptive control with feedback led to variable rates of drug infusion over time, which precluded a simple comparison of drug concentrations at the beginning and end of therapy.

Therefore all cycles of therapy were analyzed at all four dose levels and compared with the performance of the single compartment non-linear model described above with the same model modified to allow V_{max} to increase with time. The formula used to describe this increase was:

$$V_{max,t}(1)=V_{max,\infty}\times\{1.0+[(IF-1.0)\times(1.0-e^{-IR\times t})]\}$$

wherein t is the time elapsed (in hours) since the initiation of therapy, IF is an induction factor representing the maximum-fold increase in V_{max} at infinite time and IR is a first order rate constant (h^{-1}) describing the rate at which V_{max} increases over time. Each cycle of therapy ($n=21$) was evaluated by comparing the difference in the weighted sum of errors squared generated by non-linear least-squares-fitting with each model. The significance of the difference was evaluated using the F test (see statistical methods). In 9 of the 21 cycles, allowing V_{max} to increase with time yielded an improved fit (induction parameters, mean±SD: $IF=1.87\pm0.37$, $IR=0.0028\pm0.003 h^{-1}$, $p\leq0.035$). The Spearman rank correlation method did not demonstrate a correlation between rate of drug administration and the need to incorporate the two induction parameters into the model (rank correlation coefficient=0.39, $p=0.084$). The dose rates administered ranged from 450 to 1,850 mg/h.

Review of concomitantly administered medications revealed no association between specific drugs and the occurrence of a time-dependent increase in phenylacetate clearance. In seven patients with primary CNS tumors, treatment with anticonvulsants always antedated the administration of phenylacetate by months to years.

Mechanisms of Phenylacetate Clearance. As shown in FIG. 13, phenylacetate underwent rapid conversion to phenylacetylglutamine in the three patients who received 150 mg/kg of phenylacetate over 2 hours, the peak serum concentration of phenylacetylglutamine (mean±SD) was 224±81 µg/ml, 325±72 minutes post-infusion. After 60 mg/kg boluses, the peak serum phenylacetylglutamine concentration was 104±33 µg/ml at 86±33 min. The plasma glutamine concentration prior to bolus treatment with phenylacetate was 105±29 µg/ml (mean±SD, $n=16$), similar to reported values in the literature for normal volunteers. The largest reduction in circulating plasma glutamine levels (46%) was observed in a patient receiving a 150 mg/kg bolus.

The molar excretion of phenylacetylglutamine was determined from 24 hour urine collections. It accounted for 99±23% ($n=18$) of the dose of phenylacetate administered over the same period of time. The recovery of the free, non-metabolized drug was only 1.5±2.4% of the total administered dose. A strong phenylacetate odor was detectable on patients' clothes and on examiners' hands after physical examination. This suggests that phenylacetate may also be excreted to some extent transdermally.

Distribution of Phenylacetate and Phenylacetylglutamine into the CSF. Clinical circumstances required evaluation of the cerebrospinal fluid in two patients who had metastatic prostate cancer and were free of CNS metastases. The first had reached steady-state phenylacetate and phenylacetylglutamine concentrations of 141 and 199 µg/ml, respectively, the corresponding simultaneous CSF concentrations were 74 and 5 µg/ml, respectively. At the time of simultaneous serum and CSF sampling, the second patient had been off therapy for 6 hours after having reached a serum concentration of phenylacetate of 1044 µg/ml. Measurements in serum and CSF were 781 versus 863 µg/ml for phenylacetate and 374 versus 46 µg/ml for phenylacetylglutamine, respectively.

Clinical Toxicities. No toxicity was associated with bolus administration of the drug. The highest peak serum concentrations were measured after the 150 mg/kg bolus over 2 hours (533±94 µg/ml, mean±SD). Table 15 lists the average serum phenylacetate concentrations per dose level. Although those achieved at dose levels 3 and 4 are close to their target,

the large standard deviations reflect our inability to maintain serum phenylacetate concentrations within the desired range, even when using adaptive control with feedback.

Drug-related toxicity was clearly related to the serum phenylacetate concentration. Three episodes of CNS toxicity, limited to confusion and lethargy and often preceded by emesis, occurred in patients treated at dose levels 3 and 4. They were associated with drug concentrations of 906, 1044 and 1285 $\mu\text{g/ml}$ (mean: 950 ± 300 $\mu\text{g/ml}$), respectively. Symptoms were completely resolved within 18 hours of terminating the drug infusion in all instances,

Antitumor Activity. Stabilization of PSA for more than 2 months was noted in 3 of the 9 patients with prostate cancer treated at dose levels 2, 3 and 4 (mean phenylacetate concentration: 234 ± 175 $\mu\text{g/ml}$). A fourth patient experienced marked improvement in bone pain and was able to substitute a non-steroidal anti-inflammatory drug to his morphine regimen. One patient with glioblastoma multiform has had improvement in performance status (30% on Karnofsky's scale), intellectual function and expressive aphasia of greater than 5 months duration. Although no change in the size of the tumor mass was noted, reduction in peritumoral edema was documented by MRI.

Discussion. Previous descriptions of the pharmacokinetics of phenylacetate have been fragmentary. Simell et al. reported the drug to have first order elimination kinetics with a half-life of 4.2 hours following bolus dose administration 9270 mg/kg) in children [Simell, O, Sipila, I, Rajantie, J, Valle, D L, and Brusilow, S W. Waste nitrogen excretion via amino acid acylation: benzoate and phenylacetate in lysinuric Protein intolerance. *Pediatr. Res.*, 20: 1117-1121, 1986]. The failure to recognize the non-linear nature of phenylacetate pharmacokinetics probably resulted from the smaller total doses given to these patients compared to those given in our study. The saturable pharmacokinetics of phenylacetate are consistent with an enzymatic process and our calculations from the 24 hour urinary excretion of phenylacetylglutamine confirm that this is the major route of elimination. Evidence that drug clearance increases with time was derived from the comparison of drug levels on days 2 and 11 of the CIVI, adding another layer of complexity to the pharmacokinetics of phenylacetate. To explain this phenomenon, the potential role of concomitantly administered medications was first considered, but failed to demonstrate any association. Analysis of a the relationship between an increase in drug clearance with time and the rate of drug administration did not reach statistical significance and suffered from the small number of cycles of therapy available for analysis. It should also be noted that, relative to the 14 day period over which it is assessed, V_{max} tended to increase slowly, with an average half-time calculated from the induction rate (IR) of 9.6 days.

As expected for such a small molecule, phenylacetate readily penetrates into the CSF, which may explain the dose-limiting side-effects of the drug, i.e., nausea, vomiting, sedation and confusion.

The results of Table 15 indicate that attempting to maintain serum phenylacetate concentrations at either 200 or 400 $\mu\text{g/ml}$ using adaptive control with feedback was problematic, with drug concentrations that often greatly exceed the level-specific targets. All patients who exhibited CNS toxicity had serum phenylacetate concentrations in excess of 900 $\mu\text{g/ml}$. In the average patient, the drug must be infused at a rate equal to 75% of V_{max} in order to maintain a constant serum phenylacetate concentration of 400 $\mu\text{g/ml}$, which is four times greater than K_m . Thus, the slightest error in the estimation of individual pharmacokinetics or in the

rate of drug infusion results in large changes in drug concentration. Phenylacetate was delivered by CIVI in order to mimic the preclinical conditions that had demonstrated antitumor activity, namely, continuous exposure to concentrations above 475 $\mu\text{g/ml}$ for at least two weeks. Unfortunately, such concentrations cannot be practically maintained.

An alternative strategy is to deliver the drug by repeated short infusions. Our limited experience with the 150 mg/kg i.v. boluses suggests that serum phenylacetate concentrations occurring transiently above 500 $\mu\text{g/ml}$ are well tolerated. In addition, the time interval between infusions allows some drug washout to occur, thereby minimizing drug accumulation. A simulated regimen of 200 mg/kg q 12 h (1 hour infusion) is presented in FIG. 16. The simulation assumes that the pharmacokinetic parameters determined from our 17 patients are representative of the cancer population at large and that V_{max} does not change with time. It predicts that a wide range of peak drug concentrations will be present. However, it is possible that these would be sufficiently transient so as not to produce CNS toxicity and the troughs not so prolonged as to abrogate the drug's antitumor activity.

TABLE 14

| PA Standard Curve Assay Variability | | | |
|-------------------------------------|-----------|-----------------------------|-----------|
| PA ($\mu\text{g/ml}$) | CV (%) | PAG ($\mu\text{g/ml}$) | CV (%) |
| 40 | 2.6 | 40 | 4.6 |
| 400 | 1.7 | 400 | 4.3 |
| 1000 | 3.4 | 1000 | 3.1 |

TABLE 15

| PA and PAG Concentrations Per Dose Level During CIVI | | | |
|---|----------------------|---|--|
| Dose Level | PA dose level | PA ^a ($\mu\text{g/ml}$) | PAG ^a ($\mu\text{g/ml}$) |
| 1 | 150 mg/kg/d | 49 ± 19 | 90 ± 34 |
| 2 | 250 mg/kg/d | 104 ± 40 | 150 ± 63 |
| 3 | 200 $\mu\text{g/ml}$ | 178 ± 85 | 188 ± 55 |
| 4 | 400 $\mu\text{g/ml}$ | 397 ± 244 | 306 ± 51 |

^amean \pm SD

EXAMPLE 16

Effect of NaPA on Differentiation of Human Neuroblastoma Cells

The ability of NaPA to promote the differentiation of human neuroblastoma cells was studied, both alone and in combination with retinoic acid (RA), a known inducer of neuroblastoma differentiation and maturation. In the LA-N-5 cell line, phenylacetate stimulated the differentiation of human neuroblastoma cells as evidenced by dose-dependent inhibition of cell proliferation, neurite outgrowth, increase acetylcholinesterase activity, and reduction of N-myc protein levels. Furthermore, NaPA and RA synergized in inducing LA-N-5 differentiation in that combination treatment resulted in complete cessation of cell growth along with morphologic and biochemical changes indicative of the loss of malignant properties. The combined effects represent a strong differentiation response in neuroblastoma cells, both as to number of responding cells and maturational level

achieved. Transient transfection of LA-N-5 cells with a variety of CAT reporter gene plasmids including constructs containing thyroid and RA responsive regulatory elements have suggested that the pathways of action of NaPA and RA may intersect at the nuclear level through activation of common response elements. The synergistic effects, thus, may be mediated by the ability of NaPA to modulate the RA differentiation pathway so as to result in altered transactivation of RA responsive regulatory elements in relevant target genes. These in vitro antineoplastic effects were observed under drug concentrations achievable in humans without significant toxicities.

Section B: Phenylacetate and its Derivatives in the Treatment and Prevention of Aids

The etiology of human acquired immunodeficiency syndrome (AIDS) has been linked to the human immunodeficiency virus (HIV), which is capable of selective infection and suppression of the host immune system. This immune defect renders the human body susceptible to opportunistic infections and cancer development, which are ultimately fatal. The spread of HIV throughout the world is rapid, with no effective therapeutics on hand. It is suggested that NaPA, a nontoxic natural compound capable of glutamine depletion in vivo, can be used in the treatment and prevention of AIDS.

HIV is a retrovirus. The production of retroviruses is dependent on transcriptional activation by the long terminal repeat (LTR) element, and the availability of glutamine (Gln) for translational control. Experimental data obtained with chronically infected cultured cells and animal models indicate that virus replication is specifically inhibited in cells starved for glutamine, but not in those starved for other amino acids (Gloger and Panet (1986); (J. Gen. Virol. 67: 2207-2213) Roberts and McGregor, (1991), (J. Gen. Virol. 72: 2199-305). The results could not be attributed to either an effect on cell cycle or a general inhibition of protein synthesis.

The reason why glutamine depletion leads to virus suppression can be explained as follows. Replication competent murine retroviruses contain an amber termination codon at the junction of gag and pol genes, which can be recognized by amber suppressor tRNA^{Gln}. Glutamine is thus essential for the readthrough of viral mRNA transcripts [Yoshinaka et al. (1985); PNAS 82: 1618-1622]. Reduction in glutamine concentrations disrupts viral mRNA translational readthrough and protein synthesis, with subsequent inhibition of viral assembly and secondary spread. Although human retroviruses are somewhat different from the murine viruses studied, it has been shown that reduction in the levels of amber suppressor tRNA^{Gln} in human cells infected with HIV causes a significant reduction in the synthesis of viral proteins [Muller et al. Air Research and Human Retroviruses 4: 279-286 (1988)]. Such data suggest that agents which can lower glutamine levels in humans are likely to benefit patients infected with HIV. NaPA may be such an agent, since it is known to conjugate to glutamine in humans with subsequent renewed excretion of phenylacetylglutamine. Since NaPA also possesses antitumor activities, the drug is likely to affect Kaposi's sarcomas, the tumors found in as many as 30% of all AIDS patients, as well as lymphomas associated with AIDS.

EXAMPLE 17

NaPA for Treatment of AIDS Related Disorders

Evidence from experimental model systems in support of the above hypotheses includes:

(a) Preliminary findings with cultured cells indicate that NaPA can inhibit expression of genes controlled by the retroviral LTR; (b) While animal studies have been hindered by the fact that glutamine depletion by NaPA is limited to humans and higher primates, an acceptable animal model (other than primates) involves rodents treated with glutaminase. The expression of retroviral genes is under the control of the long terminal repeat (LTR) element, inhibition of LTR would prevent transcription and synthesis of viral proteins. To examine the effect of NaPA on the retroviral LTR, V7T fibrosarcoma cells carrying an LTR-dependent Ha-ras oncogene were used as a model. Results of Northern blot analysis showed markedly reduced levels of the ras RNA transcription in cells treated with NaPA compared to RNA transcription levels in untreated control cells. The results cannot be explained by a general effect on gene expression, as indicated by the increased expression of the cellular genes collagen and 2'-5' oligo adenylate synthetase (2-5 ASyn). The latter are of particular interest since collagen is a marker of fibroblast differentiation, and 2-5 ASyn is associated with growth control. Taken together, the data indicated the NaPA suppressed the activity of the retroviral LTR, while restoring growth control and differentiation to the host cells. Similarly desirable changes might occur in HIV-infected monocytes and T4 lymphocytes following systematic treatment of afflicted patients with NaPA. Glutaminase is a bacterial enzyme that causes reduction of extracellular (and presumably intracellular) glutamine concentrations. Glutaminase treatment of viremic mice infected with Rouscher murine leukemia virus (RLV) inhibited retroviral replication and the development of splenomegaly, and significantly increased animal survival [Roberts and McGregor J. Gen. Virology 72: 29-305 (1991)]. The efficacy of glutaminase therapy compared favorably with AZT, the drug currently used for treatment of AIDS. The results are of particular interest since the RLV serves as a model in the search for anti-HIV drugs (Ruprecht et al., 1986). Unfortunately, however, glutamine depletion by glutaminase in vivo is only transient due to development of neutralizing antibodies to the enzyme. Once this occurs, viral replication can resume, eventually killing the host. NaPA, unlike the bacterial glutaminase, is a natural component of the human body, and thus is less likely to induce the production of neutralizing antibodies; (c) There is clinical evidence for sustained reduction by NaPA of plasma glutamine concentrations. NaPA is currently being used for treatment of hyperammonemia associated with inborn disorders of urea metabolism. Clinical experience indicates that long-term treatment with NaPA effectively reduces glutamine levels. Such treatment is nontoxic and well tolerated even by newborns. In conclusion, NaPA might benefit patients with HIV infection. NaPA could inhibit viral replication through (among other mechanisms) inhibition of LTR and depletion of glutamine, the amino acid required for appropriate processing of viral proteins. If NaPA proves to have anti-HIV activities in humans, it could be used to prevent disease progression in asymptomatic HIV-positive individuals. The lack of toxicity, easy oral administration and relatively low cost uniquely qualify NaPA as a chemopreventive drug. In fact, the drug is so well tolerated by humans that treatment can start just a few hours after birth. In addition, NaPA could be used (alone or in combination with other drugs) in treatment of AIDS-associated disorders including opportunistic infections, HIV encephalopathy, and neoplasia.

Section C: Induction of Fetal Hemoglobin Synthesis in β -chain Hemoglobinopathy by Phenylacetate and its Derivatives

There is considerable interest in identifying nontoxic therapeutic agents for treatment of severe β -chain hemoglobinopathies. Employing the human leukemic K562 cell line as a model, we have explored the cellular responses to NaPA, an amino acid derivative essentially nontoxic to humans. Treatment of cultures with pharmacologically attainable concentrations of NaPA resulted in time- and dose-dependent inhibition of cell proliferation and caused an increase in hemoglobin production. Molecular analysis revealed accumulation of the fetal form of hemoglobin (HbF), which was associated with elevated steady-state levels of gamma globin mRNA. All NaPA effects reversed upon cessation of treatment. Interestingly, addition of NaPA to other antitumor agents of clinical interest, i.e., 5-azacytidine and hydroxyurea, resulted in superinduction of HbF biosynthesis. The results suggest that NaPA, an agent known to be well tolerated by newborns, could be used alone or in combination with other drugs for long-term treatment of some inborn blood disorders.

The pathophysiology of inherited blood disorders such as sickle cell anemia and severe β -thalassemias is based on genetic abnormalities in the β -globin gene which result in deficient or absent β -globin synthesis. The latter prevents the production of hemoglobin and results in ineffective red blood cell production and circulation. Recent data indicate that pharmacological manipulation of the kinetics of cell growth and differentiation might have a beneficial effect in patients with the β -chain hemoglobinopathies, due to the induction of fetal hemoglobin (HbF) synthesis. To date, several antitumor drugs including 5-azacytidine (5AzaC), 5-aza-2'-deoxycytidine (5AzaCdC), hydroxyurea (HU), vinblastine, and arabinosylcytosine (ara-C) have been shown to increase the production of HbF in experimental models [Dover, *Ann NY Acad. Sci.* 612: 184-190 (1999)]. Moreover, there is clinical evidence for 5AzaC and HU activity in severe β -thalassemia and sickle cell anemia, respectively. However, concerns regarding toxic and potential carcinogenic effects of the prevailing antitumor drugs raise the need to identify safe alternatives for long-term treatment of the inborn nonmalignant diseases. The accumulation of fetal hemoglobin in adults is thought to be due to changes in the kinetics of erythroid differentiation rather than a direct effect on the fetal globin genes. According to this hypothesis, other agents that can induce differentiation would also be expected to affect HbF production. The focus here is on the efficacy of a novel nontoxic differentiating agent, NaPA.

As discussed in Section A, Applicant's laboratory has found that NaPA can also affect the maturation (i.e., differentiated state) of various animal and human cell types. The drug caused growth arrest and reversal of malignant properties in a variety of in vitro tumor models including cell lines established from adenocarcinomas of the prostate and lung, malignant melanomas, and astrocytomas. Moreover, NaPA treatment was associated with adipocyte conversion in premalignant mesenchymal C3H 10T1/2 cells, and granulocyte differentiation in promyelocytic leukemia HL-60 cultures. Studies indicated that NaPA, in contrast to the chemotherapeutic differentiating drugs 5AzaC and 5AzaCdC, may be free of adverse effects such as cytotoxicity and tumor progression.

Indeed, NaPA is well tolerated by humans as indicated by the vast clinical experience with NaPA in the treatment of hyperammonemia in infants with inborn errors of ureagenesis. The clinical experience indicates that acute or long-term treatment with high doses of NaPA is essentially free of adverse effects. The lack of toxicity and the ability to induce cellular differentiation prompted Applicant to examine the effect of NaPA on HbF expression.

K562 Cells—Induction of HbF by Treatment with NaPA

The experimental system involved the human leukemic K562 cells, which carry a nonfunctional β -globin gene, but produce low levels of the fetal gamma globin and of HbF. The K562 cell line was originally established from a patient with chronic myelogenous leukemia in the blast cells transformation, and has since been extensively utilized as a model in studies of erythroid differentiation and regulation of the gamma globin gene expression. Applicant has shown that pharmacologically attainable concentrations of NaPA can promote HbF biosynthesis in the human leukemic cells, and can cause superinduction when combined with the other chemotherapeutic agents of interest, 5AzaC and HU.

Cell Culture and reagents. The human leukemia K562 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco), 50 U/ml penicillin, 50 μ g/ml streptomycin, and 2 mM L-glutamine unless otherwise indicated. The suspension cultures were kept in exponential growth phase by diluting every 3-5 days with fresh medium, and cell viability was determined by trypan blue exclusion. Phenylacetic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid (Sigma, St. Louis, Mo.) and PAG (a gift from L. Trombetta, Houston, Tex.) were dissolved in distilled water, and brought to pH 7.0 by the addition of NaOH, DON, adivicin, 5AzaCdC, 5AzaC, and HU (Sigma) were also dissolved in distilled water. All drug stock solutions were stored in aliquots at -20° C. until used.

Determination of Hemoglobin Production. K562 cells were seeded at 1×10^5 cells/ml and treated with the drugs for four to seven days prior to assay. Qualitative estimation of hemoglobin production was determined by benzidine staining of intact cells in suspension. The hemoglobin concentration within cells was determined by the protein absorption at 414 nm. Briefly, 1×10^7 cells were lysed in 1 ml of lysing buffer (0.12% Tris pH 7.4, 0.8% NaCl, 0.03% Mg-acetate, and 0.5% Np-40), vortexed and incubated on ice for 15 minutes. The lysates were then centrifuged for 15 minutes at 1500 rpm at 4° C., and the absorption of the supernatant monitored between 350 nm and 650 nm using Beckman Du-7 scanning spectrophotometer. The hemoglobin was quantitated using the relationship of 1.0 optical density (OD) at 414 nm corresponding to 0.13 mg/ml hemoglobin as described before.

Northern Blot Analysis and DNA probes. Cytoplasmic RNA was prepared from cultures at logarithmic phase of growth and separated on 1% agarose-formaldehyde gels. Gel electrophoresis, transfer of RNA onto nylon membranes (Schleicher & Schuell), hybridization with radiolabeled DNA probes, and autoradiography (Kodak X-ray film XAR5) were according to established procedures. The probe for gamma globin was a 0.6 Kb EcoRI/HindIII fragment of the human gamma globin gene. Probes were labeled with [32 P]dCTP (New England Nuclear, Boston, Mass.) using random primed DNA labeling kit (Boehringer Mannheim, West Germany).

Analysis of HbF Protein Synthesis. Newly synthesized proteins were labeled with 35 S-methionine and the HbF immunoprecipitated and analyzed as previously described. Briefly, cells (1×10^6 per point in 1 ml) were first subjected to 1 hr starvation in methionine-free medium, then incubated in the presence of 100 uCi/ml of 35 S-methionine for 2 hrs.

The labeled cells were harvested, washed and lysed in a lysing buffer containing 10 mM phosphate buffer pH 7.4, 1% Triton x100, 0.1% SDS, 0.5% deoxycholate, 100 mM NaCl, 0>1% NAN3, 2 mM PMSF, and 10 µg/ml lenpeptin. 1×10⁷ cpm of TCA precipitable count of cytoextract was incubated with rabbit anti-human HbF (Pharmacia) and protein A Sepharose at 4° C., and the immunoprecipitates were separated by electrophoresis on 12% SDS-polyacrylamide gels.

The Effect of NaPA and Analogues on Cell Growth and Differentiation. Treatment of the K562 cultures with NaPA resulted in dose dependent inhibition of cell proliferation, with 1.4 mg/ml causing 50% reduction in cell number after four days of treatment (FIG. 17). No toxicity was observed with doses as high as 2.0 mg/ml. In addition to the cytostatic effect, NaPA also induced erythroid differentiation, as indicated by an increase in the number of benzidine-positive cells (FIG. 17) and confirmed by quantitative analysis of hemoglobin production (Table 16). Similar treatment with PAG, which is the glutamine conjugated form of NaPA, had no significant effect on either cell proliferation or hemoglobin accumulation, suggesting that the changes associated with NaPA treatment are specific and not due to alterations in culture conditions.

The effect of NaPA on cell growth and differentiation could be mimicked by the use of 4-hydroxyphenylacetate (Table 16). This was in marked contrast to the analogues 3,4-dihydroxyphenylacetate and 2,5-dihydroxyphenylacetate, which were highly toxic to the cells (LD50 of 60 and 100 µg/ml, respectively), and did not induce differentiation.

Regulation of Fetal Hemoglobin Production by NaPA. K562 cells normally express low but detectable levels of HbF. Protein analysis employing anti-HbF antibodies revealed significantly increased amounts of HbF in cells treated with NaPA compared to untreated controls; this was associated with elevated steady-state levels of the fetal gamma globin mRNAB). The effect of NaPA on HbF production was time and dose dependent, and apparently reversible upon cessation of treatment.

Glutamine Starvation and HbF Production. NaPA treatment of humans can lead to depletion of circulating glutamine due to conjugation to glutamine and formation of PAG, an enzymatic reaction known to take place in the liver and kidney. The in vivo reduction in plasma glutamine was mimicked in vitro by culturing the K562 cells in the presence of lowered glutamine concentrations. Results presented in Table 17 show, in agreement with previous reports, that glutamine starvation alone can affect the growth rate as well as HbF production in the K562 cells. Addition of NaPA to the glutamine-depleted growth medium further augmented the cytostatic and differentiating effects observed. Therefore, the effect of NaPA on erythroid differentiation and HbF production in humans may be even more dramatic than that observed with the in vitro model, due to depletion of circulating glutamine and a direct effect on the erythroid progenitor cells.

Potential by NaPA of Erythroid Differentiation induced by Other Chemotherapeutic Drugs. There is considerable interest in the use of 5AzaC, 5AzadC and HU for treatment of sickle cell anemia and β-thalassemia; however, the clinical use of these drugs is often limited by unacceptable toxicities. Combination treatments with nontoxic differentiating agents like NaPA could enhance hemoglobin production while minimizing the adverse effects. Therefore the efficacy of various combinations of NaPA with the other drugs of clinical interest was tested. Results, summarized in Table 18, show that addition of NaPA 800 µg/ml, to low

doses of 5AzadC or HU act synergistically to further augment HbF production with no toxic effect to cells. The concentration of HU used in these experiments is comparable to the plasma HU levels measured in sickle cell anemia patients following an oral administration of 25 mg/kg [(Goldberg et al. New England J Med 323: 366–372 (1990)]. As to NaPA, pharmacokinetics studies in children with urea cycle disorders indicate that plasma levels of approximately 800 µg/ml can be obtained by infusion with 300–500 mg/kg/day, a treatment well tolerated even by newborns.

Discussion. Chemotherapeutic agents selected for their low cytotoxic/mutagenic potential can be used for induction of fetal hemoglobin in patients with congenital severe anemias such as sickle cell and β-thalassemia. Drug toxicity is an important consideration in view of overall health condition and the variable life-span of patients with these non-malignant blood disorders. Unfortunately, recombinant human erythropoietin, which has proved to be both nontoxic and effective therapy for anemias associated with chronic renal disease, is apparently ineffective in the treatment of sickle cell anemia. The application of other active drugs such as 5AzadC, HU, vinblastine and ara-C has been hindered by concerns regarding their carcinogenic effects. HU is also difficult to use because of the narrow margin between toxicity and the desired effect on increased HbF production [Dover, et al., *Blood* 67: 735–738 (1986)]. In contrast, NaPA, shown here to affect HbF production, is so well tolerated by humans that treatment can be initiated just a few hours after birth.

Using an in vitro model involving human leukemic K562 cells, it is shown that NaPA can promote the maturation of early erythroid progenitor cells that have an active HbF program. Addition of NaPA to other therapeutic agents currently in clinical use, i.e., 5AzaC, 5AzadC, or HU resulted in superinduction of HbF synthesis. 5AzaC has been shown to be less toxic and more effective than HU in stimulating HbF production. Moreover, 5AzaC, unlike HU, is effective in treatment of both sickle cell anemia and β-thalassemia. Such data are consistent with the interpretation that 5AzaC acts by both perturbation of erythropoiesis and by its effect on DNA methylation. However, while hypomethylation can lead to gene activation and cell differentiation, it can also promote oncogenesis and the evolution of cells with metastatic capabilities. Results obtained with the K562 erythroid progenitor cells indicate that the therapeutic effects of NaPA compare favorably with those of 5AzadC, yet NaPA (unlike the cytosine analog) did not cause tumor progression. Moreover, NaPA was shown to prevent tumor progression induced by 5AzadC.

The data show that NaPA, used alone or in combination with other drugs, is of value in treatment of leukemias and β-chain hemoglobinopathies. In addition to promoting the production of red blood cells expressing HbF through nontoxic mechanisms, NaPA may also minimize the adverse effects of other antitumor drugs currently in clinical use.

TABLE 16

| HbF Accumulation in Treated K562 Cells | | | | |
|--|----------------|-----------|----------------|----------|
| Benzidine | Positive Cells | | HbF production | |
| | | | | |
| Treatment | | fold | | fold |
| (mg/ml) | (%) | increase | (pg/cell) | increase |
| None | 2.2 ± 0.8 | 1 | 0.35 ± 0.06 | 1 |
| NaPA | 0.4 | 2.7 ± 0.2 | 0.49 ± 0.02 | 1.4 |
| | 0.8 | 7.0 ± 0.3 | 1.15 ± 0.20 | 3.3 |

61

TABLE 16-continued

| HbF Accumulation in Treated K562 Cells | | | | |
|--|----------------|---------------|----------------|---------------|
| Benzidine Treatment (mg/ml) | Positive Cells | | HbF production | |
| | (%) | fold increase | (pg/cell) | fold increase |
| 4HP | 1.6 | 14.6 ± 0.2 | 6.6 | 2.40 ± 0.16 |
| PAG | 1.6 | 14.2 ± 0.5 | 6.45 | ND |
| | 2.6 | 2.1 ± 0.5 | 0.95 | 0.37 ± 0.03 |
| | | | | 1.06 |

TABLE 17

| Glutamine Starvation and HbF Production | | |
|---|----------------------|-----------------------|
| HbF (g/cell) | | |
| Gln (mM) | Gln starvation alone | plus NaPA (0.8 mg/ml) |
| 2.0 | 0.39 ± 0.04 | 1.0 ± 0.06 |
| 0.5 | 0.56 ± 0.01 | 1.15 ± 0.01 |
| 0.2 ^a | 1.17 ± 0.12 | 1.75 ± 0.22 |
| 0.1 ^a | 1.86 ± 0.40 | 2.22 ± 0.20 |

^aThe concentration of NaPA used in this study (0.8 mg/ml) is pharmacologically attainable without toxicity. In children such a treatment is expected to cause a drop in circulating glutamine plasma levels to 0.1–0.2 mM. The results presented above indicate that under such conditions HbF production increases 4.5–5.7 fold compared to controls. We propose therefore that the effect of NaPA in children might be more dramatic than that seen under routine culture conditions (i.e., cell growth in medium with 2 mM Gln).

TABLE 18

| Potentiation by NaPA of HU's Therapeutic Effect | |
|---|--------------------------|
| Treatment | HbF (pg/cell) |
| None | 0.39 ± 0.04 |
| NaPA (0.8 mg/ml) | 1.64 ± 0.07 |
| HU (50 uM) | 1.00 ± 0.03 |
| HU (50 uM) + NaPA | 5.91 ± 0.6 ^b |
| HU (100 uM) | 2.12 ± 0.04 |
| HU (100 uM) + NaPA | 6.71 ± 0.05 ^b |

^aTo mimic the effect of NaPA in vivo, treatments involving NaPA were performed in medium supplemented with 0.2 mM Gln (see explanation to Table 17). Control untreated cells and those treated with HU or 5AzadC alone were maintained in growth medium with 2 mM Gln.

^bThe results indicate that NaPA and HU act synergistically to induce HbF Production in the erythroid progenitor cells.

Note: Similar results have been obtained for the combination NaPA 0.8 mg/ml and 5AzadC 0.3 uM.

EXAMPLE 19

HbF Induction in Nonmalignant and Malignant Cells

General ability of NaPA and its derivatives to induce production of HbF. The ability of oral administration of sodium 4-phenylbutyrate to increase fetal hemoglobin production was assayed. To do so, the percentage of red cells containing fetal hemoglobin (F cells) was measured by flow-cytometric single-cell immunofluorescent assays in 15 patients (7 females and 8 males) with hereditary urea-cycle disorders who had received sodium 4-phenylbutyrate therapy for 5 to 65 months. In determining the differences in low levels of fetal hemoglobin in persons without anemia, the measurement of the percentage of F cells is more precise than conventional measurements of fetal hemoglobin as a percentage of total hemoglobin. The mean percentage of F

62

cells was significantly higher in the patients than in normal subjects:

| Patient No. | Age yr | Dose of Phenylbutyrate g/kg/day | F Cells* % |
|----------------------------|--------|---------------------------------|--------------|
| | | | |
| 1 | 29 | 0.30 | 9.4 |
| 2 | 11 | 0.67 | 20.4 |
| 3 | 6 | 0.62 | 0.5 |
| 4 | 5 | 0.48 | 6.5 |
| 5 | 2 | 0.58 | 22.7 |
| 6 | 13 | 0.46 | 7.7 |
| 7 | 2 | 0.38 | 11.8 |
| 8 | 11 | 0.41 | 1.9 |
| 9 | 6 | 0.27 | 1.9 |
| 10 | 5 | 0.62 | 2.3 |
| 11 | 6 | 0.65 | 21.1 |
| 12 | 21 | 0.29 | 1.7 |
| 13 | 3 | 0.47 | 7.6 |
| 14 | 6 | 0.64 | 40.5 |
| 15 | 2 | 0.63 | 29.7 |
| 20 Patients, mean ± SE | | — | 12.4 ± 3.1** |
| Normal subjects, mean ± SE | | — | 3.1 ± 0.2 |

*F cells were measured with a flow-cytometric technique that counts the percentage of F cells in a total of 10,000 red cells. The difference between repeated measurements was less than 10 percent.

**P = 0.005 by the Kolmogorov-Smirnov two-sample test for the comparison of the F-cell values in the 15 patients with urea-cycle disorders and the values in 293 normal adults. The percentage of F cells reaches the range of values found in normal adults at about two years of age.

EXAMPLE 20

In vitro Study of Sick Cell and Beta-thalassemia Responses to NaPA/NaPB

An in vitro study was conducted on cells derived from patients with homozygous sickle cell disease or B-thalassemia who had been admitted to the Clinical Center of the National Institutes of Health (NIH) for routine evaluation, or normal blood donors from the Department of Transfusion Medicine (NIH). Approximately 20–25 ml of blood was obtained for erythroid cell cultures. Diagnosis of SS or B-thal was made on the basis of: (1) hemoglobin electrophoresis on alkaline cellulose acetate and on acid citrate sugar; (2) peripheral blood examination; and occasionally (3) DNA and RNA analysis of bone marrow aspirates. When possible, diagnosis was confirmed by family studies. Routine hematologic profiles were performed on a Coulter Model S.

Peripheral blood mononuclear cells were isolated by centrifugation on a gradient of Ficoll-Hypaque and cultured for 7 days (phase I) in alpha-minimal essential medium supplemented with 10% fetal calf serum (FCS) (both from GIBCO, Grand Island, N.Y.), 1 µg/ml cyclosporin A (Sandoz, Basel, Switzerland) and 10% conditioned medium collected from bladder carcinoma 5637 cultures (Myers C D, Katz F E, Joshi G, Millar J L: A cell line secreting stimulating factors for CFU-GEMM culture. Blood 64: 152, 1984). In phase II, the non-adherent cells were recultured in alpha-medium supplemented with 30% FCS, 1% deionized bovine serum albumin, 1×10⁵M 2-mercaptoethanol, 1.5 mM glutamine (unless otherwise indicated), 1×10⁶M dexamethasone, and 1 U/ml human recombinant Epo (Ortho pharmaceutical Co., Raritan, N.J.). These cultures yielded up to 10⁶ erythroid cells per milliliter of blood. Cell viability was determined by Trypan Blue exclusion. Phenylacetic acid, 4-phenylbutyric acid, phydroxyphenylacetic acid, p-chlo-

rophenylacetic acid, and butyric acid (Sigma, St. Louis, Mo.) were dissolved in distilled water and brought to pH 7.0 by the addition of NaOH. 5-Azacytidine and hydroxyurease was obtained from Sigma, and PAG was obtained from S. Brusilow (Johns Hopkins, Baltimore, Md.).

Differentiation was assessed morphologically by preparing cytocentrifuge slides stained with alkaline benzidine and Giemsa. The number of Hb-containing cells was determined using the benzidine-HCl procedure (Orkin SH, Harosi Fla., Leder P: Differentiation of erythroleukemic cells and their somatic hybrids. *Proc Natl. Acad. Sci USA* 72: 98, 1975). Hbs were characterized and quantitated by cation exchange high performance liquid chromatography (HPLC) of cell lysates as previously described (Huisman TH: Separation of hemoglobins and hemoglobin chains by high performance liquid chromatography. *J Chromatography* 418: 277, 1987). Total Hb in lysates prepared from a known number of Hb-containing (benzidine-positive) cells was measured using either the tetramethylbenzidine procedure (Sigma kit, Catalog No. 527) or by cation exchange HPLC (measuring total area under chromatogram). Standard Hb solutions (Isolab, Inc., Akron, Ohio) were used for reference. Mean cellular Hb (MCH) was calculated by dividing the total Hb content of the lysate by the number of benzidine-positive cells.

Cytoplasmic RNA was separated on 1% agarose-formaldehyde gels. RNA isolation, gel electrophoresis, transfer onto Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.), hybridization with radiolabeled DNA probes, and autoradiography (Kodak X-ray film XAR5) were described [Samid D, Yeh A, Presanna P: Induction of erythroid differentiation and fetal hemoglobin production in human leukemic cells treated with phenylacetate. *Blood*, 80: 1576, 1992]. The human globin cDNA probes included JW101 (alpha), JW102 (beta), and a 0.6 kb EcoRI/HindIII fragment of the 3' end of human G-gamma-globin gene. Probes were labeled with [³²P]dCTP (New England Nuclear, Boston, Mass.) using a random primed DNA labeling kit (Boehringer, Mannheim, Germany).

Results. Addition of NaPA or NaPB to phase II erythroid cultures resulted in reduced cell proliferation with no apparent change in cell viability. Cytostasis was associated with a decline in total Hb produced per culture; however, both Hb content per cell (MCH) and the proportion of HbF (%HbF) increased upon treatment (FIG. 18). The extent of changes observed was dose- and time-dependent: the earlier the drugs were added during the second phase of growth, the higher was the increase in % HbF, however, cell yields were proportionately decreased. For example, addition of 5 mM NaPA to normal precursors on day 2 caused approximately 90% decrease in cell number along with a 12-fold increase in % HbF, a determined on day 13. When treatment was initiated on day 67, cell number decreased on by 60% compared to controls, and % HbF increased 3.3-fold. In order to obtain sufficient cells for further analysis, subsequent experiments involved the addition of drugs on days 6-7, and cells were harvested on day 13. Under these conditions, results were reproduced in cultures derived from 6 normal donors as well as 4 patients with sickle cell anemia and 4 patients with B-thal. NaPA (5 mM) and NaPB (2.5 mM) caused a significant increase in both MCH(38-100%) and the proportion of HbF produced. In the case of homozygous SS patients, % HbF was elevated 2.0-4.1 fold (mean 3.0) by 4 mM NaPA, and 3.2-5.6 fold (mean 4.0) by 2.5 mM NaPB. The latter was associated with a 12±3% decrease in HbS levels, with no change in HbA₂ (FIG. 19).

As in K562 cells, increased HbF production by NaPA or NaPB in primary cultures of normal or SS cells appears to

be due to pre-translational regulation of gamma-globin expression. Northern blot analysis showed dose-dependent increase (up to 5 fold) in the steady-state levels of gamma globin mRNA, accompanied by a slight decrease (less than two fold) in the amounts of beta globin transcripts. There was no change in alpha globin expression.

PAG, the end-metabolite of both NaPB and NaPA, is formed by phenylacetate conjugation to glutamine with subsequent excretion in the urine. PAG was found to be inactive on erythroid proliferation and HbF accumulation. Glutamine starvation of the non-malignant erythroid cells had no effect on either cell growth or HbF production, nor did it enhance the efficacy of NaPA.

The effect of NaPA with other drugs was also assayed. When used alone in cultures derived from normal donors (HbF base levels of 0.8-2.0%), NaPA (5 mM) and hydroxyurease (0.05 mM) increase % HbF by 3.5 and 2.0-fold, respectively; the combination of the two resulted in a 4.7-fold increase in HbF. NaPA also augmented HbF stimulation by butyrate (0.5 mM) (from 3.1 to 7.15-fold), and of 5-Azacytidine (2 uM) (from 2.5 to 6.6-fold). These results indicate that NaPA when added to suboptimal, non-toxic doses of other drugs, can potentiate HbF production with significant cytostasis and no significant change in cell viability.

As exemplified below in Table 20, combination treatment comprising administration of NaPA (or a pharmaceutically acceptable derivative of phenylacetic acid) simultaneously with hemin, a known stimulator of HbF production, synergistically increases the induction of erythroid differentiation, as indicated by the increase in the number of benzidine positive cells, and HbF production. In K562 cells, the range of increase in the production of HbF with this combination treatment varied from 1.5 to 5 times that produced by treatment with 10 mM PA alone. Further, treatment with NaPB in combination with hemin also resulted in classical synergism. Similar results were also obtained with PB in non-malignant erythroid progenitor primary cells. In all cases, treatment with both drugs was maintained for 4-6 days prior to measurement of HbF.

TABLE 20

STIMULATION OF HbF BY NaPA IN COMBINATION
WITH HEMIN - K562 MODEL

| R _x | % Benzidine pos. | Hb pg/cell | Viability |
|-------------------|---------------------|------------|-----------|
| CONTROL | >0.01 | 0.26 | 97 |
| NaPA (10 mM) | 1.6-3.1 | 0.91 | 96 |
| NaPA (10 mM) + H | 25.4-32.6 | 4.03 | 92 |
| NaPA (5 mM) + H | 12.6 | 2.34 | 99 |
| NaPA (2.5 mM) + H | 8.1 | 1.95 | 94 |
| HEMIN (20 uM) | 2.9 | 1.04 | 98 |
| CONTROL | 2.1 | 0.65 | 97 |
| NaPA (2.5 mM) | 2.6 | 0.91 | 97 |
| NaPA (5 mM) | 7.7 | 1.04 | 97 |
| NaPA (10 mM) | 14.3 | nd | 96 |
| HEMIN (20 uM) | 13.8 | 2.34 | nd |
| NaPA (5 mM) + H | 42.3 | 5.2 | 97 |

Section D: Use of Phenylacetic Acid and its Derivatives in Wound Healing

Growth factors, including TGF-α, play a critical role in wound healing and repair processes. Wound healing is a

localized process that involves inflammation, wound cell migration and mitosis, neovascularization, and regeneration of the extracellular matrix. Recent data suggest the action of wound cells may be regulated by local production of peptide growth factors which influence wound cells through autocrine and paracrine mechanisms (Schultz et al., *J. Cell Biochem.* 45(4): 346 (1991); Schultz et al., *Acta Ophthalmol. Suppl. (Copenh)*, 202: 60 (1992)). Two peptide growth factors which may play important roles in normal wound healing in tissues such as skin, cornea, and the gastrointestinal tract are the structurally related epidermal growth factor (EGF) and TGF- α , whose receptors are expressed by many types of cells including skin keratinocytes, fibroblasts, vascular endothelial cells, and epithelial cells of the gastrointestinal tract. EGF or TGF- α is synthesized by several cells involved in wound healing, including platelets, keratinocytes, activated macrophages and corneal epithelial cells. Healing of a variety of wounds in animals and patients, such as epidermal regeneration of partial thickness burns, dermatome wounds, gastroduodenal ulcers and epithelial injuries to the ocular surface, is enhanced by exogenous treatment with EGF or TGF- α . TGF- α , which is a potent inducer of lysyl oxidase mRNA levels in cultures of human scleral fibroblasts, may be primarily responsible for inducing synthesis of extracellular matrix components after an injury. Furthermore, TGF- α is known to promote angiogenesis.

The lack of adequate stimulation of growth factors contributes to the nonhealing conditions of many chronic wounds. Poorly healing conditions could markedly benefit from either addition of exogenous TGF- α or stimulation of effector cells to produce TGF- α and related growth factors. It has now been discovered that PA and PB (or a pharmaceutically acceptable derivative) are capable of stimulating production of TGF- α in cells of melanocytic origin; astrocytic lineage (glioblastoma cells); and several normal human epithelial cell types, including keratinocytes (FIG. 20), which are involved in wound healing. Further, treatment with PA and PB enhances collagen- α type 1 expression. Induction of TGF- α mRNA expression upon treatment with NaPA and NaPB in human melanoma cells was observed; expression of TGF- α was confirmed following protein analysis. FIG. 20 shows the increased production of the TGF- α protein in human keratinocytes upon exposure to NaPA and NaPB. This increased production of TGF- α is maintained for a few days after which the levels return to approximately pretreatment levels. As discussed below and in FIG. 21, further support for the use these compounds in treating wounds may be found in the enhanced expression of ICAM-1, which is a cellular adhesion molecule/surface antigen, following treatment with NaPB.

Thus, the instant invention provides a method for stimulating the production of TGF- α in cells. Further, wound healing in a human or animal can be enhanced by treatment with a therapeutic amount of phenylacetic acid or a derivative of phenylacetic acid such as NaPA or NaPB, which stimulates the in-situ production of TGF- α . For instance, surface wounds can be treated by topically applying PA, PB or a derivative of either PA or PB to the skin surface, such as in a cream formulation. Likewise, ocular injuries can be treated by application of a PA or PB (or PA/PB derivative) formulation, such as eye drops, to the cornea. Similarly, internal injuries, such as injuries to the gastrointestinal tract, can be treated by administration of oral formulations. Vaginal or anal injuries can also be treated, such as with a suppository containing pharmaceutically effective amounts of PAA or a derivative. The PA/PB or derivative formula-

tions can be administered continuously or, preferably, intermittently, such as one or more doses in daily, weekly or monthly courses. For example topical administration once or twice a day of a composition containing from 0.1 to 10 mM PA, preferably 0.1 to 5.0 mM PA or from 0.1 to 5 mM PB, preferably 0.1 to 2.5 mM PB over the course of a week adequately stimulates wound repair. From the information contained herein, dosage concentrations and amounts for the various administration vehicles can be easily determined. For instance, a topical treatment, such as a cream containing PB, typically will contain approximately 0.5 to 3.0 mM PB or an equipotent (by equipotent it is meant that dosage may be varied among the different phenylacetic acid derivatives so as to achieve the equivalent effect on the subject) dose of a phenylacetic acid derivative. For instance, and without limitation, approximately one-half as much PB in a dose is needed to equal the potency of a similarly indicated PA dose. Section E: Use of Phenylacetic Acid or its Derivatives in Treatment of Diseases Associated with Interleukin-6

Interleukin-6 (IL-6), which can be produced by monocytes and keratinocytes upon stimulation, is a pleiotropic cytokine that plays a central role in defense mechanisms, including the immune-response, acute phase reaction and hematopoiesis. Activation of mature B cells can be triggered by antigen in the fluid phase. When antigen binds to cell membrane IgM in the presence of IL-1 and IL-6, mature virgin B cells differentiate and switch isotypes to IgG, IgA or IgE. Abnormal expression of the IL-6 gene has been suggested to be involved in the pathogenesis and/or symptoms of a variety of diseases, including (1) non-malignant disorders associated with abnormal differentiation programs, autoimmunity and inflammatory processes, e.g., rheumatoid arthritis, Castleman's disease, mesangial proliferation, glomerulonephritis, uveitis, sepsis, autoimmune diseases such as lupus, inflammatory bowel, type I diabetes, vasculitis, and several skin disorders of cell differentiation such as psoriasis and hyperkeratosis; (2) viral diseases such as AIDS and associated neoplasms, e.g., Kaposi's Sarcoma and lymphomas; and (3) other neoplasms, e.g., multiple myeloma, renal carcinoma, Lennert's T-cell lymphoma and plasma cell neoplasms. For instance, significantly increased IL-6 mRNA levels in lesional psoriatic tissue relative to normal tissue and elevated amounts of IL-6 in sera and peripheral blood mononuclear cells of psoriatics compared to samples from atopsics or healthy controls have been found (Elder et al., *Arch. Dermatol. Res.*, 284 (6): 324 (1992); Neuner et al., *J. Invest. Dermatol.*, 97 (1): 27 (1991)).

It has now been discovered that phenylacetic acid or a derivative of phenylacetic acid, such as NaPA or NaPB, can inhibit the expression of IL-6. For instance, PA inhibits IL-1-induced IL-6 expression in colon carcinoma cells. This reduction in RNA is confirmed by reduction in IL-6 protein. Thus, PA, PB and their derivatives can be used in the treatment of diseases involved with the abnormal overexpression of IL-6.

For instance, treatment twice daily by topical application of either 2mM NaPB in a mineral oil-based cream or 2 mM naphylacetate and Vitamin B₁ in a mineral oil-based cream directly onto the patient's psoriatic lesions resulted in disappearance of the lesions within a week. Similar treatment of a patient with a severe case of psoriasis resulted in the psoriatic lesions resolving in approximately 1-3 weeks. Obviously, the mode of administration and amount of drug can vary depending upon the IL-6-related disease being treated in order to target the drug to the cells in which reduction of IL-6 expression is desired. For example, injection of a 0.1 mM-5 mM PB solution or an equipotent

solution containing a pharmaceutically acceptable phenylacetic acid derivative into the joint region may be appropriate for treatment for rheumatoid arthritis whereas other diseases may be more appropriately treated by topical, intravenous or oral delivery. Treatment can be by either continuous or discontinuous treatment, but cessation of the drug, particularly PB, may be accomplished by ramping down the dosage amounts to prevent an overreaction to the cessation of treatment with the drug. Additionally, diseases involving the abnormal overexpression of IL-6 can be treated by administration of an effective amount of phenylacetic acid or a phenylacetic acid derivative, particularly PA or PB, in combination with an effective amount of an anti-inflammatory agent, including various vitamins such as vitamin B₁, non-steroidal inflammatory agents and steroidal anti-inflammatory agents. The anti-inflammatory agent can be combined with the phenylacetic acid derivative(s) of this invention in the same dosage form or administered separately by the same or different route as the derivative. An effective amount of the anti-inflammatory agent refers to amounts currently in clinical use for the specific disease state or less.

Section F: Use of Phenylacetic Acid or its Derivatives in the Treatment of Aids-associated CNS Dysfunction

Hallmarks of central nervous system (CNS) disease in AIDS patients are headaches, fever, subtle cognitive changes, abnormal reflexes and ataxia. Dementia and severe sensory and motor dysfunction characterize more severe disease. Autoimmune-like peripheral neuropathies, cerebrovascular disease and brain tumors are also observed. In AIDS dementia, macrophages and microglial cells of the CNS are the predominant cell types infected and producing HIV-1. However, it has been proposed that, rather than direct infection by HIV-1, the CNS disease symptoms are mediated through secretion of viral proteins or viral induction of cytokines that bind to glial cells and neurons, such as IL-1, TNF- α and IL-6 (Merrill et al., *FASEB J.*, 5(10): 1291(1991)). TGF- β is a growth factor which is released by many cell types. Among other effects, TGF- β is highly chemotactic for macrophages and fibroblasts and stimulates the release of TNF- α , TGF- α and, indirectly, a variety of other modulators from macrophages which have been implicated in the initiation of the CNS symptoms of AIDS.

It has now been discovered that phenylacetic acid or a derivative of phenylacetic acid, such as NaPA or NaPB, can inhibit the production of TGF- β 2. Because TGF- β 2 is an immunosuppressive factor, this inhibition results in a general improvement of the patient's immune system. Gene expression of TGF- β 2 in glioblastoma cells was inhibited by both PA and PB. This reduction in RNA leads to reduced TGF- β 2 protein synthesis. Thus, PA, PB or their derivatives can be used to inhibit the production of TGF- β 2 in cells, particularly to control or alleviate the CNS symptoms resulting from HIV infection. As discussed above, this treatment also inhibits the production of IL-6, further allowing for alleviation of the CNS symptoms. Amounts of drug and/or regimens of administration effective for inhibiting TGF- β 2 correspond to those appropriate for treatment or prevention of cancer as given herein, such as in SECTION C.

Section G: Use of Phenylacetic acid and its derivatives to Enhance Immunosurveillance

Immunosurveillance in an animal such as a human can be enhanced by treatment with PA, PB or their derivatives. Tumor cells are thought to escape attack by the immune system by at least two means. First, many tumors secrete immune suppressive factors that directly reduce immune activity. Additionally, some tumor cells do not express, or

have reduced expression of, appropriate surface antigens that allow the immune system to identify outlaw cells. However, the compositions of the instant invention can activate otherwise dormant genes such as fetal globin, perhaps by DNA hypomethylation. Similarly, activation of cancer suppressor genes, dormant antigens and other genes, such as (1) cellular major histocompatibility antigens (MHC Class I and II) or other surface antigens, such as ICAM-1; (2). tumor antigens such as MAGE-1; and (3) viral latent proteins such as EBV's latent membrane protein (which is implicated in numerous diseases such as T-cell neoplasms, Burkitt's lymphoma nasopharyngeal carcinoma, and Hodgkin's disease), may contribute to enhanced immunosurveillance. Thus, neoplastic cells can be treated with PA, PB or their derivatives to provide for expression of cell surface antigens that increase the effectiveness of the immune system by allowing for adequate identification and clearance of the tumor cells by the immune system. Activation of latent viral proteins could also induce a lytic cycle leading to death of the infected cell.

Evidence that the instant phenylacetic acid or phenylacetic acid derivative compositions can activate dormant genes and enhance expression of surface antigens is given by FIG. 21, which shows enhanced expression of MHC Class I, MHC Class II and the adhesion molecule ICAM-1 in melanoma cells that have been treated for 10 days with 2 mM NaPB (e.g., note the shift of the population mean from approximately 50 to 200 for MHC class I).

Furthermore, it has now been discovered that PB induces expression of EBV's latent membrane protein (LMP) in Burkitt's lymphoma cells. Cytoplasmic RNA (20 μ g/lane) was isolated from LandisP, Raji and P3HRI Burkitt's lymphoma cell lines, which had been treated with 2 mM PB for four days, and subjected to Northern blot analysis with a specific LMP probe. In all three cell lines, a positive reaction was observed compared to controls (untreated cells), indicating that PB induces the expression of EBV's latent membrane protein. In Burkitt's lymphoma cells both PA and PB cause additional molecular and cellular changes, including cytostasis, decline in myc expression and enhancement of HLA+1.

Because these surface antigens enhance tumor immunogenicity in vivo, treatment of the animal (human) with PA, PB or their derivatives can enhance the effectiveness of the immune system of the individual. Doses of approximately 0.5–3.0 mM PB or equipotent doses of pharmaceutically acceptable phenylacetic acid derivatives may be useful. This treatment can also be combined with conventional immunotherapy treatments and/or antigen targeted, antibody-mediated chemotherapy. While treatment usually is accomplished by a protocol which allows for substantially continuous treatment, discontinuous or pulsed treatment protocols are also effective, especially for cells capable of terminally differentiating upon treatment with PAA or a PAA derivative. For instance, treatment of the melanoma cells given in FIG. 21 for 10 days was sufficient to allow continued enhanced expression of the surface antigens past this 10 day period.

EXAMPLE 21

NaPA and NaPB Effects on Burkitt's Lymphoma

The Epstein Barr Virus (EBV) infected cell line is growth inhibited by NaPB more than by NaPA. This may be due to a reduction c-myc expression after about four days of treatment. Unfortunately, there is at the present time, no

known measure of differentiation in this cell line. However, treatment with NaPA causes substantial morphological changes and clumping (perhaps due to cell-cell or cell-substrate interaction alterations). The increase in proportion of cells which bind to the extracellular matrix may be due to an increase in ICAM 1. The cell produces more HLA class I antigens and more latent membrane protein which thereby enhances the visibility of the cell to the immune system. Section H: Method of Monitoring the Dosage Level of Phenylacetic Acid or its derivatives in a Patient and/or the Patient Response to These Drugs

As discussed above, administration of phenylacetic acid or a derivative of phenylacetic acid such as NaPA or NaPB to an animal (human) in amounts and over treatment courses as described herein induce a variety of molecular changes. These molecular traits can be used as biomarkers to either (1) monitor the dosage level of the drug or its bioavailability in the animal and/or (2) serve as a biomarker of the patient response to the drug. For instance, as described above, administration of an effective amount of NaPA or NaPB (or their derivatives) results in a variety of molecular effects, including a) increased levels of fetal hemoglobin in erythrocytes; b) increased production of TGF- α in various cells such as those of melanocytic origin, astrocytic lineage or epithelial cell types; c) inhibition of the production of IL-6; and d) inhibition of the production of TGF- β 2. Thus, absolute or relative (before/after treatment) concentrations of a particular biomarker can be determined in an appropriate cell population of the individual to allow monitoring of the dosage level or bioavailability of the drug. Further, this concentration can be correlated or compared with patient responses to develop a patient response scale for a desired treatment goal based upon that biomarker. For instance, the increased amount of fetal hemoglobin can be used to indicate the bioavailability of PA or PB for treatment or prevention of a neoplastic condition as well as indicating the degree of patient response to the drug.

Section I: The activation of PPAR by Phenylacetic Acid and its Derivatives

Peroxisomes are cellular organelles that contain enzymes which control the redox potential of the cell by metabolizing a variety of substrates such as hydrogen peroxide. Recent advances in this area reveal that peroxisomes can be proliferated through activation of a nuclear receptor which regulates the transcription of specific genes (Gibson, *Toxicol. Lett.*, 68 (1-2): 193(1993)). This nuclear receptor has been named the peroxisome proliferator-activated receptor (PPAR) and belongs to the steroid nuclear receptors family that have a major effect on gene expression and cell biology. Binding by peroxisome proliferators such as clofibrate, herbicides, and leukotriene antagonists with PPAR activates the nuclear receptor, which acts as a transcriptional factor, and can cause differentiation, cell growth and proliferation of peroxisomes. Although these agents are thought to play a role in hyperplasia and carcinogenesis as well as altering the enzymatic capability of animal cells, such as rodent cells, these agents appear to have minimal negative effects in human cells, as exemplified by the safety of drugs such as clofibrate (Green, *Biochem. Pharm.* 43 (3): 393 (1992)).

Peroxisome proliferators typically contain a carboxylic functional group. Therefore, PA, PB and various phenylacetic acid derivatives were tested for their ability to activate the PPAR and compared with known peroxisomal proliferators. As shown in FIG. 22, Clofibrate, a known activator of peroxisomal proliferation, caused a 4- to 5-fold increase in activation as measured by increased production of the response element for acyl-CoA oxidase, which is the rate

limiting enzyme in beta-oxidation and is contained in peroxisomes (Dreyer et al., *Biol. Cell*, 77(1): 67 (1993)). PA and PB caused mild activation (double baseline activity), naphthyl acetate was relatively more active (approximately 2.5- to 4-fold increase) while the halogenated analogs of PB were very potent stimulators. Interestingly, butyrate was not a significant peroxisomal proliferation activator.

The peroxisome proliferator-activated receptor has been shown to belong to the same family of nuclear receptors as the retinoid, thyroid and steroid receptors and PPAR is known to interact with RXR, the receptor of 9-cis-retinoic acid (a metabolite of all-trans-retinoic acid). Because the PPAR signaling pathway converges with the 9-cis retinoid receptor signal, it can be anticipated that retinoic acid or the like will significantly enhance the activity of PA or PB or other phenylacetic acid derivatives of this invention. Indeed, enhancement of the induction of HL-60 cell differentiation by NaPA in combination with retinoic acid is discussed above. Additionally, this synergistic response has been confirmed in other tumors, such as neuroblastoma, melanoma and rhabdomyosarcoma cells.

Thus, combination therapy consisting of administration (simultaneously in the same dosage form or simultaneously/sequentially in separate dosage forms) of Vitamin A, Vitamin D, Vitamin C, Vitamin E, B-carotene, or other retinoids and the like with PA, PB or other phenylacetic acid derivatives is encompassed by the instant invention for any of the treatment regimes given herein. Appropriate doses of the phenylacetic acid derivatives include approximately 0.5–10 mM PA, more preferably 0.5–5 mM PA, doses or equipotent doses of a pharmaceutically acceptable phenylacetic acid derivative. Between 0.1 and 1.0 μ M concentrations of the retinoids are expected to be effective. This combination therapy enhances, for instance, the efficacy of treatment with PA, PB or other phenylacetic acid derivatives, taken alone, for cancer, anemia and AIDS treatment, wound healing, and treatment of nonmalignant disorders of differentiation.

Agents affecting cellular peroxisomes have a major impact on oxidative stress and the redox state of a cell. Thus, further evidence that PA, PB or other phenylacetic acid derivatives activate PPAR can be found by the rapid increase of gamma glutamyl transpeptidase and catalase following cellular exposure to PA or PB as shown in FIG. 23. These antioxidant enzymes, whose activities are increased when peroxisome proliferation has been activated, were increased by 100% 24 hours after administration of sodium phenylbutyrate. This effect was reversed by approximately 48 hours and activity was maintained below control levels through 100 hours. The intracellular level of glutathione followed a similar biphasic pattern with an initial increase (20%) followed by a fall to levels below baseline at 100 h. The rapid induction with subsequent sharp decline of these antioxidant enzymes was observed in numerous tumor types from prostatic, breast and colon adenocarcinomas, osteosarcoma, and brain tumors. Molecular analysis showed changes in the rate of gene transcription of the GSH-related and antioxidant enzymes, which are consistent with activation of PPAR by PA, PB or their analogs.

Because peroxisomal enzymes are instrumental in defending against oxidative stress, experiments were undertaken to examine the effects of treatment with PA or PB on cells which were subjected to chemical or radiation stress. Pretreatment of glioblastomas (FIG. 24), breast carcinoma and metastatic prostate cells with a non-toxic dose of PA or PB 72 hours prior to ^{60}Co γ -radiation or treatment with adriamycin demonstrated a significant dose-related increase in cell killing by either modality. The surviving fraction of

cells following drug treatment was nearly one tenth the fraction surviving with no pretreatment, which suggests that PA, PB or other like analogs could be used to increase the efficacy of radiation therapy and chemotherapy substantially. As such, the instant invention encompasses combination anti-cancer therapy consisting of administration of a non-toxic effective amount of phenylacetic acid or a pharmaceutically acceptable phenylacetic acid derivative (according to any of the dosage concentration protocols given herein) in combination with radiation therapy, particularly local treatment, or chemotherapy, particularly targeted to the tumor cells. This adjuvant therapy can be administered, for instance, after approximately 24 hours, such as from 24 hours to 120 hours or more, from the initiation of the administration of the derivative.

Phenyl fatty acids such as PA and its derivatives or analogs have exhibited a wide range of activity in the treatment of glioblastomas and adenocarcinomas by inducing malignant cell differentiation at relatively nontoxic doses. Further, it has been discovered that PA and its derivatives/analogues inhibit the post-translational processing of the ras oncogene-encoded protein which was implicated in the maintenance of tumor cell radioresistance. Thus, PA and its derivatives/analogues may be useful and potent radiosensitizers. The lack of significant toxicity for PA/PB makes them particularly attractive radiosensitizers in comparison to currently used chemical sensitizers.

In vitro data demonstrate that cellular exposure to PA and PB resulted in a marked increase in cellular radiosensitivity in brain, breast, prostate, and colon human tumor cells. This increased radiosensitivity was associated with a decrease in the activity of protective antioxidant enzymes such as catalase and gamma-glutamyl transferase and a concomitant decrease in the naturally occurring radioprotector, glutathione (GSH). Interestingly, highly radioresistant prostate tumor cells that express high levels of the ras oncogene were significantly radiosensitized in comparison to tumor cells that express low levels of ras which were only slightly radiosensitized by PA exposure.

These results suggest a further consideration of a variety of pathogenic disorders. Inflammatory response to tissue injury, pathogenesis of emphysema, ischemia-associated organ injury (shock), doxorubicin-induced cardiac injury, drug-induced hepatotoxicity, atherosclerosis, and hyperoxic lung injuries are each associated with the production of reactive oxygen species and a change in the reductive capacity of the cell. Although long-term exposure to PA or other phenylacetic acid analogs depletes cellular redox protection systems, short term treatment with PA and the like may have significant implications for treatment of disorders associated with increased reactive oxygen species.

Section J: Use of Phenylacetic Acid and its Derivatives in Treatment of Cancers having a Multiple-drug Resistant Phenotype

In treating disseminated cancers, systemic treatment with cytotoxic agents is frequently considered the most effective treatment. However, a number of cancers exhibit the ability to resist the cytotoxic effects of the specific antineoplastic drug administered as well as other agents to which the patient's system has never been exposed. In addition, some cancers appear to have multiple drug resistance even prior to the first exposure of the patient to an antineoplastic drug. Three mechanisms have been proposed to explain this phenomenon: P-glycoprotein Multiple Drug Resistance (MDR), MDR due to Topoisomerase Poisons and MDR due to altered expression of drug metabolizing enzymes (Holland et al., *Cancer Medicine*, Lea and Febiger, Philadelphia, 1993, p. 618-622).

P-glycoprotein MDR resistance appears to be mediated by the expression of an energy-dependent pump which rapidly removes cytotoxic agents from the cell. High levels of p-glycoprotein are associated with amplification of the MDR gene and transcriptional activation. Increased expression of p-glycoprotein can also be stimulated by heat shock, heavy metals, other cytotoxic drugs and liver insults, and ionizing radiation in some cell lines from some species. The results are not sufficiently consistent to confirm a causal relationship but are highly suggestive.

Topoisomerases are nuclear enzymes which are responsible for transient DNA strand breaks during DNA replication, transcription and recombination. Cytotoxic agents, such as etoposide, doxorubicin, amsacrine and others are known poisons of topoisomerase II, and cause lethal DNA strand breakage by the formation of stable complexes between the DNA, topoisomerase II and drug. MDR to this type of drug is thought to be caused by changes in the nature and amount of enzymatic activity, which is thought to prevent the formation or effect of the DNA-enzyme-drug complex.

Some cytotoxic agents are able to induce increased metabolic capability which permits rapid elimination of the toxin. Among the enzymes which have been implicated are glutathione S-transferase isozymes (GSTs). These enzymes are responsible for the conjugation of the electrophilic moieties of hydrophobic drugs with glutathione, which leads to detoxification and elimination of the drug.

As discussed above, PA and other phenylacetic acid analogs have been shown to stimulate the proliferation of peroxisomes which contain some isozymes of GST. Based on that observation, it would be expected that PA and PB would also stimulate MDR. However, as shown in FIG. 43, it has now been discovered that the opposite occurs. Thus, FIG. 25 shows the inhibition by PA of the growth of cells from a line of breast cancer cells that exhibit the MDR phenotype. Up to 10mM PA in cultures, growth of cells is dramatically inhibited in a dose-dependent manner. Surprisingly, PA and PB are more highly active against adriamycin-resistant breast cancer cells than compared to adriamycin-sensitive cells. This increased sensitivity of the MDR phenotype is reproducible in other tumor models, including those that are resistant to radiation therapy.

Thus, the instant invention provides a method of treating tumor cell populations in a patient that are resistant or able to survive current conventional treatments, particularly tumors having a MDR phenotype, by administration to the patient of non-toxic amounts of PA (such as amounts that provide up to 10 mM PA or an equipotent dose of a pharmaceutically acceptable phenylacetic acid derivative) in the vicinity of the tumor or equivalently effective amounts of phenylacetic acid or a phenylacetic acid analog. PA or other analog dosage protocols similar to those described in relation to the potentiation of differentiation in tumor cells by these phenylacetic acid-related compounds, including the various combination therapies described herein, can be used to treat patients with resistant tumors such as MDR tumors. Long-term (weeks, months) or short-term (day(s)) substantially continuous treatment regimens (including continuous administration or frequent administration of separate doses) as well as pulsed regimens (days, weeks or months of substantially continuous administration followed by a drug-free period) can beneficially be employed to treat patients with MDR tumors.

Section K: Phenylacetate and its derivatives, correlation between Potency and Lipophilicity

One potential problem that could hinder the clinical use of phenylacetate is related to the large amounts of drug

required to achieve therapeutic concentrations, i.e., over 300 mg/kg/day. Studies were thus undertaken to develop analogs that are effective at lower concentrations. 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 [Muir, R. M., Fujita, T., and Hansch, C. Structure-activity relationship in the auxin activity of mono-substituted phenylacetic acids. *Plant Physiol.*, 42: 1519-1526, 1967.]. Calculated partition coefficient (CLOGP) was used to correlate the predicted lipophilicity with the measured antitumor activity of phenylacetate analogues. For these analogues, enhanced potency in inducing cytostasis and phenotypic reversion in cultured prostate carcinoma, glioblastoma, and melanoma cells was correlated with increased drug lipophilicity.

Cell Cultures. Studies included the following humans tumor cell lines: (a) hormone-refractory prostatic carcinoma PC3, DU145, purchased from the American Type Culture Collection (ATCC, Rockville, Md.); (b) glioblastoma U87, A172 (ATCC); (c) melanoma A375 and mel 1011, provided by J. Fidler (M. D. Anderson, Houston Tex.) and J. Weber (NCI, Bethesda Md.), respectively. Cells were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (Gibco Laboratories), antibiotics, and 2 mM L-glutamine. Diploid human foreskin FS4 fibroblasts (ATCC), and human umbilical vein endothelial cells (HUVC) were used for comparison. The HUVC cells, isolated from freshly obtained cords, were provided by D. Grant and H. Kleinman (NIH, Bethesda Md.).

Antitumor Agents. Sodium phenylacetate and phenylbutyrate were from Elan Pharmaceutical corp, Gainesville Ga. 4-Iodophenylacetate, 4-iodophenylbutyrate and 4-chlorophenylbutyrate were synthesized by the Sandmeyer procedure 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 (Milwaukee, Wis.) 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° C. till used.

Calculation of Relative Drug Lipophilicities. Estimation of the contribution of lipophilicity to the biological activity of a molecule was based on its calculated logarithm of octanol-water partition coefficient (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) assuming that the degree of ionization is similar for all tested compounds.

Quantitation of Cell Growth and Viability. Growth rates were determined by cell enumeration with a hemocytometer following detachment with trypsin-EDTA, and by an enzymatic assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide (MTT). These two assays produced essentially the same results. Cell viability was assessed by trypan blue exclusion.

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 per ml in growth medium containing 0.36% agar (Difco). Two ml of cell suspension were 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, and colonies composed of 30 or more cells were counted after 3 weeks.

Growth on Matrigel. Cells were first treated with drugs in T.C. plastic dishes for 4-6 days, and then replated (5 ± 10^4 cells per well) onto 16 mm dishes (Costar, Cambridge, Mass.) coated with 250 μ l of 10 mg/ml matrigel, a reconstituted basement membrane (Collaborative Research). 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 hours, while invasion into the matrigel was evident after 6-9 days.

Drug Uptake Studies. Cells were plated in 6-well T.C. dishes (Costar) at 5×10^5 cells per dish. The growth medium was replaced after 24 hrs with 750 μ l of fresh medium containing 4.5×10^5 DPM of either 14 C-phenylacetic acid (3.4 mCi/mmmol, Sigma) or 14 C-naphthylacetic acid (5.4 mCi/mmmol, Sigma), and the cultures were incubated for 10-180 minutes at 37° C. Labeling was terminated by placing plates on ice. Cells were then washed twice with 5 ml ice-cold phosphate buffer saline (PBS), detached by scraping, and the radioactivity retained by cells determined using liquid scintillation. Blank values were determined by incubating the radiolabeled compounds in an empty dish.

Correlation Between Drug Lipophilicity and Growth-Inhibitory Effect of Phenylacetate and its Analogues. The growth inhibitory effect of these compounds on prostatic carcinoma, glioblastoma, and melanoma cell lines are expressed as IC_{50} and correlated with drug lipophilicity determined using the CLOGP program. As seen in Tables 21 and 22, there is a good correlation between cytostasis and lipophilicity. In agreement with previous observations with phenylacetate (3), the cytostatic effect was selective as higher drug concentrations were needed to significantly affect the proliferation of normal endothelial cells and skin fibroblasts. No cytotoxicity (i.e., decline in cell viability) occurred during 4-6 days of continuous treatment with the tested compounds.

TABLE 21

| Phenylacetate and analogues containing alkyl-chain substitutions: Relationship of IC_{50} to CLOGP | | | | | |
|--|-------|----------------|--------------|----------|--------------|
| Rx | CLOGP | IC_{50} (mM) | | | |
| | | prostate ca. | glioblastoma | melanoma | normal cells |
| α -methoxy-PA | 2.17 | 6 | 5.8 | 6 | ND |
| PA | 2.05 | 5 | 4.3 | 5 | 12 |
| α -methyl-PA | 2.42 | 2.6 | 3.8 | 3.5 | 12 |
| α -ethyl-PA | 2.77 | 2.1 | 2.8 | 2.2 | 9 |
| PB | 2.89 | 1 | 1.8 | 1 | ND |
| 4-chloro-PB | 3.30 | 0.75 | ND | 0.8 | ND |
| 4-iodo-PB | 3.85 | 0.36 | 0.27 | 0.22 | ND |

ND, not determined

TABLE 22

| Phenylacetate and analogues containing ring substitutions: Relationship of IC ₅₀ to CLOGP | | | | | |
|---|-------|-----------------------|-------------------|---------------|-----------------|
| Rx | CLOGP | IC ₅₀ (mM) | | | |
| | | prostate ca. | glio- blastoma | mela- noma | normal cells |
| 4-Hydroxy-PA | 1.78 | 7.5 | 10 | 10 | ND |
| PA | 2.05 | 5 | 4.3 | 5 | 12 |
| 4-fluoro-PA | 2.17 | 2.8 | 4 | 2.5 | ND |
| 2-methyl-PA | 2.43 | 2.5 | ND | ND | ND |
| 3-methyl-PA | 2.45 | 2.1 | ND | ND | ND |
| 4-methyl-PA | 2.47 | 2.1 | ND | ND | ND |
| 4-chloro-PA | 2.48 | 1 | 0.9 | 1.2 | 3 |
| 3-chloro-PA | 2.54 | 1.75 | 1.7 | 1.5 | 7 |
| 2-chloro-PA | 2.56 | 2.4 | 2.1 | 2.5 | ND |
| 2,6-dichloro-PA | 2.87 | 1 | 0.8 | 1 | ND |
| 4-iodo-PA | 3.12 | 0.6 | 0.9 | 1.2 | ND |
| 1-naphthyl- acetate | 3.16 | 0.8 | 0.9 | 0.8 | 2.8 |

ND, not determined

Further analysis of structure-activity relationships was based on the method of Hansch and Anderson used for the correlation of the anesthetic and metabolic effects of barbiturates with their octanol-water partition coefficients [Hansch, C., and Anderson, S. M. The structure-activity relationship in barbiturates and its similarity to that in other narcotics. *J. Med. Chem* 10: 745-753, 1967.]. Adaptation of this method assumes that, if the relationship is simple, it will follow the equation: $\log 1/IC = \text{slope} \log P + K$. Plotting the $\log 1/IC_{50}$ values obtained with prostatic cells vs drug CLOGP (FIG. 26) shows that the best fit line is described by the equation: $\log 1/IC_{50} = 0.89 \text{ CLOGP} + 0.55$. The slope of this line (0.89) is in the range of values found for the anesthetic potencies of a series of barbiturate analogues. Hansch and colleagues also studied the effect of phenylacetate and its derivatives on plant growth. As shown in FIG. 27, the concentration range and rank order of inhibition of plant growth by phenylacetate analogues are comparable to the inhibition of growth of prostatic cancer cells by this same series of compounds.

While the overall trend of enhanced activity of phenylacetate derivatives with increased lipophilicity is clear, some small deviations occur. For both chloro- and methyl-substitutions, the para position is more potent than the ortho position. In addition, and despite their nearly equal contributions to lipophilicity, para chloro-substitution was more potent than methyl. In contrast to derivatives containing ring or alpha-carbon substitutions, those with blocked carboxyl groups exhibited a decline in cytostatic activity. The methyl ester of phenylacetate was about half as active than the free acid (IC_{50} in DU145 prostatic cells 8.8 mM versus 4.1 mM for phenylacetate). The amide forms were also less active than the parent compounds in this experimental system, with IC_{50} s of 2.0 mM for phenylbutyramide versus 1.2 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 that of the more hydrophobic analog, naphthylacetate (Table 22). After 10 minutes, relative to phenylacetate more than twice as much naphthylacetate had entered the glioblastoma U87 cells (uptake of phenylacetic acid was 41% that of naphthylacetic acid) indicating that its movement through the

plasma membrane was more than twice as fast as phenylacetate. After 20 minutes, the amount of naphthylacetate taken up by the cells was as only 26% greater than that of phenylacetate and at 180 minutes 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 concentration of phenylacetate inside and outside the cells was 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. 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 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 formation of "net"-like structures when plated on a reconstituted basement membrane (matrigel). The ability of phenylacetate and representative analogs to bring about loss of such properties is summarized in FIG. 27. Similar to the cytostatic effect, drug ability to induce reversion to a non-malignant phenotype was highly correlated with the calculated lipophilicity of the drugs. Of the tested compounds, naphthylacetate, as well as 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).

Discussion. The comparative activity of phenylacetate and its analogues against a number of tumor cell lines suggest that these compounds may form a new class of therapeutic agents whose effectiveness varies with structure. Improved anticancer activity is achieved if factors controlling their action are understood, and toward this end the effects of systematic changes in structure with changes in activity have been compared. The outstanding result is the discovery that: (a) there is a simple relationship between the lipophilicity of a phenylacetate derivative and its activity against human tumor cells, and (b) 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.

The efficacy of aromatic fatty acids was demonstrated in vitro using tumor cell lines derived from patients with hormone-refractory prostatic carcinoma, glioblastomas, and malignant melanoma. Like phenylacetate, several derivatives containing alpha-carbon or ring substitutions all induced cytostasis and phenotypic reversion at non-toxic concentrations. Changes in tumor biology included reduction in cell proliferation rate and loss of malignant properties such as invasiveness and anchorage-independence. There were, however, significant differences in potency. When compared to phenylacetate, analogs with naphthyl-, halogen- or alkyl-ring, as well as alpha-carbon alkyl substitutions exhibited increased activity, while those with alpha-methoxy or hydroxyl replacement at the phenyl ring were less effective. Drug potency was correlated with the degree of calculated lipophilicity, indicating that differences in efficacy may be due in part to the ease with which these agents enter into and cross the lipid bilayer of cell membranes. In agreement, uptake of the more hydrophobic compound, naphthylacetate, was significantly faster than that of phenylacetate. At equilibrium (about 180 minutes for phenylacetate), however,

there were no differences in either the total intracellular concentration of both compounds, or the levels inside and outside cells. These results suggest that the rates of drug uptake are balanced by proportional rates of efflux, and that 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. 26), small deviations within the phenylacetate-related series may give some clues regarding mechanisms of action. Halogen substitutions para to the alkylcarboxyl group were found to increase potency more than those in the ortho position, suggesting that orientation of the hydrophobic substituent may be important. At the para position, chlorine had a greater impact on efficacy than a 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 more lipophilic analog α -methoxyphenylacetic acid was less active. The α -methoxyphenylacetic acid is a significantly stronger acid, and this greater acidity could be important. 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). 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 to the parental compounds and failed to induce cell differentiation (unpublished data). Moreover, phenylacetylglutamine has no detectable effect on cell growth and maturation. It appears, therefore, that a free carboxyl group may be essential for some aspects of the antitumor activity of phenylacetate and derivatives.

The correlation between partition coefficients and bioactivity of the aromatic fatty acids is reminiscent of that observed for a large number of other lipophilic agents. A survey by Hansch and Anderson revealed that, in a variety of animal tissues, the anesthetic and metabolic effects of barbiturates corresponded well with their hydrophilicity, having an average slope of about 1 compared to a slope of about 0.67 for lipophilic interaction with protein. It was concluded that the critical step in initiating biological activity was entry into the lipid bilayer, probably followed by interaction with membrane proteins. Some of the subsequently identified targets of barbiturates are indeed, membrane proteins and these include the GABA receptor-chloride in neurons, the ATP-K⁺ pump in pancreatic B-cells, and the G-protein that stimulates PLC activity in leukemic cells. Despite a wide body of literature implicating phenylacetate and analogs in growth control throughout phylogeny, 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. 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. These similarities in potency, summarized in FIG. 27, suggest that some of the underlying mechanisms of negative growth control may be similar as well.

There is accumulating evidence indicating that phenylacetate and derivatives may act through multiple mechanisms

to alter gene expression and cell biology. At growth inhibitory concentrations, the aromatic fatty acids could alter the pattern of DNA methylation, an epigenetic mechanism controlling the transcription of various eukaryotic genes. Phenylacetate inhibits DNA methylation in plant and mammalian cells, and both phenylacetate and phenylbutyrate were shown to activate the expression of otherwise dormant methylation-dependent genes. DNA hypomethylation per se is not sufficient to induce gene expression. Preliminary findings indicate that phenylacetate, phenylbutyrate and several analogs activate a nuclear receptor that functions as a transcriptional factor; interestingly, the receptor 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.

In addition to affecting gene transcription, the phenylfatty acids may interfere with protein post-translational processing by inhibiting the mevalonate (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. The latter include plasma membrane G and G-like proteins (e.g., ras) involved in mitogenic signal transduction (molecular weight 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. Furthermore, compounds such as phenylacetate can assume a conformation resembling mevalonate pyrophosphate and inhibit MVA utilization specifically. It was recently demonstrated that phenylacetate activity against poorly differentiated mammalian tissues (human glioblastoma cells and the developing fetal brain) is associated with inhibition of MVA decarboxylation and a decline in protein isoprenylation. Rapidly developing mammalian and plant tissues are highly dependent upon MVA for cell replication. Inhibition of MVA utilization by phenylacetate-related compounds could thus be responsible in part for their effect documented in such highly divergent organisms.

In conclusion, phenylacetate and analogs appear to represent a new class of pleiotropic growth regulators that might 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 treatment of hyperammone-mia, and phase I clinical trials in adults with cancer confirmed that millimolar levels can be achieved in the plasma and cerebrospinal fluid with no significant toxicities (discussed herein). However, rather large doses (300 mg/kg/day or more) are required to achieve potentially therapeutic levels. The identified relationship between lipophilicity of commercially available analogs and their antitumor activity in experimental models led us to predict that analogs with greater CLOGPs, e.g., iodo derivatives of phenylacetate and phenylbutyrate, would be highly effective. Indeed, these compounds were found to be the most potent aromatic fatty acids yet tested. With this approach, it should be possible to identify highly effective and safe antitumor agents suitable for clinical application.

Section L: NAPA and NAPB—Retinoic Acid Combination Treatment

Using the LA-N-5 cell line, NaPA can stimulate the differentiation of human neuroblastoma cells. Furthermore, the results show that combination treatment with NaPA and RA results in synergistic anti-tumor and differentiating effects which may be mediated, among other mechanisms, by the ability of NaPA to impact positively on the RA differentiation program.

LA-N-5 Cells—Combination of NaPA and RA

Cell culture. The LA-N-5 human neuroblastoma cell line was grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, HEPES buffer and 50 IU/ml penicillin/streptomycin and 1 μ g amphotericin (complete medium) as previously described (Sidell, N., Altman, A., Haussler, M. R., and Seeger, R. C. Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. *Expl. Cell Res.*, 148: 21–30, 1983). NaPA was obtained from Elan Pharmaceutical Research Corporation (Gainesville, Ga.). All other chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise indicated. All-trans-RA was dissolved in dimethylsulfoxide to a concentration of 5×10^{-2} mmol and stored at -20° C.

3 H-Thymidine incorporation. Cells (3 to 5×10^3 /well) were seeded in 24-well plastic tissue-culture dishes (2 cm^2 , Corning, Palo Alto, Calif.) and grown in the absence or presence of the indicated concentration of NaPA and/or RA. One μ Ci 3 H-thymidine/well was added to the cultures after 6–7 days of growth unless otherwise indicated. After incorporation for 12 to 16 hr, cultures were extensively rinsed in PBS, TCA-precipitated for 30 min (10% TCA) and washed in absolute ethanol. Cultures were then solubilized in 1N NaOH, neutralized in 1N HCl and an aliquot was counted in Scintifluor (National, Diagnostics, Manville, N.J.) cocktail. Results from typical experiments are expressed as mean cpm (\pm SEM) of triplicate or quadruplicate wells. Each experiment was performed at least 3 times with similar results.

Acetylcholinesterase (ACHE) activity. Specific AChE activity was measured as a biochemical index of the relative state of differentiation of treated and control LA-N-5 cells (Sidell, N., Lucas, C. A., and Kreutzberg, G. W. Regulation of acetylcholinesterase activity by retinoic acid in a human neuroblastoma cell line. *Expl. Cell Res.*, 155: 305–309, 1984). For measure of AChE activity, cells were grown in 25 cm^2 tissue culture flasks for 6 days in the absence or presence of the indicated concentrations of NaPA and/or RA, washed twice with isotonic saline, and harvested by vigorous shaking of the culture flask. After removal of saline, cells were frozen at -20° C., thawed by the addition of ice-cold 10 mM sodium phosphate buffer (pH 7.4) containing 0.5% Triton X-100 (1.5 ml/ 10^6 cells), and sonicated for 10 sec. AChE activity in samples of the homogenate was determined photometrically by following the hydrolysis of acetylthiocholine as previously described (Sidell, N., Lucas, C. A., and Kreutzberg, G. W. Regulation of acetylcholinesterase activity by retinoic acid in a human neuroblastoma cell line. *Expl. Cell Res.*, 155: 305–309, 1984). Protein concentrations were determined with a BioRad Coomassie protein assay kit using bovine serum albumin as the standard. Results, in nmoles/hr/mg protein, are expressed as means (\pm S.E.M.) of triplicate wells in a typical experiment. All experiments were repeated at least 3 times.

Northern blot analysis. Total RNA was extracted from harvested cells by the guanidinium isothiocyanate method and precipitated through a CsCl gradient (Chirgwin et al., 1979). The RNA was then stored at -70° C. until use. RNA (20 – 25μ g) was denatured by glyoxal, fractionated by electrophoresis through a 1.2% agarose gel at low voltage then transferred to a Biotrans nylon membrane (ICN, Irvine, Calif.) using a positive pressure transfer apparatus (Stratagene, La Jolla, Calif.). DNA probes were labelled using a random prime kit and activities of 10^9 cpm/ μ g were nor-

mally obtained (Amersham, Arlington Heights, Ill.). Prehybridization and hybridization medium consisted of $6 \times$ standard saline citrate (SSC), $5 \times$ Denhardt's solution, 0.5% SDS and 100 μ g/ml salmon sperm DNA. After 16 hrs of incubation, filters were washed once in $1 \times$ SSC, 0.1% SDS at room temperature for 30 min and 3 times in $0.2 \times$ SSC, 0.1% SDS at 68° . Autoradiography was performed using Kodak SAR film at -70° . Quantitative densitometry was performed with a scanning laser densitometer (Biomed Instruments, Irvine, Calif.).

The nuclear retinoic acid receptor- β (RAR β) probe was obtained from clone RAR- β O as described (Brand, N., Petkovich, M., Krust, A., Chambon, P., de The, H., Marchio, A., Tiollais, P., and Dejean, A. Identification of a second human retinoic acid receptor. *Nature*, 332: 850–853, 1988). The N-myc specific probe pNB-1 was obtained from American Type Culture Collection. All filters were reprobed with A 600 base EcoRI/BamHI β -actin fragment in order to confirm comparable loading of samples.

Immunocytology. LA-N-5 cells were plated onto tissue culture chamber slides (Nunc, Inc., Naperville, Ill.) in complete medium in a humidified 5% CO_2 atmosphere. Cells were allowed to attach overnight then treated with the indicated concentrations of NaPA and/or RA. After one week of treatment, cells were washed in PBS and fixed with 2% paraformaldehyde in PBA at pH 7.0, followed by methanol, at 4° C. for 10 min each. Endogenous peroxidase activity was eliminated by incubation in 3% H_2O_2 in methanol for 10 min at room temperature. Non-specific antigenic sites were blocked by a 30 min room temperature incubation with 10% normal goat serum in PBS, followed by a 2 hr incubation with primary antibody at 37° C. Anti-N-myc monoclonal antibody NCM II 100 (Ikegaki, N., Bukovsky, J., and Kennett, R. H. Identification and characterization of the NMYC gene product in human neuroblastoma cells by monoclonal antibodies with defined specificities. *Proc. Natl. Acad. Sci. USA*, 83: 5929–5933, 1986) and a non-binding IgG control monoclonal antibody were used as primary antibodies in the reactions. Incubation with primary antibody was followed by sequential incubation with biotinylated goat anti-mouse antibody at room temperature for 30 minutes, Z-avidin-peroxidase conjugates at room temperature for 30 minutes (Zymet Laboratories, Inc., San Francisco, Calif.), and diaminobenzidine/ H_2O_2 (Sigma) at 0.5 mg/ml in 50 mM Tris pH 8 at room temperature for 10 minutes. Cells were washed for 15 minutes in PBS between all steps. Nuclear staining intensity was quantified and analyzed using a Nikon digital image microscope system and image processing and analysis software from Analytical Imaging Concepts (Irvine, Calif.).

Analysis of protein isoprenylation. Cell cultures were incubated with 10 mM phenylacetate and/or 1 μ M RA for 24 hrs in complete medium containing 5 μ M lovastatin. The cells were labeled with RS-[2 - ^{14}C]mevalonate (16 μ Ci/ml, specific activity 15 μ Ci/mmol) (American Radiolabelled Chemicals, Inc., St. Louis, Mo.) during the final 15 hours of treatment. Whole cell proteins were extracted, resolved on 10% SDS-polyacrylamide gels, and stained with Coomassie Brilliant Blue. Gels were then dried and exposed to Kodak X-Omat film for 4 days.

Morphologic differentiation. NaPA induced neurite outgrowth from LA-N-5 cells. This effect first became apparent after about 3 days of continuous exposure in culture, with maximal increases in the formation of neurites occurring at NaPA concentrations between 5–10 mM. Concentrations less than 1 mM produced no noticeable morphologic effects. No decrease in the percentage of viable cells was detected in

the treated cultures as compared with control cultures. However, higher NaPA concentrations (>10 mM) were found to be toxic over extended culture periods (>7 days). The cell soma were not markedly altered with NaPA treatment, although a slight increase in the number of intracellular inclusions resembling lipid droplets at NaPA concentrations greater than 5 mM was observable.

As extensively reported, RA also induced neurite sprouting from LA-N-5 cells with maximal effects seen at RA concentrations greater than 1 μ M (Abemayor, E., and Sidell, N. Human neuroblastoma cell lines as models for the in vitro study of neoplastic neuronal cell differentiation. *Environ. Health Perspect.*, 80: 3-15, 1989; Sidell, N., Altman, A., Haussler, M. R., and Seeger, R. C. Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. *Expl. Cell Res.*, 148: 21-30, 1983; Lando, M., Abemayor, E., Verity, M. A., and Sidell, N. Modulation of intracellular cyclic AMP levels and the differentiation response of human neuroblastoma cells. *Cancer Res.*, 50: 722-727, 1990; Sidell, N., Lucas, C. A., and Kreutzberg, G. W. Regulation of acetylcholinesterase activity by retinoic acid in a human neuroblastoma cell line. *Expl. Cell Res.*, 155: 305-309, 1984). In the presence of optimal concentrations of both agents, morphologic differentiation of LA-N-5 was profound. Indeed, treatment with 5 mM NaPA plus 1 μ M RA induced cellular clustering and neurite bundle recruitment reminiscent of pseudoganglia formation after only 6-7 days of culture (FIG. 29D). Longer periods with both agents (~2 weeks) resulted in cultures consisting predominantly of large cellular aggregates connected by thick fascicles of neurites against an elaborate background network of thin fibers. At this point, very few individual cells (those not associated with a cell cluster) could be observed.

Proliferation. (FIG. 28) shows the dose-dependent effects of NaPA treatment on 3 H-thymidine incorporation in LA-N-5 cells. It is evident that greater than 65% inhibition was achieved at NaPA concentrations of 5 mM and above, while little effect was seen below 1.25 mM. Concentrations above 10 mM were not tested since detachment of cells from the flasks suggested significant toxicity.

NaPA and RA were synergistic in their antiproliferative effects on LA-N-5 cells in that combination treatment consistently reduced 3 H-thymidine incorporation to lower levels than one would expect from the combined action of two antiproliferative agents acting through independent mechanisms. For example, in FIG. 29, 3 H-thymidine incorporation in the presence of 1.25 mM NaPA was around 90% of control while that with 10^{-7} M RA was 70%. Both agents together at these concentrations reduced 3 H-thymidine incorporation to 30% of control while 63% would be expected from the two agents acting independently of each other ($0.90 \times 0.70 = 63\%$). Combination treatment at the highest concentrations tested (NaPA—5 mM; RA—1 μ M) resulted in almost complete cessation of cell growth while each agent alone produced only a partial effect after the 6-day assay period.

Acetylcholinesterase (ACHE) activity. Concomitant with neurite outgrowth, AChE activity increases in LA-N-5 cells induced to differentiate with a variety of agents including RA (Lando, M., Abemayor, E., Verity, M. A., and Sidell, N. Modulation of intracellular cyclic AMP levels and the differentiation response of human neuroblastoma cells. *Cancer Res.*, 50: 722-727, 1990; Wuarin, L., Verity, M. A., and Sidell, N. Effects of gamma-interferon and its interaction with retinoic acid on human neuroblastoma cells. *Int. J. Cancer*, 48: 136-144, 1991). To assess whether this bio-

chemical index of neuroblastoma differentiation was associated with NaPA-induced neurite outgrowth, AChE activity was measured in cells treated with various concentrations of NaPA for 6-7 days (FIG. 30). As can be seen, AChE was significantly increased at 1.25 mM NaPA with maximal increases occurring at 5 mM AChE. In the presence of NaPA and RA, AChE activity was markedly potentiated over that seen with either agent alone. Thus, as shown in FIG. 30, while AChE activities in either NaPA or RA treated cells remained below 40 nm/min/mg protein, levels as high as 150 nm/min/mg protein could be achieved with combination treatments. This activity represents the strongest AChE induction yet observed in LA-N-5 cells using a variety of differentiating agents, either alone or in combination. Although the absolute values of AChE varied from one experiment to another, the pattern was consistent and the results shown in FIG. 30 is representative. Generally, the induced increase in AChE activity paralleled the extent and complexity of neurite formation in the LA-N-5 cultures.

Susceptibility of LA-N-5 cells to glutamine depletion. In humans, NaPA causes depletion of circulating glutamine due to conjugation of the amino acid to form phenylacetylglutamine, an enzymatic reaction known to take place in the liver and kidney (James, M. O., Smith, R. L., Williams, F. R. S., and Reidenberg, M. The conjugation of phenylacetic acid in man, sub-human primates and some non-primates species. *Proc. R. Soc. Lond. B*, 182: 25-35, 1972). The in vivo reduction in plasma glutamine levels was mimicked in vitro by culturing cells in the presence of lowered glutamine concentrations. Glutamine deprivation showed no significant effect on the growth, morphology, or AChE activity of LA-N-5 cells. Indeed, in the complete absence of exogenous glutamine, LA-N-5 cultures were essentially indistinguishable from those growing in physiological levels of glutamine. Furthermore, both NaPA- and RA-induced differentiation of LA-N-5 cells were unaffected by the absence of glutamine. Thus, glutamine depletion by NaPA could not explain the differentiation-induction effects of this compound on LA-N-5 cells.

N-myc expression. Expression of the N-myc oncoprotein has been shown to be rapidly decreased in LA-N-5 and other neuroblastoma cells during RA-induced differentiation (Thiele, C. T., Reynolds, C. P., and Israel, M. A. Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. *Nature*, 313: 404-406, 1985; RA19). Nuclei of untreated LA-N-5 cells strongly reacted with the N-myc antibody and showed a marked heterogeneity of staining intensity within the culture as previously reported for neuroblastoma cells in general (Ikegaki, N., Bukovsky, J., and Kennett, R. H. Identification and characterization of the NMYC gene product in human neuroblastoma cells by monoclonal antibodies with defined specificities. *Proc. Natl. Acad. Sci. USA*, 83: 5929-5933, 1986). This heterogeneity was observed by quantitative image analysis of the immunostained cytoslide preparations. Cells incubated for greater than 3 days with NaPA or RA showed marked reductions of N-myc expression as indicated by a decrease in the median relative staining intensity from 44 in control cultures to 36 and 29 in NaPA- and RA-treated cultures, respectively (FIG. 31). Combination treatment with both NaPA and RA resulted in a further profound decrease in N-myc levels with a mean relative staining intensity of 16.

Although NaPA induced a reduction in N-myc protein, no changes were observed in N-myc mRNA expression in short term (3-6 day) cultures. Six day-treatment of LA-N-5 cells with 5 mM NaPA (a condition which consistently reduced

N-mFc protein levels) demonstrated no apparent difference from untreated cultures in N-myc mRNA levels as assessed by Northern blotting. On the other hand, RA treatment caused a dramatic decrease in the amount of message produced as previously reported while combination treatment resulted in message levels similar to that seen with RA alone. In contrast to this lack of effect of NaPA on N-myc RNA in short term cultures, longer treatment with NaPA (>8 days) induced both a moderate decrease in N-myc mRNA levels and a profound further reduction when combined with RA.

Nuclear retinoic acid receptor- β (RAR β) expression. In human neuroblastoma, as in many other tissues, RAR β shows low constitutive expression in untreated cells but is rapidly induced by RA (de The, H., Marchio, A., Tiollais, P., and Dejean, A. Differential expression and regulation of the retinoic acid receptor α and β genes. *EMBO J.*, 8: 429–433, 1989; Wuarin, L., Chang, B., Wada, R., and Sidell, N. Retinoic acid upregulates nuclear retinoic acid receptor- α expression in human neuroblastoma. *Int. J. Cancer* (in press)). RAR β mRNA levels were moderately increased by NaPA, while in the presence of both NaPA and RA, the expression of this receptor was markedly enhanced over that seen with RA alone. This effect occurred prior to the morphologic or other phenotypic changes induced by NaPA in LA-N-5 cells.

Effects of NaPA on protein isoprenylation. Active de novo synthesis of cholesterol and isoprenoids from precursors such as acetyl-CoA and mevalonate (MVA) is an important feature of developing neuronal tissue (Grossi, E., Paoletti, P., and Paoletti, R. An analysis of brain cholesterol and fatty acid biosynthesis. *Arch. Int. Physiol. Biochem.*, 66: 564–572, 1958). As such, protein prenylation has been shown to be an important post-translational process critical for normal regulation of cell growth and differentiation (Marshall, C. J. Protein prenylation: A mediator of protein-protein interactions. *Science*, 259: 1865–1866, 1993; Braun, P. E., De Angelis, D., Shtybel, W. W., and Bernier, L. Isoprenoid modification permits 2',3'-cyclic nucleotide 3'-phosphodiesterase to bind to membranes. *J. Neurosci. Res.* 30: 540–544, 1991). As discussed elsewhere herein, NaPA inhibits protein isoprenylation and MVA decarboxylation in human glioblastoma cells. This effect is also observed in embryonic brain in phenylketonuria, an inborn error of phenylalanine metabolism which is associated with excessive production of phenylacetate (Scriver, C. R., and Clow, C. L. Phenylketonuria: epitome of human biochemical genetics. *N. Engl. J. Med.*, 303: 1394–1400, 1980; Castillo, M., Zafra, M. F. and Garcia-Peregrin, E. Inhibition of brain and liver 3-hydroxy-3-methylglutaryl-CoA reductase and mevalonate-5-pyrophosphate decarboxylase in experimental hyperphenylalaninemia. *Neurochem. Res.*, 13: 551–555, 1988). NaPA, but not RA, inhibited protein isoprenylation in LA-N-5 neuroblastoma. Furthermore, in the presence of NaPA and RA, these effects were similar to that induced with NaPA alone.

NaPA induced the differentiation of the LA-N-5 human neuroblastoma cell line as assessed by dose-dependent growth inhibition, neurite outgrowth, increased AChE activity, and reduction of N-myc expression. As discussed herein, NaPA was shown to reduce the malignant phenotype of promyelocytic leukemia, prostate cancer, and glioblastoma cells. The ability of NaPA to selectively arrest tumor growth and promote differentiation was confirmed using rats with malignant brain tumors (described herein). The data confirms and extends data showing NaPA-induced morphologic differentiation, growth inhibition, and reduction of N-myc

protein levels in two other human neuroblastoma cell lines (Cinatl, J., Cinatl, J., Mainke, M., Weissflog, A., Rabenau, H., Kornhuber, B., and Doerr H-W. In vitro differentiation of human neuroblastoma cells induced by sodium phenylacetate. *Cancer Lett.*, 70: 15–24, 1993). NaPA and RA synergized in inducing LA-N-5 differentiation. Thus, combination treatment with NaPA and RA at concentrations that were saturating in terms of a differentiation response with each agent alone caused further marked enhancement of all parameters measured. Furthermore, at all concentrations, combination treatment consistently induced LA-N-5 differentiation to a level that was greater than would be expected from two agents acting independently of each other. Indeed, the combined effects of both agents represent the strongest differentiation response yet observed by neuroblastoma cells, both in terms of number of cells responding and maturational level achieved.

These results suggest that NaPA and RA do not work through coincidental pathways of action but that their pathways must, nevertheless, intersect at some level to synergistically potentiate cellular differentiation and growth arrest. This contention was supported by the finding that NaPA markedly enhanced RA induction of RAR β mRNA levels in LA-N-5 cells. Upregulation of this receptor has been shown to be a necessary event in RA-induced differentiation of embryonal carcinoma cells (Kruyt, F. A., van der Brink, C. E., Defize, L. H., Donath, M. J., Kastner, P., Kruijer, W., Chambon, P., and van der Saag, P. T. Transcriptional regulation of retinoic receptor beta in retinoic acid-sensitive and resistant P19 embryocarcinoma cells. *Mech. Dev.*, 33: 171–178, 1991), and aberrant expression or regulation of RAR β has been suggested in the pathogenesis of certain lung and hepatocellular carcinoma (Nervi, C., Volberg, T. M., George, M. D., Zelent, A., Chambon, P., and Jetten, A. M. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. *Exp. Cell Res.*, 195: 163–170, 1991; Hu, L., Crowe, D., Rheinwald J, Chambon P, and Gudas L. Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res.*, 51: 3972–3981, 1991), and head and neck tumors (de The, H., Marchio, A., Tiollais, P., and Dejean, A. A novel steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma. *Nature*, 330: 667–670, 1987). Thus, by modulating expression of RAR β NaPA might impact on the RA differentiation program in such a way as to lead to enhanced retinoid activity. However, NaPA can induce LA-N-5 differentiation in cultures consisting of delipidated FCS and hence devoid of serum retinoids. Therefore, an ability to alter cellular responses to available retinoid concentrations cannot adequately account for both the direct action of NaPA, and its synergy with RA. In this regard, it is possible that the direct action of NaPA in inducing differentiation is mediated by different intracellular events than those responsible for its interaction with RA.

A consistent biochemical change induced by NaPA, but not by RA, is a decline in protein isoprenylation. This effect occurs within 24 hrs of LA-N-5 treatment with NaPA, preceding changes in DNA synthesis and differentiation. As recently documented in malignant glioblastomas, inhibition of protein prenylation by NaPA is due primarily to a decrease in MVA decarboxylation, a key step regulated by MVA-5-pyrophosphate decarboxylase. MVA is a precursor of several isopentenyl moieties required for progression through the cell cycle such as sterols, dolichol, the side chains of ubiquinone and isopentenyladenine, and prenyl

groups that modify a small set of proteins (Marshall, C. J. Protein prenylation: A mediator of protein-protein interactions. *Science*, 259: 1865-866, 1993; Braun, P. E., De Angelis, D., Shtybel, W. Wo, and Bernier, L. Isoprenoid modification permits 2', 3'-cyclic nucleotide 3'-phosphodiesterase to bind to membranes *J. Neurosci. Res.* 30: 540-544, 1991; Goldstein, J. L., and Brown, M. S. Regulation of the mevalonate pathway. *Nature*, 343: 425-430, 1990). The latter include plasma membrane G and G-like proteins (e.g. ras) involved in mitogenic signal transduction (molecular weight 20-26 kDa), and nuclear envelope lamins that play a key role in mitosis (44-74 kDa). The present studies show a decline in prenylation of corresponding proteins in NaPA-treated LA-N-5 cells, although the specific targets have yet to be identified. Lovastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (which catalyzes the synthesis of MVA from acetyl-CoA) has also been shown to induce the differentiation of neuroblastoma cells as described elsewhere herein (Maltese, W. A., and Sheridan, K. M. Differentiation of neuroblastoma cells induced by an inhibitor of mevalonate synthesis: Relation of neurite outgrowth and acetylcholinesterase activity to changes in cell proliferation and blocked isoprenoid synthesis. *J. Cell. Physiol.*, 125: 540-558, 1985). The experience with NaPA and lovastatin suggested that growth arrest and maturation may not be directly related to a decline in cholesterol, dolichol, or ubiquinone, but rather due to depletion of isoprenoid compounds essential for protein processing and maintenance of the malignant phenotype (Maltese, W. A., and Sheridan, K. M. Differentiation of neuroblastoma cells induced by an inhibitor of mevalonate synthesis: Relation of neurite outgrowth and acetylcholinesterase activity to changes in cell proliferation and blocked isoprenoid synthesis. *J. Cell. Physiol.*, 125: 540-558, 1985). Since isoprenylation was not affected by RA treatment, it is unlikely that inhibition of this process by NaPA is a direct result of any NaPA-induced changes in the RA differentiation program (e.g. an increase in RARB expression). Thus, reduction of protein isoprenylation by NaPA reflects either early events leading to (rather than a consequence of) alterations in the retinoid pathway of action or a totally independent effect that could mediate some of the cellular responses to NaPA apart from its interaction with RA.

The effect of NaPA on N-myc mRNA levels in LA-N-5 cells was especially intriguing. In short term (3-6 day) cultures, NaPA had no apparent effects on N-myc mRNA levels, either alone or in the presence of RA even through N-myc protein was found to be decreased. However, a significant decline in N-myc RNA expression was seen in NaPA-treated cultures after longer-term treatment. This finding suggests a posttranscriptional level of regulation by NaPA that ultimately results in highly differentiated cells expressing lowered levels of N-myc RNA. Indeed, in the combined presence of NaPA and RA for 10 days, the decrease in N-myc is dramatic, with very little mRNA detectable by Northern blotting. In studies in HL60 cells (see supra), NaPA caused a rapid decline in amounts of c-myc mRNA, which occurred within 4 hr of treatment, preceding phenotypic changes associated with differentiation. Thus, alteration by NaPA of c-myc in HL60 cells and N-myc in LA-N-5 cells may involve different mechanisms of action. Similarly, modulation of these two oncogenes by RA has been found to have distinct mechanisms in that down-regulation of N-myc was shown to be at the level of transcription (Wada, R. K., Seeger, S. C., Reynolds, C. P., Alloggiamento, T., Yamashiro, J. M., Ruland, C., Black, A. C., and Rosenblatt, J. D. Cell type-specific expression and

negative regulation by retinoic acid of the human N-myc oncogene with rapid progression of neuroblastoma. *N. Engl. J. Med.*, 313: 111-116, 1985) while that of c-myc is post-transcriptional (Doty, C., Kessel, M., and Gruss, P. Post-transcriptional control of myc and p53 expression during differentiation of the embryonal carcinoma cell line F9. *Nature*, 317: 636-639, 1985). In any case, since expression of N-myc has been positively correlated with tumor progression and poor prognosis (Seeger, R. C., Brodeur, G. M., Sather, H., Dalton, A., Siegel, S. E., Wong, K. y., and Hammond, D. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastoma. *N. Engl. J. Med.*, 313: 111-116, 1985; Grady-Leopardi, E. F., Schwab, M., Ablin, A. R., and Rosenau, W. Detection of N-myc oncogene expression in human neuroblastoma by in situ hybridization and blot analysis: relationship to clinical outcome. *Cancer Res.*, 46: 3196-3199, 1986), the dramatic reduction in the levels of this oncoprotein seen in the presence of NaPA and RA underscores the potentially increased clinical efficacy of this combination treatment.

An interaction between NaPA and RA in inducing cellular differentiation and growth inhibition does not appear to be a unique phenomenon for LA-N-5 cells. NaPA-induced maturation of human leukemia, breast carcinoma, and malignant melanoma can be enhanced by the addition of low doses of RA (Samid, D., Shack, S., and Sherman, L. T. Phenylacetate: A novel nontoxic inducer of tumor cell differentiation. *Cancer Res.*, 52: 1988-1992, 1992). Furthermore, Gorski et al. noted synergistic growth inhibition with NaPA and RA of human rhabdomyosarcoma cells (Gorski, G. K., Donaldson, M. H., and McMorro, L. E. Synergistic inhibition of human rhabdomyosarcoma cells by sodium phenylacetate and tretinoin. *In Vitro Cell Dev. Biol.*, 29A: 189-191, 1993). Experiments indicated that NaPA induced growth inhibition and neurite outgrowth from the LA-N-2 and IMR32 human neuroblastoma cell lines, and that these effects were potentiated with RA. Taken together, these findings suggest that the synergistic phenomena detailed here with LA-N-5 cells may be a general feature of NaPA/RA treatment or many tumor cell types.

EXAMPLE 23

Antiproliferative effect on Neuroblastoma Cell Lines

Cell lines. In a related experiment, seven human neuroblastoma cell lines were used with varying characteristics relating to neurotransmitter phenotype, N-myc amplification, N-ras and p53 expression, neurocrest lineage response, and sensitivity to retinoic acid-induced differentiation. Table 23 below summarizes some important characteristics of the seven lines.

TABLE 23

| Neuroblastoma Cell Lines | | | | |
|--------------------------|----------------------------|--|------------------------|-------------------|
| Cell Line | Neurotransmitter phenotype | Changes induced by retinoic acid | Number of N-myc copies | Expression of p53 |
| SK-N-AS | cholinergic | resistant | 1 | n.d. ^a |
| LA-N-5 | mixed | neuronal differentiation (4+) ^b | 50 | 1+ ^c |
| LA-N-2 | cholinergic | neuronal differentiation (2+) | 25 | n.d. |

TABLE 23-continued

| Neuroblastoma Cell Lines | | | | |
|--------------------------|----------------------------|----------------------------------|------------------------|------------------------|
| Cell Line | Neurotransmitter phenotype | Changes induced by retinoic acid | Number of N-myc copies | Expression of p53 |
| SK-N-SH-F | adrenergic | schwannian transformation | 1 | + and ras ^a |
| SK-N-SH-N | adrenergic | neuronal differentiation (3+) | 1 | + and ras |
| LA-N-6 | adrenergic | growth inhibition only | 1 | n.d. |
| Lan-1-15N | adrenergic | growth inhibition only | 100 | |

^anot determined
^brelative differentiation response
^crelative expression of p53
^dalso possesses a mutationally activated N-ras

The prototype and best characterized line is LA-N-5, which, like the majority of human neuroblastoma cell lines, contains amplified copies of N-myc and is sensitive to differentiation induction by retinoic acid. As such, LA-N-5 was the best defined model system to address specific questions relating to the reversibility of PA and PB and the relationship of treatment duration to agent concentration.

Antiproliferative effects of PA and PB on human neuroblastoma cells. FIG. 32 shows the concentration-dependent effects of PA and PB on incorporation of [³H]thymidine in the seven cell lines after 7 days of treatment. As can be seen, in six of seven lines, greater than 95% inhibition was achieved at the highest PB concentration tested (4 mM) with the same cell lines exhibiting greater than 70% inhibition at 2 mM PB. One line, LA-N-6, was found to be less sensitive to PB by showing little growth inhibition at concentrations less than 4 mM.

In all cases, the neuroblastoma lines were less sensitive to growth inhibition by PA than by PB. Table 24 extrapolates from FIG. 32, the concentrations of PA and PB that can induce a 50% growth inhibition in the cell lines (GI₅₀). As is seen, the three lines least sensitive to PA (LA-N-2, SK-N-SH-N, and LA-N-6) were also the least sensitive to PB. However, the converse was not true; SK-N-SH-N was the most sensitive to growth inhibition by PA but was not the most sensitive to PB. No obvious correlation was seen between the general characteristics of the cell lines as shown in Table 24 and their growth inhibition by PA or PB.

TABLE 24

| Growth Inhibition of Cell Lines | | |
|---------------------------------|-----------------------------|-----------------|
| Cell Line | GI ₅₀ (mM) PA | Values of PB |
| SK-N-SH-N | 1.8 | 0.8 |
| LA-N-5 | 3.5 | 0.6 |
| LA-N-2 | 10.0 | 1.6 |
| SK-N-SH-F | 4.5 | <0.5 |
| SK-N-SH-N | 8.5 | 1.5 |
| LA-N-6 | 10.0 | 3.0 |
| Lan-1-15N | 4.5 | 0.5 |

These results indicate that, as a histological group, human neuroblastoma cells are very-sensitive to growth inhibition by PB and PA. In observing morphologic changes that occurred during the PA and PB treatments, general features of neurite extension, and cellular clustering in all of the cell

lines were also noted. Thus, although PB- and PA-induced neurotransmitter changes in the various lines have not yet been quantitated, the morphologic changes observed suggest that neuronal differentiation is a general feature in the response of human neuroblastoma cells to PB and PA.

Dose- and time-dependent effects of PB and PA on neuroblastoma cells. LA-N-5 cells were treated with PB and PA at doses ranging from 0.5 to 4 mM and 1.25 to 10 mM respectively, and [³H]thymidine incorporation (as a measure of growth) and acetylcholinesterase (as a measure of differentiation) were assessed over the next 8 days. As seen in FIG. 33A, cells incubated with 2 or 4 mM PB demonstrated a decrease in [³H]thymidine incorporation as early as two days after the start of culturing while those incubated with 0.5 and 1 mM PB showed significant inhibition only after four days of treatment. The highest concentrations of PB (4 mM) seemed to induce a cytotoxic effect which was reflected by a progressive decrease in cell viability (as assessed by trypan blue exclusion) starting on the fourth day of culturing. No decrease in the percentage of viable cells was detected in cultures treated with the other concentrations of PB. A similar inverse relationship between agent concentration and treatment time needed to show significant growth inhibition was noted for PA (FIG. 33B). However, in agreement with FIG. 32, PA was less effective than PB in that significant growth inhibition was first noted only after 3 days at the highest (10 mM) PA concentration tested. It took at least four days for all other concentrations of PA to induce growth inhibition of LA-N-5 cells.

Induction of AChE activity by PB and PA generally occurred quicker than did growth inhibition induced by these agents (FIG. 34). LA-N-5 cells cultured with all concentrations of PB showed a dose-dependent enhancement in AChE activity starting on the very first day of treatment and progressively increased to day 4. After this time (day 6 and 8), AChE levels generally leveled off with the exception of a sharp decrease seen in the presence of 4 mM PB. This decrease probably reflected the reduced viability of these cultures as described above. With PA, AChE activity in LA-N-5 cultures also showed a time- and dose-dependent increase, but this effect progressed with all concentrations up to the last day of measurement (day 8). Studies on the reversibility of PA and PB treatment (below) suggest that AChE levels continue to increase in PA-treatment cultures up to 2 weeks or beyond.

Reversibility of PB and PA effects on growth and AChE activity. LA-N-5 cells were cultured with either solvent control, 2 mM PB, or 5 mM PA for 6 days, then washed and refed with either control medium or medium containing the agent at the original treatment concentration. [³H]thymidine incorporation (FIG. 35) and specific AChE activity (FIG. 36) were then assessed starting 1 day after washing (posttreatment) and various days thereafter. As can be seen in FIG. 35, up to 7 days after treating cells with PA, [³H]thymidine incorporation in cultures refed with control medium continued to slowly increase at basically the same rate as those refed with PA. However, after 1 week posttreatment, cells refed in control medium showed a higher rate of proliferation than those cultured in the continuous presence of PA. A similar slow reversibility was seen with PB in that very little growth was seen for up to 7 days posttreatment in cultures refed with either control or PB-containing medium, but following this time period the two growth curves diverged. The slow steady increase in [³H]thymidine incorporation seen in the continuous presence of 5 mM PA for posttreatment periods even up to 14 days (20 days total treatment time) suggests either that this concentration of PA is not

totally suppressing cell growth or that discrete populations of LA-N-5 cells with variable resistance to the agent are present in the culture. Although it is difficult to distinguish between these two possibilities, the initial impression by observing the gross dynamics of cell populations during culture is that situations are true. In contrast, cells grown in the continuous presence of PB for up to 20 days did not show any growth, suggesting a more complete suppression than with PA and/or a lack of resistant populations. However, even the profound antiproliferative effect of PB appeared to be somewhat reversible under the conditions of this experiment.

Measurements of AChE activity following the 6 days of culturing with the agents supports a conclusion that the effects of both PA and PB on the growth and differentiation of LA-N-5 is reversible under the conditions imposed. As seen in FIG. 36, the increased AChE activity originally induced by PA and PB returned to baseline values after 1 week posttreatment. In those cultures refed with PB, AChE activity remained elevated to a more or less constant level. On the other hand, in cultures refed with PA, specific AChE activity continued to increase even up to the 7-day post-treatment time point, eventually reaching levels greater than that achieved with PB. This latter observation suggests that induction of AChE (and presumably differentiation) by PA is a relatively slow process and may require many weeks of continuous exposure to the drug before its full differentiation-inducing potential is realized. Whether or not such longer treatment times might result in irreversible differentiation is presently unknown.

The results of the experiments performed to date have established the following: 1) Human neuroblastoma cells are, as a histologic group, sensitive to growth inhibition and morphologic differentiation by PA and PB. These effects show a 3- to 10-fold greater sensitivity to PB than to PA and are not correlated with any known characteristics of the cells such as neurotransmitter phenotype, N-myc amplification and expression, or susceptibility to differentiation by retinoic acid. 2) Using LA-N-5 neuroblastoma cells as a model system, differentiation by PB and PA was first evident after one day of treatment at the higher concentrations tested, reached plateau levels of induction after 4 days in the case of PB but continued to progress for at least 2 weeks in the case of PA. 3) Differentiation by PA and PB following a 6-day treatment protocol was reversible. The possibility that longer treatments may induce irreversible differentiation is not suggested in the case of PB since its effects on the cells seemed to plateau after around 4 days of exposure. However, since induction by PA progresses for at least 2 weeks to eventually reach a greater stage of maturation than with PB (as assessed by specific AChE activity), future PA reversibility experiments with longer exposure times would appear to be warranted.

Based on the above-described discovery of the synergistic activity between retinoic acid (RA) and PA in inducing neuroblastoma differentiation, the idea that combination treatment with these compounds holds the most potential for their positive therapeutic use is central. Indeed, the interaction of PA with retinoids might be one reason by PA appears to be a much more potent anticancer agent in *in vivo* versus *in vitro* models. Thus, *in vitro* cultures have a limited supply of endogenous retinoids that are carried along with the fetal calf serum and are degraded during shelf storage, heat inactivation, light exposure, and culturing. On the other hand, serum retinoid concentrations are tightly regulated *in vivo* and remain relatively constant (in the serum, retinol levels are in the 1 μ M range with RA levels being around

0.01 μ M). As such, the addition of exogenous retinoids to *in vitro* culture systems and the study of the interaction of these compounds with PA and its derivatives may actually be a much more "physiologic" model for understanding potential single agent applications *in vivo*.

EXAMPLE 24

NaPA in combination with Flavonoids and Lignins

Furthermore, PA and its analogs may similarly also be used in combination with flavonoids and lignins, as well as with retinoids as described above. For instance, FIG. 37 shows that PA and apigenin (a flavonoid) act in a synergistic manner to suppress the growth of human prostatic carcinoma PC3 cells. Similar results were observed when PA was used in combination with 9-cis-retinoic acid. The 9-cis-RA (and its precursors, including all-trans-RA as discussed above) activate the nuclear receptor RXR, which is required for the stimulation of PPAR (described herein and in the copending application) by PA and its analogs.

PC3 cells were treated with 1) 10 μ M EtOH (control), 2) PA 4 mM, 3) apigenin 10 μ M, 4) apigenin 10 μ M in combination with PA 4 mM, 5) 9-cis-RA 2 μ M and 6) 9-cis-RA 2 μ M in combination with PA 4 mM. The combination therapies showed significant, potentiated reduction (apigenin/PA approximately 50% and 9-cis-RA/PA approximately 50%) in PC3 cell survival rates.

In addition, the flavonoid quercetin, which is known to block the efflux of PA from plant cells, may be used in combination with PA to enhance the therapies described herein.

SECTION M: NAPA AND NAPB—LOVASTATIN COMBINATION TREATMENT

Malignant gliomas are highly dependent on the mevalonate (MVA) pathway for the synthesis of cholesterol and intermediates critical to cell replication. Targeting MVA synthesis and/or utilization thus inhibits tumor growth without damaging normal brain tissues, in which the MVA pathway is minimally active. Human glioblastoma cells were found to be uniquely vulnerable to lovastatin (LOV) and sodium phenylacetate (NaPA) which act as inhibitors of the key regulatory enzymes HMG-coA reductase and MVA-PP decarboxylase, respectively.

EXAMPLE 25

NaPA combination therapies with vastatins such as lovastatin

In vitro testing. Monotherapies of both LOV (see Table 25) and NaPA are effective (induction of cytostasis and phenotypic reversion) against gliomas in laboratory models and in man (described herein). However, when combined, the two drugs act synergistically to suppress glioma cell proliferation and induce reversion to a benign phenotype. Specifically, treatment of human glioblastoma A172 cells with pharmacologically achievable, yet suboptimal concentrations of LOV (0.1–0.5 μ M) combined with NaPA (1–3 mM) resulted in: (a) complete arrest of tumor cell replication (see FIG. 38); (b) over 90% decline in invasive capacity (see FIG. 39); and (c) profound inhibition of expression of TGF- β 2, coding for a 12.5-kD protein implicated in glioma autocrine growth, angiogenesis, and tumor-induced immunosuppression. Synergy between NaPA and LOV could be due to the ability of each to block the MVA pathway at distinct regulatory sites, leading to inhibition of protein

isoprenylation. Furthermore, NaPA may further induce tumor cytostasis and differentiation through additional mechanisms such as DNA hypomethylation, activation of nuclear receptors involved in growth control and glutamine depletion.

TABLE 25

| Gliomas are Uniquely Vulnerable to Lovastatin Treatment | |
|---|---|
| Cell Type more | Conc. of LOV required for 50% or inhibition of tumor replication |
| Glioblastoma (A172, U87, U251) | 0.2–2 μ M |
| Melanoma (1011) | 20 μ M |
| Lung Adenocarcinoma (A549) | 7 μ M |
| Prostate Cancer (PC3) | 5–10 μ M |
| Neuroblastoma (C1300) | 25 μ M ^a |
| EJ Bladder Carcinoma | 16 μ M ^b |

^aMaltese et al., 1985.
^bSebtii et al., 1991.

In vivo testing. Lovastatin was administered orally to 13 patients with refractory grade 3 and 4 astrocytomas at doses ranging from 30 to 35 mg/kg/day for seven consecutive days every four weeks. Activity was documented in four patients: 1 partial and 1 minor response, as well as disease stabilization for over six months in 2 additional patients. Performance status improved from 20 to 40% on Karnofsky's scale. NaPA (dose range 15 to 40 grams/24 h) was administered by continuous or interrupted intravenous infusions (CIVI) to 12 patients with similar histologies and clinical courses. Four patients exhibited clinical improvement: 2 minor responses and 2 disease stabilizations with significant improvement in performance status (1 for over 8 months, the other for over 1 month). Both modes of administration were associated with clinical activity (daily serum concentrations, mean \pm S.D.: 174 \pm 97 μ g/ml).

Early clinical experience with NaPA and LOV, individually, showed activity in patients with high grade gliomas at well tolerated doses. Interestingly, one patient who failed to respond to LOV, showed objective and clinical improvement upon treatment with NaPA, indicating that there may be no cross-resistance to these drugs. NaPA and LOV apparently do not have overlapping toxicities. While the dose-limiting toxicity of NaPA (serum concentration over 900 μ g/ml) is reversible CNS toxicity, rhabdomyolysis-induced myopathy was seen with LOV (at 35 mg/kg/day) (readily controlled by oral ubiquinone supplementation). Thus administration of NaPA in combination with LOV is beneficial to glioma patients without significant side effects or risk of toxicity.

SECTION N: NAPA AND NAPB EFFECTS ON
MELANOMA CELL LINES

The increased incidence of melanoma and the poor responsiveness of disseminated disease to conventional treatments call for the development of new therapeutic approaches. Phenylacetate, a nontoxic differentiation inducer can suppress the growth of other neuroectodermal tumors, i.e., gliomas, in laboratory models and in man. This led to exploration of the efficacy of phenylacetate and related aromatic fatty acids in malignant melanoma. Phenylacetate and phenylbutyrate were found to: (a) induce selective cytostasis and maturation of cultured human melanoma cells, (b) modulate the expression of genes implicated in

tumor metastasis (collagenase type I, TIMP) and immunogenicity (HLA class I); and, (c) enhance the efficacy of other agents of clinical interest, including retinoids, interferon alpha, suramin, and 5-aza-2'-deoxycytidine. Reflecting on the phenotypic heterogeneity of melanoma, the degree of biological alteration induced by phenylacetate/phenylbutyrate varied significantly among the tumor cell lines tested. While losing invasive capacity and tumorigenicity in athymic mice, poorly differentiated cells exhibited only a marginal changes in morphology, remained amelanotic, and resumed growth after treatment was discontinued. By contrast, treatment of melanoma cells that were in a more advanced stage of maturation resulted in profound alterations in cell growth, morphology and pigmentation consistent with terminal differentiation. Concentrations of phenylacetate and phenylbutyrate affecting tumor cell biology in vitro have been achieved in humans with no significant toxicities, suggesting clinical efficacy of these drugs in the treatment of malignant melanomas.

Sodium phenylacetate has been described herein as a nontoxic differentiation inducer (Samid, D., Shack, S., Sherman, L. J. (1992): Phenylacetate: a novel nontoxic inducer of tumor cell differentiation. *Cancer Research* 52: 1988–1992). Phenylacetate, a common metabolite of phenylalanine, regulates cell growth in diverse organisms throughout phylogeny (Kishore, G., Sugumaran, M., Vaidyanathan, C. S. (1976): Metabolism of DL-phenylalanine by *Aspergillus niger*. *J. Bacteriol.* 128: 182–191). At millimolar concentrations, phenylacetate selectively suppresses the growth of poorly differentiated plant, rodent, and human tissues (Wightman, F., Lighty, D. L. (1982): Identification of phenylacetic as a natural auxin in the stools of higher plants. *Physiol. Plant.* 55: 17–24). Cancerous cells, which are highly reminiscent of rapidly developing, immature tissues, are likewise vulnerable. NaPA promotes differentiation of human leukemic cells (Samid, D., Yeh, A., Prasanna, P. (1992): Induction of erythroid differentiation and fetal hemoglobin production in human leukemic cells treated with phenylacetate. *Blood* 80: 1576–1581) and brings about reversal of malignancy of various solid tumor cell lines, including hormone-refractory prostatic carcinoma, glioblastoma, neuroblastoma (Jindrich Cinat, Jaroslav Cinat, Marion Mainke, Albrecht Weibflog, Holger Rabenau, Bernhard Kornhuber, Hans-Wilhelm Doerr (1993): In vitro differentiation of human neuroblastoma cells induced by sodium phenylacetate. *Cancer Letters* 70: 15–24), and rhabdomyosarcoma (Grzegorz K. Gorski, Lydia E. Mc Morrow, Milton H. Donaldson (1993): Letter to the editor: synergistic inhibition of human rhabdomyosarcoma cells by sodium phenylacetate and tretinoin. *In Vitro Cell. Dev. Biol.* 29: 189–191). The central observation is that NaPA is active in tumor models at concentrations that can be achieved in children and adults with no significant adverse effects (Brusilow, S. W., Danney, M., Waber, L. J., Batshow, M., Burton, B., Levitsky, L., Roth, K., McKeethren, C., Ward, J. (1984): Treatment of episodic hyperammonemia in children with inborn errors of urea synthesis. *N. Eng. J. Med.* 310: 1630–1634; Simell, O., Sipila, I., Rajantie, J., Valle, D. L., Brusilow, S. W. (1986): Waste nitrogen excretion via amino acid acylation: benzoate and phenylacetate in lysinuric protein intolerance. *Pediatric Res.* 20: 1117–1121). Moreover, the activity against central nervous system (CNS) tumors, observed in cell cultures and rat models, has recently been confirmed in patients with advanced disease. The responsiveness of brain tumors that are refractory to conventional systematic therapies suggested that other neoplasms of neuroectodermal origin, including melanoma, may be susceptible as well.

Phenylacetate induces selective tumor cytostasis and phenotypic reversion of human melanoma cells when used at pharmacological nontoxic concentrations. Moreover, the present studies demonstrate for the first time, that phenylbutyrate, a precursor of phenylacetate, is a potent inducer of tumor cytostasis and differentiation.

EXAMPLE 26

NaPA treatment of melanoma cells

Cell Cultures and Reagents. The SKMEL 28, G361 and RPMI melanoma cell lines were from the American Type Culture Collection (ATCC, Rockville, Md.). A375 was a gift from J. Fidler (MD Anderson, Houston, Tex.). Melanoma lines 624 mel, 501 mel, 888 mel and 1011 mel were established from patients seen at the Surgery Branch NCI. These tumor cell lines as well as normal human melanocytes were kindly provided by J. Weber (NCI, Bethesda Md.). All tumor cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco Laboratories), antibiotics, and 2 mM L-glutamine. Primary melanocyte cultures were maintained in Melanocyte Basal Medium (Clonetics, San Diego, Calif.). The sodium salts of phenylacetic acid (NaPA) and phenylbutyric acid (NaPB) were provided by Elan Pharmaceutical Research Corp., Gainesville Ga.

PAG was synthesized by the reaction of phenylacetylchloride with glutamine (Thierfelder, H., and Sherwin, C. P. (1914): Phenylacetyl-Glutamin, ein Stoffwechsel-Product des Menschlichen Körpers (o") nach Eingabe von Phenyl-essigsäure (a"). *Ber. chem. Ges.* 47: 2630-2634). Briefly, 7.5 g glutamine (Aldrich, Milwaukee, Wis.) and 8.4 g NaHCO₃ were added to 200 ml H₂O, and the mixture was adjusted to pH 10. While stirring vigorously, 7.5 g of phenylacetylchloride (Milwaukee, Wis.) was added dropwise over the course of 1 hr. When the last of the phenylacetylchloride had dispersed, the solution was adjusted to pH 2, extracted twice with hexane, and taken to dryness. The dried powder was rinsed with hexane and dissolved in a minimum of boiling H₂O. Crystallized PAG having the convert melting range and a lean HPLC profile was confirmed. Suramin was from Mobay Chemical Corp. (New York, N.Y.). Interferon-alpha (Referon-A) was purchased from Hoffmann La-Roche Inc. (Nutley, N.J.). 5AzadC and all-trans-RA were from Sigma (St. Louis, Mo.).

Analysis of Cell Proliferation and Viability. Growth rates were determined by cell enumeration using a hemocytometer following detachment with trypsin/EDTA, and by an enzymatic assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide (MTT) (Alley, M. C., Scudiero, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Schoemaker, R. H., Boyd, M. R. (1988): Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* 48: 589-601). The two assays produced essentially the same results. DNA synthesis was determined by metabolic labeling with [³H] deoxythymidine (6.7 Ci/mmol) (New England Nuclear). Cell viability was assessed by trypan blue exclusion.

Immunocytochemistry. Cells were immunostained with anti-vimentin monoclonal antibodies using Dako PAP kit K537 (Dako Corporation, Calif.).

Invasion Through Matrigel. The ability of tumor cells to degrade and cross tissue barriers was assessed by an in vitro invasion assay that utilizes matrigel, a reconstituted basement membrane. Quantitative analysis was performed using

Biocoat Matrigel invasion chamber (Becton Dickinson Labware, Bedford Mass.) according to manufacturer's instructions.

Tumor Formation in Athymic Mice. Cells (5×10^5 cells per site) were injected s.c. into 4-6 week old female athymic nude mice (Division of Cancer Treatment, NCI animal Program, Frederick Cancer Research Facility). The number and size tumors were recorded after 4 weeks.

Quantitation of Melanin Production. Melanin content was measured by the calorimetric method described by Whittaker (Whittaker, J. R. (1963): Changes in melanogenesis during the dedifferentiation of chick retinal pigment cells in cell culture. *Dev. Biol.* 8: 99-127). Briefly, tumor cells were plated at $1-2 \times 10^6$ cells per point) by the addition of 0.5 ml. of deionized water with 2 cycles of freezing and thawing. Perichloric acid was added to a final concentration of 0.5N and the suspension was kept on the ice for 10 min, then centrifuged at 15,000 rpm for 5 min. The pellets were extracted twice more with 0.5N HClO₄ followed by two extractions with a cold mixture of ethyl alcohol:ethyl ether (3:1, v/v) and final extraction with ethyl ether. The pellets were air dried, 1 ml 0.85N KOH was added, and then dissolved by heating to 100° C. for 10 min. After insoluble residue was pelleted and the supernatant was cooled to room temperature and the absorbance at 400 nm was read in a double beam spectrophotometer (UV-160A, Shimadzu Corporation, Kyoto, Japan). A standard curve was constructed by using synthetic melanin (Sigma) dissolved in hot KOH at concentrations ranging from 5 to 150 µg/ml. The relative melanin content is expressed as the absorbance at 400 nm per 5×10^6 cells/mi.

Northern Blot Analysis. Messenger RNA was extracted from treated and control cells by Invitrogen mRNA isolation kit (San Diego, Calif.). Samples (5 µg/lane) were electrophoresed through 1% agarose/formaldehyde gels and blotted onto nytran membranes (Schleicher & Schuell, Keene N.H.), UV-cross-linked, and hybridization with ³²P-labeled specific probes. Tyrosinase cDNA probe was kindly provided by Y. Kawakami (NCI, Bethesda Md.). The probe for collagenase IV was 305 base pair PCR insert of p16SPT19-1 (human 72 kDa type IV collagenase cDNA), subcloned into the EcoRI/BamHI site of Bluescript SK-(2961 Base pairs). The β-actin probe (Oncor Inc., Gaithersburg Md.), was used as control to ensure equal loading of the samples.

Probes were labeled with ³²P dCTP (NEN) using a random primed DNA labeling kit (Ready-To-Go, Pharmacia P-L-Biochemicals, UK). Membranes were hybridized with probes (according to the Quikhyb protocol provided by Stratagene, La Jolla, Calif.) at 68° C. for 1 hour and washed twice for 15 min each at room temperature with 2X standard saline-citrate/0.1X sodium dodecyl sulfate, and once at 60° C. for 30 min with 0.1X standard saline-citrate/0.1X sodium dodecyl sulfate. Autoradiography was performed using Kodak XAR5 films at -70° C. with intensifying screens.

Inhibition of Melanoma Cell Proliferation by NaPA and NaPB. Exposure of human melanoma cells to NaPA or NaPB resulted in a dose-dependent growth arrest evident after three or more days of continued treatment (Table 26). The decline in cell proliferation was accompanied by similarly reduced DNA synthesis, but there was no change in cell viability. Compared to NaPA, NaPB was significantly more potent in inducing cytostasis, with IC₅₀ values ranging in seven of the eight tested cell lines between 0.15 and 1.0 mM, versus 2.0-5.7 mM for NaPA. Reflecting on the heterogeneous response of melanoma cells, RPMI cells were more resistant, requiring 8.4 mM NaPA or 1.5 mM NaPB to cause

50% inhibition of growth. However, primary normal melanocytes were even less sensitive, with IC₅₀ of NaPA and NaPB being 11.0 mM and 2.8 mM, respectively. Phenylacetylglutamine (PAG), the end-metabolite of NaPB and NaPA in humans, had no significant effect on tumor cell growth even at doses as high as 10 mM.

TABLE 26

| Cell Line | Growth Inhibition of Human Melanoma Cells by NaPA and NaPB | |
|-----------|--|------|
| | IC ₅₀ , mM | |
| | NaPA | NaPB |
| 624 mel | 5.0 | 1.0 |
| 501 mel | 2.0 | 0.15 |
| 888 mel | 5.2 | 1.6 |
| 1011 mel | 4.7 | 0.6 |
| A375M | 4.5 | 0.65 |
| SKMEL 28 | 5.7 | 0.75 |
| G361 | 4.2 | N.D. |
| RPMI | 8.4 | 1.5 |

Data represents the mean number of cells in treated versus control cultures, determined after 4 days of continuous exposure to drugs. Cell viability was over 95% in cultures treated with 10 mM NaPA or 3 mM NaPB.

Alterations in Morphology and Pigmentation. In addition to affecting tumor cell proliferation, the aromatic fatty acids induced morphological alterations consistent with melanocyte differentiation, including reduced nuclear to cytoplasm ratio, cell flattening with enhanced cytoskeletal organization, and in some cases, development of dendritic processes and increased melanogenesis. The degree of differentiation induced by NaPB/NaPA varied significantly depending on the phenotype of cells at the time treatment was initiated. For example, in A375 cultures, which are composed of amelanotic epithelial-like cells, only marginal morphological changes could be induced with occasional appearance of dendritic cells, while dramatic alterations were observed in the more differentiated 1011 cultures. Within 3–4 days of NaPB treatment, 1011 cells appeared enlarged with a markedly increased cytoplasm to nuclear ratio. Moreover, the cells had well organized cytoskeletons (evidenced by staining for vimentin), developed long dendritic processes, and became highly melanotic. Differentiation was progressive, involved the great majority of the cell population, and became a stable trait after 2 weeks of continuous treatment (see below). Consistent with its relative potency as a cytostatic agent, NaPB was more effective than NaPA in promoting terminal differentiation of 1011 cells. Yet, neither drug was capable of inducing major morphological changes nor melanin production in A375M cultures, suggesting endogenous resistance to differentiation.

Reversibility of Antitumor Effects. The stability of NaPB-induced cytostasis and differentiation appeared to depend not only on the cells treated but also the duration of exposure to the drug. In 1011 cultures, the doubling time increased from 26±2 hrs to approximately 110 hrs following 4-day or longer treatment with 1.5 mM NaPB. If treatment was discontinued after one week, the doubling time was reduced to 67 hr. However, if the duration was increased to 14 days of continuous exposure to phenylbutyrate, the replication rate was not changed significantly upon cessation of treatment (dell doubling every 96 hrs). This contrasted the finding with A375M cells, which resumed growth in the absence of the drug (doubling time of 20–24 hrs). As shown in FIG. 40, 1011 mel cells exposed to 1.5 mM NaPB for 14 days had 31.1 µg/ml melanin per 10⁶ cells; three days after treatment was discontinued melanin levels further increased

to 39.9 µg/ml. The loss of proliferative capacity, stable morphological change and persistent melanogenesis in 1011 cells are all indicative of a terminal differentiation induced by NaPB.

Loss of Invasiveness and Tumorigenicity. Malignant melanoma are highly invasive and metastatic in vivo. Since biologically aggressive cell populations could still exhibit some differentiation markers, including melanogenesis, it was important to further examine the effect of NaPB and NaPA on the malignant phenotype. The ability of A375M, SKMEL 28 and 1011 cells to degrade and cross tissue barriers was assessed by an in vitro invasion assay using a modified Boyden chamber with a matrigel-coated filter. After 3–5 days of continuous treatment with the aromatic fatty acids there was a dose-dependent loss of invasive capacity (Table 27). This in vitro indication of phenotypic reversion correlated with loss of tumorigenicity in vivo: A375M cells treated with NaPA for one week in culture, in contrast to untreated cells, failed to form-tumors when transplanted s.c. into athymic mice (Table 27). Almost complete inhibition of invasiveness and tumorigenicity were observed with 5 mM NaPA, a concentration that caused only partial cytostasis, indicating that malignant properties may be more vulnerable than cell proliferation in tissue culture dishes.

TABLE 27

| Treatment In vitro | Reduced Invasiveness In vitro and Tumorigenicity in Athymic Mice | | |
|--------------------|--|------------------------------|----------------------------|
| | Invasion | Tumor Formation ^b | |
| | Through Matrigel ^a (% of control) | incidence | tumor diam., mean (range). |
| None | 100 | 8/8 | 8.4 (2–20) |
| NaPA 2.5 mM | 59 ± 5.5 | 3/8 | 4.7 (0–12) |
| NaPA 5 mM | 22.5 ± 4 | 1/8 | 2.0 |
| NaPB 0.5 mM | 44.1 ± 6.7 | N.D. | N.D. |
| NaPB 1.0 mM | 16.1 ± 2.9 | N.D. | N.D. |

^aA375 cells pre-treated for 4 days were detached and assayed for their ability to invade a reconstituted basement membrane using a matrigel Invasion Chamber. Comparable inhibition of invasiveness was documented with SKMEL 28 and 1011 mel cells. Under the experimental conditions used, 2–3% of the untreated cells invaded the matrigel within 40 hrs (A375M, 1011) or 24 hrs (SKMEL 28).

^bA375M cells were pre-treated for one week with NaPA prior to being injected (5 × 10⁵ cells/animal) s.c. into athymic mice. Results indicate tumor incidence (tumor bearing/injected animals) and size of tumors, as determined 4 weeks after cell transplantation.

Modulation of Gene Expression. The profound changes in tumor biology were associated with alterations in expression of genes critical to maintenance of the malignant phenotype, as well as those that could affect immunogenicity in vivo. Specifically, cells treated with either NaPA or NaPB had reduced levels of collagenase type IV mRNA, coding for a 72,000 dalton metalloprotease. The latter is involved in degradation of extracellular stroma and basal lamina structures, with the potential to facilitate tumor invasion and metastasis. As with other tumor responses, the degree of changes in gene expression varied among the different cells lines tested.

For example, while 5 mM NaPA completely abrogated collagenase IV expression in SKMEL 28 cells, the specific transcript levels were reduced only 2 fold in A375M cells. The decline in collagenase IV in A375 treated with NaPA or NaPB was accompanied by increased expression of its inhibitor TIMP II, suggesting that the net proteolytic activity and, consequently, invasiveness may be significantly reduced by the aromatic fatty acids.

Synthesis of the differentiation marker melanin is regulated by the enzyme tyrosinase. Both NaPB and NaPA increased (albeit to a different degree) the steady-state levels of tyrosinase mRNA in 1011 cells, which correlated with increased pigmentation. The Northern blot analysis indicated that the treated cells also had approximately 2 fold elevated levels of HLA-A3 mRNA. The results are consistent with previous findings of increased MHC class I antigen expression in human leukemic and prostatic carcinoma cells following treatment with NaPA.

Potential of Activity of Other Antitumor Agents. Phenotypic heterogeneity is characteristic of tumor lesions in patients with melanoma. The diversity in therapeutic responses of heterogeneous tumor masses would require appropriate combination treatment protocols. Data summarized in FIG. 41 indicate that NaPA can significantly enhance the efficacy of other antitumor agents of clinical interest. Over 70% inhibition of melanoma cell proliferation was observed when NaPA was combined with nontoxic, yet suboptimal concentrations of IFN- α , all-trans-RA, suramin, or 5AzadC.

Melanoma is becoming an increasingly important cause of disease and death worldwide, with estimated 70,000 new cases occurring each year. Despite major advances in cytotoxic chemotherapy, immunotherapy, or biological therapy, the five year survival rate for individuals with disseminated disease is 14%, and the median survival for patients with only one site of metastasis is about seven months. In pursuit of developing new approaches to systemic therapy of advanced disease, the efficacy of the differentiation inducers phenylacetate and phenylbutyrate was explored. The two aromatic fatty acids have already been established as safe and effective in treatment of children and adults suffering from hyperammonemia (Brusilow, S. W., Danney, M., Waber, L. J., Batshow, M., Burton, B., Levitsky, L., Roth, K., McKeethren, C., Ward, J. (1984): Treatment of episodic hyperammonemia in children with inborn errors of urea synthesis. *N. Eng. J. Med.* 310: 1630-1634; Simell, O., Sipila, I., Rajantie, J., Valle, D. L., Brusilow, S. W. (1986): Waste nitrogen excretion via amino acid acylation: benzoate and phenylacetate in lysinuric protein intolerance. *Pediatric Res.* 20: 1117-1121), both have recently been approved by the FDA as investigative new drugs for the treatment of adults with cancer. Experimental data indicate that these relatively nontoxic compounds can induce cytostasis and phenotypic reversion of human melanoma cells.

The degree of changes by NaPA and NaPB varied significantly among the tested cell lines, as would be expected considering the highly heterogeneous nature of melanoma cells. There were however several common alterations in tumor cell and molecular biology observed, including: (a) reduced proliferative capacity; (b) loss of invasiveness associated with a decline in collagenase type IV and enhanced TIMP II expression; and, (c) increased expression of MHC class I antigens known to affect tumor immunogenicity in vivo. As with other differentiation inducers (Jardena Nordenberg, Lina Wasserman, Einat Beery, Doron Aloni, Hagit Malik, Kurt H. Stenzel, Abraham Novogrodsky (1986): Growth inhibition of murine melanoma by butyric acid and dimethylsulfoxide. *Experimental Cell Research* 162: 77-85), the ability of cells to undergo terminal differentiation following exposure to NaPA/NaPB appeared to depend on their state of cell maturation at the time treatment was initiated. For example, in poorly differentiated A375 cells, the drugs induced cytostasis with occasional appearance of dendritic cells, a slight increase in HLA expression, and loss of tumorigenicity in athymic mice; however, these cells

remained nonpigmented and tyrosinase-negative, and resumed growth in culture once treatment of cultures was discontinued. By contrast, exposure of the more mature 1011 cells to the aromatic fatty acids resulted in profound and stable changes in cell growth and morphology consistent with terminal differentiation. NaPB-treated 1011 cells became heavily pigmented and polydendritic, and had elevated levels of tyrosinase and class I HLA mRNAs. Preliminary protein analysis indicates that NaPB causes significant increase in both MHC class I and II surface antigens. These surface proteins are necessary, if not sufficient, to evoke proliferative and cytotoxic T-cell responses against malignant melanomas (Guerry, D. IV, Alexander, M. A., Herlyn, M. F., Zehngelot, L. M., Mitchell, K. F., Zmijewski, C. M., Lusk, E. J. (1984): HLA-DR histocompatibility leukocyte antigens permit cultured human melanoma cells from early but not advanced disease to stimulate autologous lymphocytes. *J. Clin. Invest.* 73: 267-271; Fossati, G., Taramelli, D., Balsari, A., Bogdanovich, J., Ferrone, S. (1984): Primary but not metastatic human melanomas expressing DR antigens stimulate autologous lymphocytes. *Int. J. Cancer* 33: 591-597).

NaPB and NaPA are closely related aromatic fatty acids. Phenylbutyrate is metabolized by mitochondrial β -oxidation to form phenylacetate. Phenylacetate, in turn, can be converted back to phenylbutyrate through the action of medium-chain fatty acid elongase. Reminiscent other differentiation inducers such as retinoids and vitamin D derivatives, NaPA and NaPB were recently found to activate a member of the steroid nuclear receptor family, which functions as a transcriptional factor. In addition, both compounds can inhibit the mevalonate pathway of cholesterol synthesis, and thus interfere with post-translational processing of proteins critical to signal transduction and mitogenesis. At growth inhibitory concentrations, NaPB and NaPA also alter the pattern of DNA methylation, an epigenetic mechanism controlling the transcription of various eukaryotic genes. NaPB's ability to activate the expression of otherwise dormant methylation-dependent genes was documented in experimental models and in man (Samid, D., Yeh, A., Prasanna, P. (1992): Induction of erythroid differentiation and fetal hemoglobin production in human leukemic cells treated with phenylacetate. *Blood* 80: 1576-1581; Brusilow, S. W., Danney, M., Waber, L. J., Batshow, M., Burton, B., Levitsky, L., Roth, K., McKeethren, C., Ward, J. (1984): Treatment of episodic hyperammonemia in children with inborn errors of urea synthesis. *N. Eng. J. Med.* 310: 1630-1634). Thus, NaPA and NaPB affect some common pathways leading to restored growth control and cell maturation.

The findings with melanoma cells lines indicate, however, that NaPB is a more potent modulator of gene expression and cell biology compared to NaPA. The relative potency was confirmed in various hematopoietic and solid tumors including lymphomas, gliomas, and adenocarcinomas of the prostate, breast, ovarian, lung and colon. The latter could be due to the higher lipophilicity of NaPB, or be related to some differences in mechanisms of action. It is possible that, prior to being metabolized to phenylacetate, phenylbutyrate may act in an analogous way to the short fatty acid, butyrate, a well characterized differentiation inducer with therapeutic potential. There are, however, several differences in tumor responses to phenylbutyrate versus butyrate. While the former increased vimentin organization in human melanoma cells, butyrate was reported to decrease vimentin expression in B-16 melanoma cells (Ryan and Higgs 1988). Furthermore, in contrast to NaPB, butyrate failed to induce, and in some cases even inhibited tyrosinase activity and melaniza-

tion of melanoma cells (Jardena Nordenberg, Lina Wasserman, Einat Beery, Doron Aloni, Hagit Malik, Kurt H. Stenzel, Abraham Novogrodsky (1986): Growth inhibition of murine melanoma by butyric acid and dimethylsulfoxide. *Experimental Cell Research* 162: 77-85). Additional unique attributes of phenylbutyrate with potential clinical implications include its extended half-life (hours, versus minutes for butyrate), and the conversion to phenylacetate, itself a cytostatic and differentiation inducer. Before excretion in the urine, phenylacetate must first be conjugated with glutamine to form PAG. High rates of urinary excretion of PAG associated with NaPA-related therapies, can beneficially deplete plasma glutamine (Simell, O., Sipila, I., Rajantie, J., Valle, D. L., Brusilow, S. W. (1986): Waste nitrogen excretion via amino acid acylation: benzoate and phenylacetate in lysinuric protein intolerance. *Pediatric Res.* 20: 1117-1121), the amino acid critical to tumor growth (Hiroyuki Takahashi, Peter G. Parsons (1990): In vitro phenotypic alteration of human melanoma cells induced by differentiating agents: Heterogeneous effects on cellular growth and morphology, enzymatic activity, and antigenic expression. *Pigment Cell Research* 3: 223-232).

It appears therefore that NaPB and NaPA cause reversion of malignant melanoma cells and may be of value in management of this fatal disease.. In developing these drugs for clinical use, it will be important to consider the heterogeneity of melanomas. Metastatic melanoma cells vary widely in their growth rate, morphology and degree of pigmentations; such diversity was observed in different metastases and even among cells within a single lesion (Weber, G. (1983): Biochemical strategy of cancer cells and the design of chemotherapy:G.H.A. Clowes Memorial Lecture. *Cancer Res.* 43: 3466-3492; Hiroyuki Takahashi, Peter G. Parsons (1990): In vitro phenotypic alteration of human melanoma cells induced by differentiating agents: Heterogeneous effects on cellular growth and morphology, enzymatic activity, and antigenic expression. *Pigment Cell Research* 3: 223-232). In view of the reversibility of effect seen with poorly differentiated subpopulations, it is likely that prolonged duration of treatment would be required in order to benefit patients. Enhanced surface antigen expression and reduced production of tumor-secreted immunosuppression factors could eventually result in tumor rejection by the host immune system. In cases where it might be necessary to target highly divergent tumor cell populations, the aromatic fatty acids could be combined with other antitumor agents to enhance efficacy, minimize adverse side effects, and prevent disease relapse. To this end, the potential of combining NaPA with other differentiation inducers (retinoids), DNA hypomethylating drugs (5AzadC), growth factor antagonists (suramin), and cytokines (interferons) is demonstrated.

Both NaPA and NaPB are currently in clinical trials at the NCI. Ongoing phase I studies with NaPA, involving primarily patients with malignant glioma and hormone-refractory prostate cancer, indicate that therapeutic levels can be achieved with no significant adverse effects and result in objective clinical improvement in patient with advanced disease (described elsewhere herein).

SECTION O: NAPA AND
NAPB—HYDROXYUREA COMBINATION
TREATMENT

Previous clinical trials have indicated that hydroxyurea could possess some activity against prostate cancer. The in vitro activity of hydroxyurea was evaluated in three hormone-refractory prostate cancer cell lines, PC-3, DU-145,

and PC-3M (as measured by the MTT method). Cytotoxicity was noted at concentrations $\geq 100 \mu\text{M}$ of hydroxyurea, requiring at least 120 hours of drug exposure ($100 \mu\text{M}$ was the approximate IC_{50} for all three cell lines). Based on clinical pharmacokinetic data, a dosing regimen was simulated to produce a hydroxyurea plasma concentration greater than $100 \mu\text{M}$ for 120 hours (1 g loading dose, followed by 500 mg every 6 hours for 5 days in a 70 kg man). Since this plasma concentration may result in an unacceptable degree of myelosuppression, in vitro combinations studies were conducted with hydroxyurea and phenylbutyrate, a new differentiating agent. These studies resulted in a reduction of the hydroxyurea concentration necessary for a 50% growth inhibition ($50 \mu\text{M}$ of hydroxyurea plus 0.5 mM of phenylbutyrate). A regimen designed to achieve that hydroxyurea concentration (400 mg loading dose, followed by 200 mg every 6 hours for 5 days) should be clinically achievable. The combination was further evaluated for use in treatment of patients with Stage D prostate cancer.

TABLE 30

| Clinical Trials Using Hydroxyurea in Prostate Cancer | | | | | |
|---|------|----|----------------|----------------|--------------------------------|
| Author | Year | N | OR | SR | R HU Dose |
| Lerner | 1977 | 30 | 63% (19/30) | 76% (23/30) | N 80 mg/kg q3d x 6 wks |
| Kvols | 1977 | 5 | 60% (3/5) | NR | N 3 gm/m ² q3d |
| Loening | 1981 | 28 | 14% (4/28) | 21% (6/28) | Y 3 gm/m ² q3d |
| Mundy | 1982 | 22 | 36% (8/22) | 68% (15/22) | N 80 mg/kg q3d |
| Stephens | 1984 | 69 | 4% (1/24)* | 13% (9/69) | Y 3.6 gm/m ² 2 d/wk |

N = Number of patients receiving hydroxyurea
OR = objective response (stable, partial, complete)
SR = subjective response
R = randomized (N = No, Y = Yes)
HU Dose = Dose of hydroxyurea
*Stable response not reported, and only evaluated patients with soft tissue disease
Response criteria varied between trials

EXAMPLE 27

NaPA in combination with hydroxyurea—PC-3 and DU-145 cells

Two of the human prostate cell lines (PC-3 and DU-145) were obtained from American Type Culture Collection, Rockville, Md. PC-3M was obtained from James Kozlowski, M. D. at the University of Wisconsin via the NCI Cancer Treatment Screening Program at Frederick, Md. Hydroxyurea was purchased from Sigma Chemical Co., St. Louis, Mo. Phenylbutyrate was obtained from Elan Pharmaceutical Research Corporation (Gainesville, Ga.). Cell culture medium used was RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin 5,000 units/mL, streptomycin 5,000 $\mu\text{g/mL}$ and 2 mM L-glutamine (Gibco Laboratories, Grand Island, N.Y.). The prostatic carcinoma cell lines were propagated in RPMI-1640, which was supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). The cells were grown to 80% confluent monolayers (6×10^5 cells/sq. cm) and cultivated in 75-sq. cm flasks (Nunc, Denmark). Cells were harvested with trypsin (0.05%):EDTA (0.02%) (Gibco Laboratories, Grand Island, N.Y.) solution and counted in a hemocytometer. The cells were then seeded into 96 well microtiter plates (CoStar, Cambridge, Mass.) at a density of

3,000 cells per well in RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin solution and reincubated for 24 hours to allow for cell reattachment (37° C., 5% CO₂ atmosphere). Hydroxyurea, diluted in tissue culture medium to yield a final well volume of 200 μ L, was then added at specified concentrations (0.01, 0.1, 1, 10, 100, 1,000, 10,000, and 100,000 μ M) and left undisturbed for 120 hours in the tissue culture incubator. Control cells were grown in an equal volume of medium. The 3-(4,4-dimethyl-2-thiazolyl)-2,5-di-phenyl-2H-tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, Mo.) assay was used to estimate cell number (Beckloff GL, Lerner HJ, Frost D, et al. Hydroxyurea (NSC-32065) in biologic fluids: dose-concentration relationship. *Cancer Chemother Rep* 1965;48: 57-8). After exposure to hydroxyurea, MTT (20 μ L, 5 mg/mL in PBS) was added to the wells, incubated for 4 hours, and centrifuged at 2,000 g, and the medium was decanted. Dimethyl sulfoxide (150 μ L) was added to each well, the plates were shaken for 30 seconds, and the optical density at 540 nm was determined on a kinetic microplate reader (Bio-Tek EC340 Immunoplate-reader).

The synergistic activity studies were conducted with PC-3 cells by adding 0.5 mM and 1 mM of phenylbutyrate to 50 μ M and 100 μ M of hydroxyurea. Similar in vitro conditions and durations of drug exposure were utilized as described above. Cell numbers were determined by hemocytometer.

Statistical analysis was performed using the SAS computer program (version 6.02, SAS Institute, Inc., Cary, N.C.). The mean optical density for the various concentrations of hydroxyurea versus control were compared by an adjusted two-sided Wilcoxon:rank-sum test. An alpha of 0.05 was used to detect statistical difference. To determine the relationship between hydroxyurea dose and cell survival, the PC-3 experiments were conducted five times for the PC-3 cell line and three times for both DU-145 and PC-3M. In each experiment there were six wells were concentration. The experiments to determine the optimal duration were performed once with 12 wells per fixed concentrations (five plates for both methods).

For all three cell lines there was a dose-dependent decrease in the percentage of cells surviving with hydroxyurea concentrations between 10 and 1,000 μ M (see FIG. 42). At a concentration of 100 μ M there was an approximate 50% reduction in cell survival (42.7%, 45.5%, and 52% for PC-3, DU-145, and PC-3M, respectively) for all three cell lines, compared to untreated cells ($p=0.016$). Increases in the hydroxyurea concentration above 1,000 μ M exerted little further effect on the inhibition of cell growth. (see FIG. 42). At concentrations less than 100 μ M, hydroxyurea was only cytostatic, but cytotoxicity increased with increasing concentrations less than 100 μ M, 86% of the total cells were viable at 100 μ M and 41.9% at 1,000 μ M, determined by trypan blue staining (Gibco Laboratories, Grand Island, N.Y.).

Two additional experiments were conducted with PC-3 to investigate the relationship between the duration of hydroxyurea exposure and inhibition of cell growth. In the first experiment cell survival was assessed daily, from 24 to 120 hours of exposure, with four different concentrations of hydroxyurea (0.1, 1.0, 10, and 100 μ M); the MTT assay was performed immediately upon completion of the specified duration of hydroxyurea exposure. In the second experiment cells were exposed to the same concentrations of hydroxyurea for varying periods of time (24, 48, 72, 96 and 120 hours), but grown for a total of 120 hours before performing the MTT assay. Drug exposure was terminated at the various intervals by replacing drug-containing medium with drug-

free medium. This later experiment was performed to detect the possibility of a recovery in cell growth following brief periods of drug exposure. (see FIG. 43) In both experiments there was decrease in the percentage of surviving cells with increasing duration of exposure. There was no evidence of a plateau in the effect by 120. hours, nor of a resurgence in cell growth following drug washout.

With increasing concentrations of hydroxyurea there was a marked alteration in PC-3 cell morphology. Specifically, it appears that there was a significant degree of cellular selectivity in the PC-3 cell line when exposed to 100 μ M hydroxyurea concentrations compared to control cells. The PC-3 cells are heterogenous and hydroxyurea may have selected cells with a low nucleus/cytoplasm ratio (i.e. large flat cells).

The pharmacokinetic model and model parameters used to describe the disposition of hydroxyurea in humans were derived from concentration versus time data previously reported from clinical trials of this drug (Beckloff GL, Lerner HJ, Frost D, et al. Hydroxyurea (NSC-32065) in biologic fluids: dose-concentration relationship. *Cancer Chemother Eep* 1965; 48:57-8; Veale D. Cantwell BMJ, Kerr N. et al. Phase I study of high-dose hydroxyurea in lung cancer. *Cancer Chemother Pharmacol* 1988;21: 53-6; Adamson RH, Ague SL, Hess SM, et al. The distribution, excretion and metabolism of hydroxyurea-C14. *J. Pharmacol and Exp Therapeut* 1965; 150: 322-7; Belt RJ, Haas CD, Kennedy J, et al. Studies of hydroxyurea administered by continuous infusion. *Cancer* 1980;46: 455-62; Rosner F, Rubin H, Parise F. Studies on the absorption, distribution, and excretion of hydroxyurea (NSC-32065). *Cancer Cehmother Rep* 1971;55: 167-73; Creasey WA, Capizzi RL, DeConti RC. Clinical and biochemical studies of high-dose intermitten therapy of solid tumors with hydroxyurea (NSC-32065). *Cancer Chemother Rep* 1970;54: 191-4; Bolton BH, Woods LA, Kaung DT, et al. A simple method of colorimetric analysis for hydroxyurea (NSC-32065). *Cancer Chemother Rep* 1965;46: 1-5; Davidson JD, Winter TS. A method of analyzing for hydroxyurea in biological fluids. *Cancer Chemother Rep* 1963;27: 97-110). Curve-stripping techniques (Abbottbase™ Pharmacokinetic System, ver. 1.0) applied to the concentration versus time data following a single oral dose of hydroxyurea (80 mg/kg) indicated that these data were best described by a single exponential decay, allowing a single compartment open linear model to be used in predicting hydroxyurea's pharmacokinetics. The bioavailability of hydroxyurea has never been precisely determined but is reported to be "complete" (Donchower RC. Hydroxyurea. In: Chabner BA, Collins JM, eds *Cancer Chemotherapy, Principles and Practice*, Philadelphia: JB Lippincott 1990: 225-33) hence a bioavailability of 100% was assumed. Estimates of hydroxyurea's total body clearance were obtained using the trapezoidal rule. Using the average parameter values derived from this model (volume of distribution, half-life, and clearance) the hydroxyurea dosing regimen used by Lener et al. (80 mg/kg every third day; see FIG. 44A) was simulated. This regimen provides only very brief exposure to hydroxyurea concentrations with in vitro antiproliferative activity. A regimen of 1.0 g loading dose followed by 500 mg every 6 hours for 5 days in a 70 kg man is shown in FIG. 44B; this regimen approximates the conditions required in vitro for 50% inhibition of prostate cancer cell growth.

Because hydroxyurea plasma concentrations of 100 μ M or greater may cause an unacceptable degree of myelosuppression in some patients, the ability of phenylbutyrate to potentiate the efficacy of hydroxyurea was examined.

Hydroxyurea at the suboptimal concentration of 50 μ M was combined with phenylbutyrate at concentrations of 0.5 mM and 1 mM. A 58% reduction in PC-3 cell growth was achieved when 0.5 mM of phenylbutyrate was added to 50 μ M of hydroxyurea. As monotherapy, hydroxyurea (50 μ M) reduced cell proliferation by only 38% and phenylbutyrate, 0.5 mM and 1 mM, by 38% and 55%, respectively. Approximately 80% inhibition of PC-3 growth was observed when 50 μ M hydroxyurea was combined with 1 mM of phenylbutyrate, without affecting cell viability. (see FIG. 45).

The results of previous clinical trials indicate that hydroxyurea could possess some activity against hormone refractory prostate cancer. Lerner et al. initially evaluated hydroxyurea in 1977 for the treatment stage D prostate cancer (Lerner HJ, Malloy TR. Hydroxyurea in stage D carcinoma of prostate. *Urol* 1977;10:35-8). The drug was administered as a single oral dose (80 mg/kg) every third day. A partial objective tumor response was reported in 15 of the 30 patients treated. Subsequent to Lerner's initial study there have been four additional clinical reports of hydroxyurea in hormone-refractory prostate cancer (Kvols LK, Eagan RT, Myers RP. Evaluation of melphalan, ICRF-159, and hydroxyurea in metastatic prostate cancer: a preliminary report. *Cancer Treat Rep* 1977;61: 311-2; Loening SA, Scott WW, deKernion J, et al. A Comparison of hydroxyurea, methylchloroethyl-cyclohexylnitrosourea and cyclophosphamide in patients with advance carcinoma of the prostate. *J Urol* 1981;125: 812-6; Mundy AR. A pilot study of hydroxyurea in hormone "escaped" metastatic carcinoma of the prostate. *Br J Urol* 1982;54:20-5; Stephens RL, Vaughn C, Lane M, et al. Adriamycin and cyclophosphamide versus hydroxyurea in advanced prostatic cancer. *Cancer* 1984;53: 406-10). Mundy studies the same regimen of hydroxyurea administration (80 mg/kg every third day) in 22 patients failing hormonal therapy and reported improvement in bone pain and performance status in 15 of these patients (68%) within 6 weeks of starting therapy (Mundy AR. A pilot study of hydroxyurea in hormone "escaped" metastatic carcinoma of the prostate. *Br J Urol* 1982;54: 20-5). Kvols et al. reported a single partial objective response with hydroxyurea in a group of five patients (Kvols LK, Eagan RT, Myers RP. Evaluation of melphalan, ICRF-159, and hydroxyurea in metastatic prostate cancer: a preliminary report. *Cancer Treat Rep* 1977;61: 311-2). The activity of hydroxyurea in hormone-refractory prostate cancer was evaluated in a randomized study carried out by the National Prostatic Cancer Project. In this study 125 patients failing hormonal therapy were randomly assigned to treatment with either methyl-chloroethyl-cyclohexylnitrosourea (semustine), cyclophosphamide or hydroxyurea (Loening SA, Scott WW, deKernion J, et al. A Comparison of hydroxyurea, methylchloroethyl-cyclohexylnitrosourea and cyclophosphamide in patients with advance carcinoma of the prostate. *J Urol* 1981;125: 812-6). Objective response was observed in 30% of the patients receiving semustine, 35% of the patients receiving cyclophosphamide, and 15% of the patients receiving hydroxyurea. Nonetheless, improvement in performance status was greater in the group of patients receiving hydroxyurea (18%, 5 of 28 patients) than in the other two groups (1 of 27 patients for the semustine group and 5 of 43 patients for the cyclophosphamide group), and it was equivalent in relieving bone pain. Another randomized, multicenter trial compared the regiment of doxorubicin plus cyclophosphamide to hydroxyurea in 158 patients with stage D prostate cancer (Stephens RL, Vaughn C, Lane M, et al. Adriamycin and cyclophosphamide versus hydroxyurea in advanced prostatic cancer. *Cancer* 1984;53: 406-10). No

statistically significant difference in antitumor activity could be demonstrated between the two regimens (objective response rate 6 of 19 patients versus 1 of 24 patients, $p=0.06$). However, response was evaluated only in patients with measurable soft tissue disease, who represent a minority (15%) of the patients with prostate cancer and whose biology may differ from that of patients having disease limited to bone (Hanks GE, Myers CE, Scardino PT. Cancer of the Prostate. In: DeVita VT, Hellman S, Rosenberg SA, eds. *Cancer: Principles and Practice of Oncology*, Fourth Edition. Philadelphia: JB Lippincott 1993;1104). Unfortunately, comparing the response rates for these trials is impeded by the use of heterogeneous, imprecise and vague criteria at the time the trials were conducted.

If the modes of antitumor activity observed in previous clinical trials of hydroxyurea in hormone-refractory prostate cancer were the result of the drug's ability to inhibit DNA synthesis during S-phase, then it is clear that the dosing regimens used in those trials were suboptimal. It is reasonable to assume that the regimen simulated in FIG. 44B would undoubtedly result in neutropenia. In order to reduce the toxic effects of hydroxyurea, a reduced concentration was combined with a clinically achievable concentration of phenylbutyrate, a relatively non-toxic differentiation inducer currently undergoing clinical trials. Phenylbutyrate undergoes rapid conversion to phenylacetate in vivo by B-oxidation (Knoop F. Der Abbau aromatischer fettsaure Tierkörper. *Beir Chem Physiol Pathol* 1905;6: 150-62). Phenylbutyrate and phenylacetate have been used in children with urea cycle abnormalities and appear well tolerated in high-doses (Brusilow SW, Horowich AL. Urea cycle enzymes. In: Scriber C, Beaudet A. Sly W, ValleDr, eds. *Metabolic Basis of inherited Diseases*, New York: McGraw Hill 1987: 629). Both agents have been recently identified as antineoplastic agents affecting tumor growth and maturation. In addition to causing selective cytostasis, both induce malignant cells to undergo reversions to a more benign phenotype. The data indicate that a combination of hydroxyurea and phenylbutyrate each used at cytostatic concentrations results in significant inhibition of cell proliferation of the PC-3 cell cultures.

In conclusion, the results indicate that a much greater hydroxyurea exposure (i.e. >100 μ M for at least 120 hours), as a single agent, is required for cytotoxic cell death than has been achieved in previous clinical trials. A regimen designed to achieve that concentration would most likely result in unacceptable side effects. However, hydroxyurea in combination with phenylbutyrate has a clinical role in patients with hormone refractory metastatic prostate cancer. This combination regimen requires a relatively low-dose of hydroxyurea (400 mg loading dose followed by 200 mg every 6 hours for 5 days, see FIG. 44C) to produce a concentration of 50 μ M, as well as a reduced concentration of phenylbutyrate. The hydroxyurea dose employed may cause a mild reduction in neutrophils, but this reduction should be clinically tolerated. The adverse effects associated with phenylbutyrate should not be additive (i.e., CNS depression) to the myelosuppression associated with hydroxyurea. Based on these results, this combination deserves further evaluation in patients with Stage D prostate cancer.

SECTION P: NAPA AND NAPB EFFECTS ON MEDULLOBLASTOMA & ASTROCYTOMA DERIVED CELLS

Medulloblastoma, and other malignant brain tumors, are heterogeneous with regard to cell morphology, antigenic

phenotype and biochemical features. A proportion of cells express a more differentiated phenotype. Malignant transformation of cells does not necessarily abrogate their potential for expression of differentiated characteristics including cessation of proliferation (Sartorelli, AC: Malignant cell differentiation as a potential therapeutic approach. *Br. J. Cancer* 52: 293-302, 1985; Marks PaA, Sheffery M, Rifkind RA: Induction of transformed cells to terminal differentiation and the modulation of gene expression. *Cancer Res.* 47: 659-666, 1987). Spontaneous differentiation of medulloblastoma to a non-proliferative state occasionally does occur (DeChadarevian J-P, Montes JL, Govman AM, Freman CR: Maturation of cerebellar neuroblastoma into ganglioneuroma with melanosis. *Cancer* 59: 69-76, 1987), indicating that neuroectodermal tumors may be capable of differentiation to an effective signal. These biological features and the current lack of sufficiently effective therapeutic strategies make agents which may induce differentiation of these tumors particularly interesting.

The mechanisms by which differentiating agents affect the growth and phenotypic characteristics of responsive cell lines have been studied extensively and although their actions are incompletely understood, a substantial body of evidence indicates that agents such as all-trans retinoic acid (ATRA) interact with autocrine growth control pathways (Falk LA, DeBenedetti F, Lohrey Net al.: Induction of TGF β 1 receptor expression and TGF β 1 protein production in retinoic acid treated HL-60 cells. *Blood* 77: 1248-1255, 1991). A variety of growth factor molecules and their receptors are affected by exposure to differentiating agents such as ATRA. In a number of neoplastic cell lines as well as non-neoplastic cell types, ATRA induces production and secretion of one or more isoforms of TGF β (Falk LA, DeBenedetti F, Lohrey Net al.: Induction of TGF β 1 receptor expression and TGF β 1 protein production in retinoic acid treated HL-60 cells. *Blood* 77: 1248-1255, 1991).

The TGF β autocrine growth regulatory pathways are of particular interest in primary central nervous system tumors. This pluripotential growth regulator is produced by both primary malignant astrocytoma tissue (Clark WC, Bressler J: TGF β -like activity in tumors of the central nervous system. *J Neurosurg* 68: 920-924, 1988; Samuels V, Barrett JM, Brochman Set al.: Immunocytochemical study of transforming growth factor expression by benign and malignant gliomas. *Am J Pathol* 134: 895-902, 1989) and by cell lines derived from such tumors (Jennings MT, Macina RJ, Carver R et al.: TGF β 1 and TGF β 2 are potential growth regulators for low grade and malignant gliomas in vitro with evidence in support of an autocrine hypothesis. *Int. J. Cancer* 49: 129-139, 1991). The immunosuppressive effects of malignant astrocytoma cells on cocultured lymphocytes in vitro has been convincingly linked to TGF β production and can be partially neutralized by antibodies against TGF β . TGF β has been shown to be a growth regulator for gliomas in vitro (Jennings MT, Macina RJ, Carver R et al.: TGF β 1 and TGF β 2 are potential growth regulators for low grade and malignant gliomas in vitro with evidence in support of an autocrine hypothesis. *Int. J. Cancer* 49: 129-139, 1991). The role of the TGF β pathway in growth regulation of medulloblastoma is less well established than for malignant astrocytomas. The antiproliferative effect of ATRA on Daoy medulloblastoma cells is associated with increased secretion of TGF β 2 and with induction of TGF β receptor expression. Because the TGF β family of growth factors plays such an important role in the biology of malignant astrocytomas, the initial focus has been on this potential autocrine pathway.

The effects of a nontoxic differentiation inducer, phenylacetate (PA), on neuroectodermal tumor-derived cell lines

were examined. Treatment of medulloblastoma (Daoy, D283) and glioma (U251, C6, RG2) cell lines resulted in a dose-dependent decline in DNA synthesis and cell proliferation, with ID₅₀ values ranging from 6.3 to 14.6 mM. PA increased TGF β 2 production by medulloblastoma Daoy cells; however, neutralizing antibodies against either TGF β 2 or TGF β 1 failed to block the growth arrest observed, suggesting that, unlike other differentiation agents, such as retinoic acid, the antiproliferative effect of PA is not mediated by a TGF β pathway. In addition to cytostasis, PA induced marked morphological changes in U251 and C6 glioma cells associated with increased abundance of GFAP-positive processes. Although the morphology of PA-treated medulloblastoma cells was not significantly altered, the D283 cells exhibited increased expression of neurofilament proteins and Hu antigen, indicative of differentiation along a neuronal pathway. The effects of PA on the medulloblastoma cell lines were compared to the effects of PA on the well established human neuroblastoma differentiation model BE(2)C, which is capable of a bidirectional differentiation towards a neuronal or a glial/schwann cell pathway. In BE(2)C cells PA induced differentiation towards a schwann/glia cell like phenotype, suggesting that the choice of differentiation pathway is cell type and agent specific.

EXAMPLE 28

NaPA efficacy on Astrocytoma Cells

Cell culturing techniques. Human glioma (U251), rat glioma (C6, RG2), human medulloblastoma (Daoy, D283), human neuroblastoma (BE(2)C) (all from ATCC) and the murine lung fibroblast cell line MuLv1 (provided by Dr. S. Cheifetz) were routinely maintained in Eagles minimal essential media (MEM) (GIBCO) supplemented with 10% fetal calf serum (FCS), nonessential amino acids (NEAA), L-glutamine (2 mM), penicillin (100U/ml) and streptomycin (100 ug/ml) (all medium constituents were purchased from Gibco, Grand Island, N.Y., USA). Adherent cell lines were plated in T-150 tissue culture flasks (Becton-Dickenson) and passaged using trypsinization when 70% confluent. The cell line growing in suspension (D283) was maintained at a cell density of 10^5 - 10^6 cells per ml. Cells were grown at 37° C. in a humidified 5% CO₂ incubator. For glutamine depletion studies U251 cells were adapted to serum- and glutamine-free Ultraculture medium (Hyclone). For proliferation studies with exogenous TGF β 1 and TGF β 2 and neutralizing antibodies against TGF β Daoy cells were grown in MEM containing 0.2% FCS and U251 cells were cultured in serum-free Ultraculture medium (Hyclone).

PA- and ATRA-treatment of cell lines. Phenylacetic acid (Sigma) was dissolved in MEM to make a 100 mM stock solution and the pH was adjusted to 7.2 with NaOH. Then the MEM was supplemented with 10% heat-inactivated FCS. All-trans-retinoic acid (ATRA) was dissolved in ethanol to prepare a 10^{-3} M stock solution and added to the media to yield final concentrations ranging from 10^{-7} to 10^{-6} M.

Cell proliferation assay. For proliferation studies tumor cells in log phase growth were harvested and resuspended at a concentration of 20,000 cells/ml; 100 ul/well of the cell suspension was placed into 96-well microtiter plates (Costar). After 24 h the cells were incubated with an equal volume of the serial dilutions of PA to yield final concentrations ranging from 5 to 20 mM PA. MEM containing 10% FCS served as a control. Cell proliferation was determined at 24 h intervals by ³H-thymidine incorporation. One μ Ci of

³H-thymidine (Amersham) was added to each well and the microtiter plates were incubated for 4 h at 37° C. Then the cells were harvested onto glass fiber filters using a semi-automated cell harvester (Cambridge Biotechnologies) and ³H-thymidine activity was quantitated using a liquid scintillation counter (Hewlett Packard). All experiments were set up in quadruplicates.

Effects of exogenous TGFβ. To determine the effects of exogenous TGFβ on Daoy cells recombinant human TGFβ1 and TGFβ2 (R&D Systems) at concentrations ranging from 0.5 pM to 1 nM were added to similarly prepared microtiter plates for 24 h prior to determining ³H-thymidine uptake.

Neutralizing studies with anti-human TGFβ1 and TGFβ2 antibodies. Polyclonal antibodies against human TGFβ1 and TGFβ2 (R&D Systems) were coincubated in concentrations of 10 to 40 μg/ml with Daoy cells grown in 0.2% FCS or U251 cells grown in serum-free medium (Ultraculture, Hyclone) exposed to PA 10 mM. Proliferation was determined by ³H-thymidine incorporation as described above.

Washout studies. Cells were seeded similar to proliferation studies as described before except for a number of 250 cells per well. Two days after treatment with PA at various concentrations the agent containing medium was replaced by plain culture medium and ³H-thymidine uptake was measured after 4, 8, 24, 48 and 72 h.

Immunocytochemistry. Adherent cell lines were grown on Lab-Tek 8-chamber slides (Nunc) under the same conditions previously described for cell cultures. After 24 h the cells were treated with PA 20, 10 or 5 mM for 4 to 7 days. After fixation in cold acetone immunoreactivity was determined using the avidin-biotin-peroxidase complex method of Hsu and colleagues (Hsu SM, Raine L and Fanger H: Use of avidin-biotin-peroxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem Cytochem* 29: 577-580, 1981). For nonadherent cell lines, cytocentrifuged preparations were made using a Cytospin II (Shandon) and the cells fixed essentially as described above.

Mouse mAbs specific against GFAP (clone G-A-5), NF triplet proteins (68 kD (clone NR4), 160 kD (clone BF10) and 200 kD (clone RT97)), synaptophysin (clone SY38) or vimentin (clone V9) were purchased from Boehringer-Mannheim. Anti-TGFα (clone 213-4.4) and EGF-receptor (clone) antibodies were obtained from Oncogene Science. Anti-HLA class I (clone) and II (clone L243) mAbs were purchased from Becton-Dickenson. Irrelevant mouse antibodies (MOPC-21, Sigma) served as a negative control.

Anti-Hu IgG (provided by Dr. J. B. Posner) was prepared from a high titer anti-Hu serum and conjugated to biotin as reported previously (Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E, Henson J, Posner JB, Furneaux H: HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. *Cell* 67: 325-333, 1991). Frozen sections (7 μm thick) of human brain served as a positive control. Incubation with biotinylated IgG from a normal individual was taken as negative control.

Western blotting. Tumor cells were solubilized in 50 mM Tris-HCl, pH 7.2, containing 0.5% NP-40 and proteinase inhibitors (phenylmethylsulfonylfluoride, pepstatin, leupeptin, soybean trypsin inhibitor, aprotinin and benzamidin). Equal aliquots (15-30 μg) of the protein extracts were electrophoresed in 8 or 10% SDS-PAGE gels under denaturing conditions. The proteins were then electroblotted (Hoeffer Transblot system) from the gel onto 0.2 μm porous nitrocellulose. Blots were blocked in 5% milk for 2 h,

incubated with the primary antibody (mAb in mouse or human serum) for 1 h and detected using HRP-conjugated sheep anti-IgG (Amersham) directed against the appropriate species of primary antibody and the chemiluminescent technique (Amersham).

For Western analysis of Hu a high titer serum of a patient with anti-Hu syndrome was used and serum from a healthy individual served as a negative control.

Cell cycle analysis. For cell cycle studies, Daoy cells were plated in T-150 culture flasks in serum-containing medium. After 24 h of incubation, the culture medium was replaced by serum-free medium (Ultraculture, Hyclone) for 24 h to synchronize the cell cultures; then the cultures were refed medium containing PA 10 mM or plain serum containing medium. Cells were harvested after 48, stained with acridine orange and analyzed on a FAC-Scan (Becton-Dickenson).

RT-PCR assay of HuD-expression. Total RNA (1 μg) from tumor cell lines was reverse transcribed with random hexamers (2.5 μM) for 50 min at 37° C. in a reaction volume of 20 μl containing the following: 1 mM each dNTP, 1 U/μl RNase inhibitor, and 2.5 U/μl MMLV-RT (Gibco). RT reactions were terminated by incubation at 99° C. for 5 min. One-twentieth of the RT reaction product was used for PCR amplification. PCR was carried out in a 1xPCR buffer (50 mM Tris pH 9.5, 1.5 mM MgCl₂, 20 mM ammonium sulfate), 0.2 mM each dNTP, 5 μCi of (³²P)dCTP, 0.5 μM each upstream and downstream primer (CCAGGCCCT-GCTCTCCC, AGGCTTGTCATTCCATC) for HuD and for β2-microglobulin, and 1 U of Taq polymerase (Perkin-Elmer Cetus). PCR analysis was performed in a Perkin-Elmer Cetus DNA cycler with the following temperature profile: 1 min at 90° C.; 35 cycles of 30 s at 55° C., 1 min at 72° C., and 1 min at 92° C.; 2 min at 55° C.; and 10 min at 72° C. One-tenth of the PCR product was electrophoresed in 6% acrylamide gel, and the PCR product was analyzed by autoradiography.

TGFS bioassay. Conditioned media was produced by incubating Daoy cells in the presence of PA 10 mM in T-150 culture flasks. The culture media was aspirated and concentrated 20 fold to a final volume of 200 μl using Amicon centrifugal concentrators. The concentrated media was transferred to silicon treated polypropylene microfuge tubes (Danville Scientific) and protease inhibitors (same as mentioned before) added. To activate latent TGFβ, the samples were acidified using acetic acid and subsequently neutralized with 5N NaOH prior to the assay.

The MuLvl cells were harvested by scraping flasks, washed in serum free MEM, resuspended in MEM+0.2% FCS and seeded into 96 well microtiter plates at a cell density of 2000 cells per well in 50 μl aliquots. After the plates were incubated for 4 h, 150 μl of conditioned media from cell lines to be tested for TGFβ production was added per well. The plates were incubated for 24 h and then cell proliferation was measured by ³H-thymidine incorporation as described previously. A standard curve relating TGFβ concentration to rate of ³H-thymidine incorporation was generated using human recombinant TGFβ1 (R&D systems), ranging from 0.1 pM to 10 nM.

Elisa for TGFβ2. Conditioned media was concentrated 20 fold using Amicon concentrators to a final volume of 200 μl. All the concentration steps were performed at 420 C. Protease inhibitors (same as listed for Western analysis) were added to the concentrated samples prior to storage at -70° C. Latent TGFβ was activated by adding glacial acetic acid to the samples to yield a final concentration of 1M acetic acid. Then the samples were evaporated to dryness using a

Speed-Vac and redissolved in 200 µl of distilled water. The reconstitution/drying step was repeated for a total of 3 washes. Then the samples were redissolved in the sample treatment buffer RD5B provided in the kit and the capture ELISA protocol performed following the manufacturer's instructions. All data points were performed in duplicate. The colorimetric reaction was quantitated by measuring absorbance at 450 nm using a Bio-RAD Microplate reader and the Biorad Elisamatic software. A standard curve was generated using human recombinant TGF μ 2 as the protein standard.

Effects of PA on cell growth and cell cycle. All 6 cell lines showed a dose-dependent growth inhibition after incubation with PA (FIG. 46) with a maximum effect after 48 to 96 h. The human malignant glioma cell line U251, which was previously reported to be ATRA resistant (Yung WKA, Lotan R, Lee P, Lotan D, Steck PA: Modulation of growth and epidermal growth factor receptor activity by retinoic acid in human glioma cells. *Cancer Res* 49: 1014-1019, 1989), also responded to PA. The human tumor cell lines as well as the rat glioma cell line C6 showed ID₅₀ values for PA between 5.8 and 9.5 mM, whereas the rat glioma cell line RG2 appeared to be less sensitive with an ID₅₀ of 14.6 mM (Table 28). Trypan blue exclusion-demonstrated greater than 90% viability of PA treated cell cultures. When PA was removed after 48 h a clear washout effect was noted in 3 cell lines studied (Daoy, U251 and C6).

TABLE 28

| ID ₅₀ Determinations for PA induced growth inhibition of various tumor cell lines | |
|--|-----------------------|
| Cell Line | ID ₅₀ [mM] |
| D-283 MED | 9 |
| Daoy MED | 6.3 |
| U-251 MG | 8.2 |
| RG-2 rat glioma | 14.6 |
| C6 rat glioma | 8.5 |
| BE(2)C | 9.5 |

Cell cycle analysis of Daoy cells treated with 10 mM PA for 2 days showed an accumulation of cells in G0/G1 with an equivalent reduction in the percentage of cells in the S- and G2/M-phase. Prior to treatment 34.2% of Daoy cells were in G0/G1, 55.5% in S, and 10.3% in G2/M phase. After treatment with PA 51.9% were in G1, 39.9% in S, and 8.2% in G2/M phase.

Effects of PA on morphology and phenotype of glioma cell lines. PA induced marked morphological changes in U251 cells (FIG. 74). Prior to treatment, the cells were characterized by spindly bipolar GFAP positive processes. After PA treatment for 4 days, cytoplasmic processes were more extensive and complex. The cytoplasmic/nuclear ratio was increased greatly with enlargement of the cell body and elaboration of a radiating stellate array of cytoplasmic processes resembling those of hyperplastic astrocytes. The extent of these morphological changes were dose-related. Similar effects were seen in C6 rat glioma cells. Most untreated cells were GFAP negative, and in the few positive cells the immunoreactivity was localized in the perinuclear region. Although Western analysis for GFAP expression in both cell lines failed to show a clearcut quantitative change after exposure to PA, the fraction of cells staining for GFAP increased and filamentous immunoreactivity appeared in the cellular processes. Untreated U251 cells also expressed vimentin, EGFr, TGF α and ELA class I antigens. None of these cellular markers was modified by PA treatment.

After discontinuation of PA treatment after 4 days all morphological changes of U251 cells reverted to the pretreatment state within 3 to 5 days.

Effects of PA on the differentiation state of medulloblastoma cell lines. In short term cultures of the medulloblastoma cell lines Daoy and D283 growth inhibition by PA was not accompanied by gross morphological changes. Daoy cells expressed both class I and class II HLA immunoreactivity; these phenotypic markers remained unchanged after PA (10mM) treatment. Neither of the 2 cell lines expressed synaptophysin and GFAP before or after treatment with PA. Untreated Daoy cells were reactive for EGFr and TGF α ; immunohistochemical staining for EGFr and TGF α was not affected by PA treatment.

Expression of Hu is one of the earliest markers of neuronal phenotype in the peripheral nervous system of the chick (See Marusitch) and one of the earliest markers of neuronal phenotype in the developing central nervous system of the mouse. Previous studies of the expression of Hu in human normal tissue and tumor tissue demonstrated that the Hu antigen is highly restricted to the nervous system, small cell lung cancer tumors and neuroblastomas (Dalmau J, Furneaux HM, Cordon-Cardo C, Posner JB: The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues. *Am J Pathol* 141: 881-886, 1992). Immunocytochemistry using biotinylated anti-Hu antisera showed Hu antigen expression in D283 and BE(2)C cells, whereas Daoy cells and all glioma cell lines were negative. Results of immunostaining were confirmed by Western analysis and RT-PCR.

For Western analysis, cells were solubilized in 50 mM Tris buffer containing 0.5% NP-40 and oiled for 7 min in the presence of 2-mercaptoethanol. Proteins were separated on 10% SDS-PAGE gels with 20 µg of protein loaded per lane. Proteins were transferred from the gel to nitrocellulose by electroblotting. Immunoreactivity was determined by exposing the blots to a human serum with high titer anti-Hu antibodies or serum from a normal donor and then incubated with HRP-conjugated sheep-anti human IgG. The blots were then autoradiographed using the enhanced chemiluminescent system (Amersham). Hu-fusion protein and protein extracts of cortical neurons served as positive and normal human serum as a negative control. For RT-PCR analysis 1 µg of total RNA from tumor cell lines was reverse transcribed with random hexamers and one-twentieth of the RT reaction product was used for PCR amplification with HuD and β 2-microglobulin specific primers. The PCR product was electrophoresed in a 6% acrylamide gel and analyzed by autoradiography.

The Hu antigen was detectable as a band with a molecular weight of 35 to 40 kDa on blots of denaturing SDS-PAGE gels. None of the cell lines reacted with normal human serum.

Daoy cells were negative for NFs whereas D283 cells expressed all 3 NF proteins, determined immunocytochemically. By immunocytochemistry quantitative changes after treatment with PA were seen for NF-M and Hu expression. After exposure to PA (10 mM) for 7 days NF-M and Hu immunoreactivity were enhanced. Western analysis of untreated and PA treated D283 cell homogenates confirmed these quantitative changes in NF-M and Hu protein expression. Western analysis was performed for Hu and NF-M expression of untreated and PA (10 mM, 7 d) treated D283 cells lysed in 50 mM Tris containing 0.5% NP-40 and separated under denaturing conditions by electrophoresis through 10% SDS-PAGE gels and transferred to nitrocellu-

lose paper by electroblotting. Specific immunoreactivity was determined using human serum containing high titer anti-Hu antibodies and murine anti-NF-M respectively and the enhanced chemiluminescent technique (Amersham). Normal human serum or the appropriate isotype matched irrelevant mAb served as control. Immunostaining for NF-L revealed similar but less obvious changes after PA treatment and NF-H remained unchanged.

Effects of PA on the human neuroblastoma differentiation model BE(2)C. The effects of PA on medulloblastoma cell lines were compared to the human neuroblastoma cell line BE(2)C, which represents a well established bipotential differentiation model for neuronal and glial/schwann cell differentiation, indicated by changes in phenotypic markers, such as NF proteins, Hu and Vimentin, with ATRA inducing a neuronal and BUdR a glial/schwann cell phenotype (Biedler JL, Casals D, Chang T, Meyers MB, Spengler BA, Ross RA: Multidrug resistant human neuroblastoma cells are more differentiated than controls and retinoic acid further induces lineage-specific differentiation. *Advances in Neuroblastoma Research* 3: 181-191, 1991, Wiley-Liss, Inc.; Ross RA, Bossart E, Spengler BA, Biedler JL: Multipotent capacity of morphologically intermediate (I-type) human neuroblastoma cells after treatment with differentiation-inducing drugs. *Advances in Neuroblastoma Research* 3: 193-201, 1991, Wiley-Liss, Inc.; Ciccarone-V, Spengler BA, Meyers MB, Biedler JL, Ross RA: Phenotypic diversification in human neuroblastoma cells: Expression of neural crest lineages. *Cancer Res* 49: 219-225, 1989; Ross RA, Spengler BA, Rettig WR, Biedler JL: Permanent phenotypic conversion of human neuroblastoma I-type cells. *Proc Am Assoc Cancer Res* 35: 44, 1993). BE(2)C neuroblastoma cells showed a decrease in Hu and NF-M protein expression and an increase in vimentin expression following PA treatment (10 mM) for 7 days by both immunocytochemistry and Western analysis. Cells were lysed using 50 mM Tris containing 0.5% NP-40 and then the proteins were separated under denaturing conditions by electrophoresis through 8-10% SDS-PAGE gels and transferred to nitrocellulose paper by electroblotting. Specific immunoreactivity was determined using murine anti-vimentin mAb, human serum containing high titer anti-Hu antibodies, and murine anti-NF-M respectively and the enhanced chemiluminescent technique (Amersham). Normal human serum or the appropriate isotype matched irrelevant mAb served as control.

Role of TGF β in PA-mediated growth inhibition. To test the hypothesis that TGF β is involved in mediating the growth inhibitory effects of PA conditioned media of Daoy cells was studied before and after treatment with PA. Incubation of Daoy cells with PA 10 mM led to increased amounts of TGF β in the conditioned media determined by the mink lung cell (MuLv1) bioassay. After 4 days of incubation, the control media contained 0.5 pM and the PA treated conditioned media contained 3.0 pM TGF β . Further characterization of the secreted bioactive TGF β protein using an ELISA assay specific for TGF β 2 indicated that the secretion of TGF β 2 was stimulated by PA.

Addition of exogenous human recombinant TGF β 1 or TGF β 2 at concentrations ranging from 0.5 pM to 1 nM did not inhibit growth of untreated Daoy cells maintained in MEM containing 0.2% FCS. These TGF β proteins inhibited the growth of MuLv1 cells profoundly after a 24 h exposure with an ID₅₀ of 2 pM. Blocking studies with neutralizing antibodies against TGF β 1 and TGF β 2, at concentrations up to 20 μ g/ml, had no effects on PA induced inhibition on Daoy and U251 cell growth (FIG. 47). However, these concentrations of antibodies were capable of neutralizing the

growth inhibitory effects of exogenous TGF β 1 and TGF β 2 on MuLv1 cells.

As described herein and in the copending application, PA can induce differentiation of various hematological malignancies as well as solid tumors. These in vitro effects on human cell lines were noted at concentrations that have been achieved without toxicity in children treated with PA for urea cycle disorders. The results demonstrate that PA is a growth inhibitor for glioma and medulloblastoma-derived cell lines. PA also showed antiproliferative effects in the U251 glioma cell line, which was previously reported to be resistant to ATRA (Yung WKA, Lotan R, Lee P, Lotan D, Steck PA: Modulation of growth and epidermal growth factor receptor activity by retinoic acid in human glioma cells. *Cancer Res* 49: 1014-1019, 1989). Cell cycle analysis performed in Daoy cells showed an accumulation of cells in the G0/G1 phase, which was similar to ATRA, induced effects on cell cycle previously reported for glioma cell lines (Rutka JT, DeArmond SJ, Giblin J, McCulloch JR, Wilson CB, Rosenblum ML: Effect of retinoids on the proliferation, morphology and expression of glial fibrillary acidic protein of an anaplastic astrocytoma cell line. *Int J Cancer* 42: 419-427, 1988). The rapid onset of growth inhibition is not due to cytotoxicity, since cells resume proliferation and revert to their previous morphology after washout of PA.

PA induced morphological and immunocytochemical changes consistent with astrocytic differentiation in glioma cell lines. Both glioma cell lines studied showed marked morphological changes after PA treatment with increased cytoplasmic/nuclear ratio and the formation of GFAP positive processes. GFAP immunoreactivity appeared more intense in these newly formed processes. However, Western analysis failed to demonstrate a clear quantitative change in GFAP expression after PA treatment. Previous studies of differentiation agents in glioma cell lines showed similar findings on GFAP expression in U251 and C6 cells after treatment with mycophenolic acid (Lipsky RH, Siverman SJ: Effects of mycophenolic acid on detection of glial filaments in human and rat astrocytoma cultures. *Cancer Res* 47: 4900-4904, 1987). The enhanced immunoreactivity for GFAP without increase in GFAP levels might be due to a modification of existing proteins by affecting the organization of assembled GFAP filaments. The discrepancy between immunoblotting and immunocytochemistry may reflect the inability of the blotting technique to detect changes occurring in a subpopulation of the cells in a heterogeneous population. ATRA induced phenotypic changes in responsive glioma cell lines which are similar to those produced by PA (Yung WKA, Lotan R, Lee P, Lotan D, Steck PA: Modulation of growth and epidermal growth factor receptor activity by retinoic acid in human glioma cells. *Cancer Res* 49: 1014-1019, 1989). Human glioma cell lines were found to be more sensitive to ATRA compared to rodent cell lines. Interestingly, the least PA sensitive cell line was also of rodent origin.

In short term cultures (up to 7 days) of both medulloblastoma cell lines the growth inhibitory effects of PA were not associated with morphological changes and the Daoy cells showed no changes in cellular expression of the numerous markers tested, including NF proteins, synaptophysin, Hu, TGF α , EGFR, GFAP and HLA class I and II. This temporal dissociation of antiproliferative effects from phenotypic changes has been reported for other differentiation agents (Ross RA, Spengler BA, Rettig WR, Biedler JL: Permanent phenotypic conversion of human neuroblastoma I-type cells. *Proc Am Assoc Cancer Res* 35: 44, 1993; Pleasure SJ, Page C, Lee VM-Y: Pure, postmitotic, polarized

human neurons derived from Ntera2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *The J of Neuroscience* 12: 1803–1815, 1992). Daoy cells also revealed no morphological changes after treatment with dBcAMP (Jacobsen PF, Jenkyn DJ, Papadimitriou JM: Establishment of a human medulloblastoma cell line and its heterotransplantation into nude mice. *J. Neuropathol Exp Neurol* 44: 472–495, 1985). ATRA treatment showed profound growth inhibitory effects in medulloblastoma cell lines without demonstrable concurrent morphologic changes (Agrawil A, Martell LA, Ross DA, Muraszko KM: Retinoic acid modulation of proliferation and differentiation in brain tumors. *Proc Am Assoc Cancer Res* 34: 20, 1993 (abstract)), which is in agreement with data on ATRA studies in medulloblastoma cell lines (Lieberman F, Finzi D, Ferro J: Retinoic acid decreases proliferation and Hu expression in medulloblastoma cells. *Ann Neurol* 32: 286, 1992).

Conflicting reports about the clinical relevance of glial or neuronal differentiation in medulloblastoma as indicated by GFAP and NF protein expression and morphologic criteria (Schofield D: Diagnostic histopathology, cytogenetics, and molecular markers of pediatric brain tumors. *Neurosurg Clin N Amer* 3: 723–738, 1992) reflect an incomplete understanding of the biochemical changes correlated with growth inhibition. In addition, the cell of origin still remains unclear. Phenotypic characterization of medulloblastoma cell lines suggests at least 2 distinguishable phenotypic patterns, with relative differences in expression of neuronal- or glial-associated antigens, with D283 representing a neuronal and Daoy a nonneuronal phenotype of medulloblastoma cell lines (He X, Skapek SX, Wikstrand CJ, Friedman HS, Trojanowski JQ, Kemeshead JT, Coakham HB, Bigner SH and Bigner DD: Phenotypic analysis of four human medulloblastoma cell lines and transplantable xenografts. *J Neuropathol Exper Neurol* 48: 48–68, 1989).

NF protein expression has been used to identify neuronal differentiation (Tremblay FG, Lee M-Y, Trojanowski JQ: Expression of vimentin, glial filament and neurofilament proteins in primitive childhood brain tumors. *Acta Neuropathol (Berl)* 68: 209–244, 1985). In mature, post-mitotic neurons, NF proteins consist of 3 constituent proteins, termed light, medium and heavy based on different molecular weights in denaturing SDS gels. A previously proposed ontogenic schema suggests that the appearance of NF-H correlates with the post-mitotic state (Molenaar WM, Jansson D, Goild VE et al.: Molecular markers of pediatric neuroectodermal tumors and other pediatric nervous system tumors. *lab Invest* 61: 635–643, 1989). Previous studies of NF-expression in medulloblastoma cell lines (Baker DL, Reddy UR, Pleasure S, Hardy M, Williams M, Tartaglione M, Biegel JA, Emanuel BS, Presti PL, Kreidler B, Trojanowski JQ, Evans A, Roy AR, Venkatakrishnan G, Chen J, Ross AH, Pleasure D: Human central nervous system primitive neuroectodermal tumor expressing NGF receptors: CHP707m. *Ann Neurol* 28: 136–145, 1990) have demonstrated that the CHP707m PNET line expresses only NF-L suggesting that this cell line reflects an immature state relative to D283 cells. The D283 cell line expressed all 3 forms, although in an abnormal way (Trojanowski JA, Friedman HS, Burger PC, Bigner DD: A rapidly dividing human medulloblastoma cell line (D283) expresses all 3 neurofilament subunits. *Am J Pathol* 126: 358–363, 1987), suggesting this defect indicates aberrant NF protein construction and disordered intermediate filament function, which may be a consequence of neoplastic transformation (He X, Skapek SX, Wikstrand CJ, Friedman HS, Trojan-

owski JQ, Kemeshead JT, Coakham HB, Bigner SH and Bigner DD: Phenotypic analysis of four human medulloblastoma cell lines and transplantable xenografts. *J Neuropathol Exper Neurol* 48: 48–68, 1989). Disordered NF protein expression can be found in PC12 cells, derived from a rat pheochromocytoma (Baker DL, Reddy UR, Pleasure S, Hardy M, Williams M, Tartaglione M, Biegel JA, Emanuel BS, Presti PL, Kreidler B, Trojanowski JQ, Evans A, Roy AR, Venkatakrishnan G, Chen J, Ross AH, Pleasure D: Human central nervous system primitive neuroectodermal tumor expressing NGF receptors: CHP707m. *Ann Neurol* 28: 136–145, 1990; Trojanowski JA, Friedman HS, Burger PC, Bigner DD: A rapidly dividing human medulloblastoma cell line (D283) expresses all 3 neurofilament subunits. *Am J Pathol* 126: 358–363, 1987). In this NGF-responsive cell line, NGF treatment enhanced the expression of NF-L and NF-M. ATRA induced neuronal differentiation of NT2 cells, derived from a human teratocarcinoma, includes induction of expression of all 3NF constituents as the cells leave the mitotic cycle and produce neurites (ATRA induced neuronal differentiation of NT2 cells, derived from a human teratocarcinoma, includes induction of expression of all 3NF constituents as the cells leave the mitotic cycle and produce neurites) (Pleasure SJ, Page C, Lee VM-Y: Pure, postmitotic, polarized human neurons derived from Ntera2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *The J of Neuroscience* 12: 1803–1815, 1992; Lee M-Y, Andrews PW: Differentiation of NTERA-2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. *The J of Neuroscience* 6: 514–521, 1986). This induction of NF protein expression is accompanied by morphological changes resembling normal neurons (Lee M-Y, Andrews PW: Differentiation of NTERA-2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. *The J of Neuroscience* 6: 514–521, 1986) and these cells even show action potentials (Younkin DP, Tang C-M, Hardy M, Reddy UR, Shi Q-Y, Pleasure SJ, Lee VM-Y: Inducible expression of neuronal glutamate receptor channels in the NT2 human cell line. *Proc Natl Acad Sci USA* 90: 2174–2178, 1993). The Hu antigen is a novel neuronal differentiation antigen not previously studied in medulloblastoma-derived cell lines. This antigen, originally identified by antibodies in patients with paraneoplastic encephalomyelitis/sensory neuronopathy (Dalmau J, Furneaux HM, Gralla RJ, Kris MG, Posner JB: Detection of the antiHu antibody in the serum of patients with small cell lung cancer—A quantitative Western blot analysis. *Ann Neurol* 27: 544–552, 1990; Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E, Henson J, Posner JB, Furneaux H: HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. *Cell* 67: 325–333, 1991; Furneaux HM, Reich L, Posner JB: Autoantibody synthesis in the central nervous system of patients with paraneoplastic syndromes. *Neurol.* 40: 1085–1091, 1990), has been shown to be expressed in small cell lung tumors and neurons (Dalmau J, Furneaux HM, Gralla RJ, Kris MG, Posner JB: Detection of the anti-Hu antibody in the serum of patients with small cell lung cancer—A quantitative Western blot analysis. *Ann Neurol* 27: 544–552, 1990). The gene encoding the protein which reacts with anti-Hu antibodies has been sequenced and cloned (Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E, Henson J, Posner JB, Furneaux H: HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and SeX-lethal. *Cell* 67: 325–333, 1991). The remarkable

homology to the *Drosophila* proteins Elav and Sex-lethal suggests a role for Hu in the maturation of mammalian neurons. It may be a neuron-specific RNA processing protein (Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E, Henson J, Posner JB, Furneaux H: HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. *Cell* 67: 325-333, 1991). Hu was detectable in both cell lines with features of neuronal differentiation, D283 and BE(2)C cells, and was found to be modulated by PA. This developmental antigen, which is expressed at a very early stage of neuronal commitment way be an indicated for distinct differentiation pathways and a new marker to better define the differentiation state and subgroups of medulloblastomas.

The effect of PA administration on NF protein expression in D283 cells was characterized by an increase of NF-L and NF-M accompanied by an increase in the Hu antigen. ATRA induced opposite effects on NF-M and Hu suggesting that ATRA does not induce differentiation of D283 cells along the neuronal pathway (Lieberman F, Finzi D, Ferro J: Retinoic acid decreases proliferation and Hu expression in medulloblastoma cells. *Ann Neurol* 32: 286, 1992).

Hu expression did not correlate with synaptophysin expression. None of the medulloblastoma cell lines expressed synaptophysin before or after treatment. This 28 kDa glycoprotein, a component of presynaptic vesicles, is characteristic of neuroendocrine cells and neuronally differentiated tumors (Molenaar WM, Baker DL, Pleasure D, Trojanowski JQ: The neuroendocrine and neural profile of neuroblastomas, ganglioneuroblastomas and ganglioneuromas. *Am J Pathol* 136: 375-382, 1990). Synaptophysin has been reported in a large fraction of medulloblastomas (Cofin CM, Braun JT, Wisk MR, et al.: a clinicopathologic and immunohistochemical analysis of 53 cases of medulloblastoma with emphasis on synaptophysin expression. *Mod Pathol* 3: 164, 1990). It is considered a marker of neuronal differentiation, although the prognostic relevance of such expression is unclear.

Also studied were the effects of PA in a neuroblastoma cell line, which represents a well characterized bipotential differentiation model capable of differentiation towards a neuronal or a schwann/glia cell phenotype (Biedler JL, Casals D, Chang T, Meyers MB, Spengler BA, Ross RA: Multidrug resistant human neuroblastoma cells are more differentiated than controls and retinoic acid further induces lineage-specific differentiation. *Advances in Neuroblastoma Research* 3: 181-191, 1991, Wiley-Liss, Inc.; Ross RA, Bossart E, Spengler BA, Biedler JL: Multipotent capacity of morphologically intermediate (I-type) human neuroblastoma cells after treatment with differentiation-inducing drugs. *Advances in Neuroblastoma Research* 3: 193-201, 1991, Wiley-Liss, Inc.; Ciccarone V, Spengler BA, Meyers MB, Biedler JL, Ross RA: Phenotypic diversification in human neuroblastoma cells: Expression of neural crest lineages. *Cancer Res* 49: 219-225, 1989; Ross RA, Spengler BA, Rettig WR, Biedler JL: Permanent phenotypic conversion of human neuroblastoma I-type cells. *Proc Am Assoc Cancer Res* 35: 44, 1993). These effects were studied in order to compare the response to medulloblastoma and glioma cell lines. The cell line, BE(1)C, established by Biedler (Biedler JL, Helson L, Spengler BA: Morphology and growth, tumorigenicity of human neuroblastoma cells in continuous culture. *Cancer Res* 33: 2643-2652, 1973), represents an intermediate type subclone with features of both neuronal (N-) and glial/schwann (S-) type cells and can be neuronally differentiated by ATRA and differentiated towards a S-phenotype with BUdR and dBcAMP (Ross RA,

Bossart E, Spengler BA, Biedler JL: Multipotent capacity of morphologically intermediate (I-type) human neuroblastoma cells after treatment with differentiation-inducing drugs. *Advances in Neuroblastoma Research* 3: 193-201, 1991, Wiley-Liss, Inc.). During ontogenesis, the appearance of NF proteins coincides with a switch away from the expression of vimentin in precursor cells to the expression of NF proteins in nascent neurons derived from these precursors (Tapscot 1981, supra). PA- and ATRA-induced growth inhibition in BE(2)C cells was accompanied by changes in the expression of NF proteins, Hu and vimentin with PA and ATRA inducing opposite effects. In the BE(2)C differentiation model PA caused similar phenotypic changes as reported with BUdR and dBcAMP with differentiation towards the S-phenotype (Ross RA, Bossart E, Spengler BA, Biedler JL: Multipotent capacity of morphologically intermediate (I-type) human neuroblastoma cells after treatment with differentiation-inducing drugs. *Advances in Neuroblastoma Research* 3: 193-201, 1991, Wiley-Liss, Inc.). The choice of pathway appears cell line dependent, since D283 differentiate in a neuronal direction with PA but BE(2)C differentiate in a schwann/glia direction. The underlying mechanisms at this switch point are unknown.

The mechanisms by which PA produces its effects are not understood. The hypothesis that glutamine depletion is responsible seems unlikely. PA is effective in rat and murine systems, in which PA conjugates glycine rather than glutamine (James MO, Smith RL, Williams RT, et al.: Conjugation of phenylacetic acid in man, subhuman primates and some non-human species. *Proc R. Soc Lond* 182: 25-35, 1972) and PA's effect on in vitro growth of U251 human malignant astrocytoma cells could not be blocked by glutamine supplementation. These findings are in agreement with previous data described herein on the interaction of glutamine with PA in non-neuroectodermal derived cell lines. PA appears to cause hypomethylation of DNA in vitro (Anjusin BF, Bashkite EA, Freidrich A. et al.: Metilirovanie DNK V prostkach psenicy Ivligjanije fyto-gormonor. *Biochemia* 46: 47-53, 1981), but the significance of this observation is unclear. In HL-60 cells, PA treatment is associated with decreased c-myc expression. See supra.

The transforming growth factors beta (TGFβ1, β2 and S3) are polypeptides that influence the proliferation and differentiation of many cell types (Sporn MB, Roberts AB: Peptide growth factors and their receptors I:419-439, 1990, Springer Verlag). As described herein, there is a decrease in TGFβ2 mRNA levels after PA treatment of PC3 prostate carcinoma cells. This family of growth regulatory molecules has been shown to be important to the biology of astrocytoma growth. Both numerous cell lines (Falk LA, DeBenedetti F, Lohrey Net al.: Induction of TGFβ1 receptor expression and TGFβ1 protein production in retinoic acid treated HL-60 cells. *Blood* 77: 1248-1255, 1991) and primary tumor tissue (Clark WC, Bressler J: TGFβ-like activity in tumors of the central nervous system. *J Neurosurg* 68: 920-924, 1988; Samuels V, Barrett JM, Brochman Set al.: Immunocytochemical study of transforming growth factor expression by benign and malignant gliomas. *Am J Pathol* 134: 895-902, 1989) have been shown to express mRNA for one or more of the 3 TGFβ isoforms. Malignant glioma derived cell lines demonstrate variable proliferative responses to TGFβ in vitro (Falk LA, DeBenedetti F, Lohrey Net al.: Induction of TGFβ1 receptor expression and TGFβ1 protein production in retinoic acid treated HL-60 cells. *Blood* 77: 1248-1255, 1991) and the responses may correlate with hyperdiploid or aneuploid state, and morphologic criteria of anaplasia. TGFβ appears to be a growth inhibitory

factor for some astrocytoma cells and a mitogenic factor for others. The molecular basis for the different response is unclear. The role of TGF β in the biology of medulloblastoma growth and differentiation has not been studied, in part due to the difficulty in establishing medulloblastoma cell lines and the relative paucity of cases relative to malignant astrocytomas.

PA does appear to modulate TGF β 2 production by Daoy medulloblastoma as well as PC3 prostate carcinoma cells (described supra). Increased amounts of TGF β 2 were detected by quantitative ELISA after Daoy cells were exposed to PA. However, the data suggest that these changes do not mediate the antiproliferative effects. Exogenous TGF β 1 and TGF β 2 had no influence on Daoy cell growth. Neutralizing Abs against TGF β 1 and TGF β 2 did not block PA-mediated growth inhibition of U251 and Daoy cells.

The in vitro data suggest that PA and related compounds may be useful agents in the treatment of medulloblastomas and other primary CNS tumors. These agents warrant further studies in animal systems and in human clinical trials.

To define the capability of PA to inhibit growth and induce differentiation in neuroectodermal tumor cell lines, the effects of PA in 1 human (U251) and 2 rodent (C6, RG2) cell lines of glial origin as well as 2 human medulloblastoma cell lines (Daoy, D283) were studied. The effects of PA on these cell lines were compared to the human neuroblastoma cell line BE(2)C since this cell line represents a well characterized bidirectional differentiation model for neuronal and schwann/glial cell differentiation.

SECTION Q: NAPA AND NAPB HUMAN STUDIES

The growth inhibition and differentiating effects of sodium phenylacetate against hematopoietic and solid tumor cell lines has aroused clinical interest in its use as an anticancer drug. The non-linear pharmacokinetics, metabolism, toxicity, and clinical activity of PA when administered by continuous i.v. infusion (CIVI) for 2 weeks have recently been described. In this phase I, PA was administered i.v. twice daily (BID) in an attempt to minimize drug accumulation while maximizing peak serum drug concentrations. Twenty-seven cycles of therapy were given to 18 patients at two dose levels (125 and 150 mg/kg BID for 14 days). Detailed pharmacokinetic studies in 8 patients conclusively showed that PA induces its own clearance by a factor of 44% over two weeks. Dose-limiting toxicity consisted of reversible central nervous system depression associated with nausea and hypoacusis. Clinical improvement was observed in eight patients. Three of seven patients with refractory malignant glioma had improved performance status for up to 9 months. One had a partial response and another a minor response on MRI. Improvement was also seen in five of nine patients with hormone-independent prostate cancer (HIPC). One had a greater than 50% decline in PSA, one had resolution of disseminated intravascular coagulation and one noted decreased bone pain that lasted for more than 1 month. Two patients maintained an improved performance status for more than 8 months, one of whom demonstrated healing of blastic bone metastases. These results suggest that PA has activity in malignant gliomas and HIPC. The recommended schedule for phase II testing is 125 mg/kg BID (one hour infusion) given in monthly cycles of 14 days duration.

A first phase I trial of phenylacetate has been described above. In that trial, the drug was administered by CIVI in an attempt to maintain drug concentrations in the range asso-

ciated with preclinical activity. Under these conditions, phenylacetate displayed saturable kinetics and evidence for induction of its own metabolism (pharmacokinetic parameters, mean \pm SD: K_m =105.1 \pm 44.5 μ g/ml, V_{max} =24.1 \pm 5.2 mg/kg/hr, V_d =19.2 \pm 3.3 L, and IR =0.0028 \pm 0.003 h $^{-1}$). Clinical improvement was noted in several patients with metastatic hormone-independent prostate cancer and malignant glioma who achieved serum phenylacetate concentrations of 1–2 mM. Infusion rates close to the V_{max} of the metabolizing enzyme were required, however, in order to achieve concentrations of 3 mM or more. This often resulted in rapid drug accumulation, associated with neurological toxicity once phenylacetate levels exceeded 6 mM. These limitations led to the investigation of a different dosing schedule wherein the drug was given as a one-hour infusion twice daily, based on the assumption that elevations in phenylacetate concentrations might be well tolerated if transient, and that intermittent dosing might help control drug accumulation.

EXAMPLE 29

In vivo trials with NaPA B.I.D.

Patient Population. Adults with advanced solid tumors refractory to conventional therapy, a performance status greater than 60% on Kamofsky's scale, normal hepatic transaminases and bilirubin, a serum creatinine less than 1.5 mg/dl, and normal leucocyte and platelet counts were eligible for this study. The clinical protocol was reviewed and approved by the NCI Institutional Review Board and all patients gave written informed consent prior to participating in the study. Eighteen patients, 15 men and 3 women, with a mean age of 54 years (range, 32 to 76) were enrolled between July and October 1993. Disease distribution included metastatic, hormone-independent prostate cancer (9 patients), primary CNS tumors (7 patients), renal cell cancer (1 patient) and sarcoma (1 patient). Four patients, two with gliomas and two with prostate cancer, had received prior treatment with phenylacetate given by CIVI.

Drug Preparation and Administration. Sodium phenylacetate for injection (Elan Pharmaceutical Research Co., Gainesville, Ga.) was prepared from sterile sodium phenylacetate powder by the Pharmacy Department of the Clinical Center, NIH, in vials containing a drug concentration of 500 mg/ml in sterile water for injection, USP. Sodium hydroxide or hydrochloric acid was added to adjust the final pH to 7.4. Doses of sodium phenylacetate to be infused over 1 hour were prepared in 250 ml of sterile water for injection, USP, and were administered using an infusion pump. The experiment used the one compartment, non-linear pharmacokinetic model and population parameters derived from previous experience with phenylacetate to stimulate the course of several intermittent dosing regimens. The primary objective was to design one that would expose most patients to transient phenylacetate concentrations in excess of 3 mM and maintain trough concentrations of approximately 2 mM. These appeared necessary for antitumor activity in vitro and had been well tolerated by patients for as long as 2 weeks. The secondary objective was to pharmacokinetically determine the optimal dose of phenylacetate to achieve the above and minimize the number of escalation steps in the trial by beginning at that dose.

Phenylacetate was delivered at two dose levels: ten patients were treated at 125 and eight at 150 mg/kg/dose, BID, for 14 consecutive days. Cycles of therapy were repeated every 4 weeks. The dose was increased in sequen-

tial cohorts of at least three patients. Individual patients could escalate from one dose level to the next with sequential cycles provided they had experienced no drug-related toxicity and their disease was stable or improved.

Sampling Schedule. Serum drug concentrations were measured twice a day in all patients, immediately prior to and 15 minutes following the administration of the 05:00 PM infusion. To assess the possibility that phenylacetate induces its own clearance, 8 patients underwent more intensive drug level monitoring on days 1, 2 or 3 and days 12, 13 or 14 of the two weeks of therapy. In these patients, blood was also obtained at 0, 65, 90, 105, 120, 150, 180, 210, 240, 300 and 360 minutes from the beginning of the 08:00 AM infusion. This allowed for a comparison to be made between AUCs generated from identical doses of phenylacetate at the beginning and at the end of therapy, any difference reflecting a change in drug clearance over this period of time.

Analytical Method. Blood was drawn into plain glass Vacutainer® tubes and refrigerated. Serum was separated and frozen at -85°C . within 12 hours of collection. The HPLC method for measuring serum concentrations of phenylacetate and phenylacetylglutamine is described elsewhere herein. Briefly, 100 μl of 10% perchloric acid were added to 200 μl of serum to precipitate the proteins. After centrifugation, the supernatant was neutralized with 25 μl of a 20% solution of potassium bicarbonate. Following a second centrifugation, 20 μl of supernatant were injected onto a 300 \times 3.4 mm C-18 column heated at 60°C . Elution was performed with an increasing gradient of acetonitrile in water from 5% to 30% over 20 minutes. Its progress was followed by monitoring UV absorbance at 208 nm. Characteristic elution times for phenylacetate and phenylacetylglutamine under these conditions were 17.1 and 9.8 minutes, respectively.

Determination of Responses to Treatment. Patients were seen at least monthly by a physician at the NCI. The response status of malignancies other than prostate cancer and primary CNS tumors was determined prior to each cycle of therapy, using conventional anatomic criteria. For prostate cancer patients, criteria from the NPCP trials and published criteria of PSA decline were used. A technetium bone scan was obtained every three months if initially positive or in the presence of new bone symptoms. The assessment of patients with gliomas is complicated by the frequent microscopic multicentricity of the tumor, the variability in tumor-associated edema and its response to steroid therapy, and technical factors which precluded using the intensity of gadolinium enhancement on MRI to determine tumor response. For these reasons, special attention was paid to changes in performance status and steroid requirements, which were assessed at each visit. Complete response was defined as complete disappearance of lesions on MRI (assessment done in two different planes), and weaning from steroids. Partial response was defined by conventional anatomic criteria, absence of deterioration in performance status and stable or decreased corticosteroid requirements. Minor response was defined similarly, using 25% as the minimal limit of size reduction. Progressive disease was defined either by anatomic criteria, deterioration in performance status by at least 20 points on Karnofsky's scale or the need for increasing steroid doses in order to maintain function. Disease stabilization was defined as the absence of a significant (more than 25%) increase or decrease in tumor size while the patient maintained or improved his performance status at his pre-treatment level. To be scored as significant, disease stabilization in these patients had to be maintained for at least three months.

Statistical Methods. To determine whether phenylacetate induces its own clearance, the AUCs following a single dose of the drug at the beginning and end of PA therapy in 8 patients were compared using the Wilcoxon signed rank test for paired data.

Pharmacokinetic Simulation and Clinical Findings. Several intermittent dosing schedules were modeled using the pharmacokinetic parameters derived from a previous trial of phenylacetate. The pharmacokinetic course of a 70 kg man given phenylacetate at 125 mg/kg/dose BID (08:00 AM and 05:00 PM) was modeled. The simulation predicted peak levels between 1.5 and 3.5 mM with trough concentrations below 1 mM and no drug accumulation (95% confidence intervals).

Analysis of drug levels in 18 patients shows peak serum concentrations (mean \pm SD) of 3.0 ± 1.2 mM ($n=10$) and 3.7 ± 0.8 mM ($n=8$) at the two dose levels. Corresponding trough concentrations were 0.2 ± 0.2 mM and 0.7 ± 0.5 mM, respectively. On average, patients spent 40% of their treatment time above a concentration of 2 mM. Drug accumulation associated with neurologic toxicity (highest phenylacetate concentration: 7.3 mM) occurred in a single patient treated at the second dose level. Population pharmacokinetic parameters were determined by fitting a one compartment non-linear model to each patient's dosing and drug concentration data, using as initial parameter estimates of the mean parameter values described elsewhere above. The pharmacokinetic parameters of each individual patient were then averaged and expressed as mean parameter values with associated standard deviation:

$$K_m = 112 \pm 34 \mu\text{g/ml},$$

$$V_{max} = 25.8 \pm 5.0 \text{ mg/kg/hr, and}$$

$$V_d = 21.4 \pm 4.6 \text{ L.}$$

Phenylacetate Clearance. The molar excretion of phenylacetylglutamine was determined from 24 hour urine collections. It accounted for $76\pm 15\%$ (mean \pm SD, $n=24$) of the dose of phenylacetate given over the same period of time. The recovery of free, non-metabolized drug was $3\pm 1\%$ of the total administered dose.

Induction of Phenylacetate Clearance. The hypothesis that phenylacetate induces its own clearance was tested by comparing AUCs following the AM infusion of phenylacetate at the beginning and at the end of therapy in 8 patients. All exhibited a decrease in AUC, with a mean value of $44\pm 27\%$ (p value=0.008) between the two ends of each cycle of therapy.

Clinical Assessment of Antitumor Activity. Three of seven patients with brain tumors displayed evidence of anti-tumor activity or disease stabilization. One had a partial response accompanied by subjective improvement in short-term memory. This response was confirmed by radiographic evidence of healing osteoblastic metastases (6 months follow up) despite rising serum PSA concentrations over the same period of time in a 57 year old man with metastatic, hormone-independent prostate cancer. She was also able to reduce her daily corticosteroid dose by 50%, from 8 to 4 mg of dexamethasone. Two patients, who initially presented with confusion, had received phenylacetate by CIVI prior to being treated on the intermittent schedule. One had a minor response documented radiologically after the change in regimen, while tumor size remained stable in the second. Both maintained a 20 and 30% improvement in performance status for six and seven months, respectively. Their dose of corticosteroids was held constant throughout their treatment.

Five of nine patients with hormone-independent prostate cancer showed clinical improvement. One experienced a

greater than 50% decline in PSA sustained for a month and has maintained a performance status of 100% on Karnofsky's scale for more than five months. One with tumor-associated disseminated intravascular coagulation (prolonged prothrombin time, low fibrinogen, normal platelet count)-stopped taking flutamide concurrently with starting phenylacetate. His hematological parameters normalized during the first week of therapy while his performance status improved by 30%, due to reduction of bone pain. Two who had already been treated with phenylacetate given by CIVI with an associated improvement in bone pain and performance status, have maintained it for five additional months. One of them also had evidence of healing osteoblastic metastases on CT scan. Comparison of pre-treatment gadolinium-enhanced brain MRI in a patient with recurrent glioblastoma multiforme versus post-treatment gadolinium-enhanced MRI after 3 cycles of phenylacetate (150 mg/kg BID), confirmed this partial response. This evidence of healing metastases continued despite steadily rising serum PSA concentrations over the same period of time from 1.0 to 80 ng/ml. Finally, one patient has had subjective amelioration of bone pain for 1 month.

Clinical Toxicities. Reversible neurological toxicity was the most common side effect associated with the administration of phenylacetate twice daily. All patients experienced mild (grade I) somnolence (peak serum concentration, mean \pm SD: 2.9 \pm 0.5 mM). Three patients who received 150 mg/kg BID developed dose-limiting neurotoxicity (serum concentrations, mean \pm SD: 5.3 \pm 1.7 mM). One with glioblastoma multiforme experienced transient spatial disorientation and hypoacusis, associated with drug accumulation (phenylacetate concentration: 7.3 mM). Another with anaplastic astrocytoma experienced grade II somnolence that was temporarily related to peak concentrations of phenylacetate (mean peak concentration: 4.7 mM). A third patient with prostate cancer who suffered from suramin-induced sensory neuropathy experienced gradual deterioration of his condition over the first ten days of phenylacetate administration (peak and trough levels, mean \pm SD: 3.9 \pm 0.4 and 0.9 \pm 0.4 mM), at which time therapy was discontinued. In this patient, the side effects improved gradually over 3 months.

Three patients with a history of angina pectoris, supraventricular tachycardia or palpitations associated with mitral valve prolapse reported reversible exacerbation of their usual symptoms during the infusion of phenylacetate. This was likely related to significant fluid shifts induced by the high sodium content of the drug formulation. No such symptoms were noted in patients free of cardiovascular impairment.

This trial was designed to overcome the problem of rapid drug accumulation associated with the delivery of high doses of phenylacetate by CIVI. The goal was to achieve transient peak phenylacetate concentrations in the range found to be active preclinically (≥ 2 mM) and allow enough time for drug elimination between each dose of phenylacetate. The pharmacokinetic information derived from the first trial of phenylacetate was used to stimulate several intermittent dosing regimen. Administering 125 mg/kg/dose of phenylacetate twice daily (9 hours apart) as 60 minute infusions, was predicted to achieve serum drug concentrations between 3 and 5 mM without drug accumulation, in more than 95% of patients. This dose was therefore chosen as the starting point for the trial.

The results confirm that most patients treated with 125 and 150 mg/kg/dose of phenylacetate achieve peak serum drug concentrations in the range of 3–5 mM. No patient treated at the first dose level experienced undesirable drug

accumulation, which occurred in 1 of 8 patients treated at the higher level. Patients' exposure to potential active concentrations of phenylacetate was equal to 40% of their total treatment time. The accuracy of the pharmacokinetic simulation successfully eliminated the need for multiple escalation steps and allowed the clinical questions to be answered rapidly.

The trial enabled the characterization of the toxicity of phenylacetate with respect to peak drug levels. The 125 mg/kg dose level was associated with a mean peak serum concentration of 3.0 mM and grade I neurocortical toxicity (somnolence). The temporal relationship between drug infusion and the onset of somnolence was noted in all patients treated at this dose level. Gradual recovery between each infusion was the rule. The second dose level (150 mg/kg BID) was associated with grade I neurotoxicity in five patients (mean peak serum concentration: 3.7 mM) and more severe toxicity in three patients whose mean peak drug concentration was 5.3 mM. Except for the deterioration seen in a patient with pre-existing suramin-induced sensory neuropathy, neurotoxicity from phenylacetate has been acute and reversible.

Evidence for the induction of phenylacetate clearance is available from the first trial of phenylacetate. The demonstration was indirect, however, due to frequent adjustments in drug dosage within each cycle of therapy. With the current fixed-dosing regimen, a 44% mean decline in the AUC associated with identical doses of drug given at the beginning and end of the two week treatment was shown. This verifies the hypothesis that PA induces its own clearance by the hepatic enzyme phenylacetyl Coenzyme A:glutamine acyltransferase. The induction of metabolism is not sustained after treatment is discontinued, lending further support to the hypothesis that metabolism of phenylacetate is self-induced. The observation of autoinduction of clearance is relevant to the optimal duration of phenylacetate therapy and the eventual need for dose modification over time.

The partial and minor responses noted in patients with malignant gliomas were not expected from a differentiating agent causing cytostasis *in vitro*. There is evidence (described herein), however, that phenylacetate inhibits the mevalonate pathway of cholesterol synthesis, which malignant astroglia rely on predominantly for their growth. This could result in cell death and decreased tumor size. An alternative mechanism relates to the depletion of circulating glutamine, a major source of energy for various tumor cell types. The metabolism of phenylacetate yields phenylacetylglutamine and causes significant reductions of plasma glutamine after rapid infusion. In this respect, although repeated administration of phenylacetate was not associated with sustained declines in plasma glutamine concentrations, a 70 kg patient receiving 125 mg/kg/dose twice daily would excrete more than 90 moles of glutamine a day under as urinary phenylacetylglutamine. At present, whether this is reflected at the tumor site is not known. Assessing tumor response in patients treated with differentiating agents may be problematic. The difficulty is especially acute in patients with hormone-independent prostate cancer, for whom PSA has been proposed as the best follow up tool available. Since PSA production is organ-specific and directly correlated with the degree of tumor differentiation, phenotypic reversion induced by phenylacetate could potentially be associated with rising serum levels of the marker. This would render PSA clinically useless as a marker of disease burden and emphasize the role of the more traditional anatomic criteria. The latter are unfortunately inappropriate for evaluating the vast majority of patients with advanced prostate cancer who

lack soft tissue metastases. Therefore, conventional anatomic criteria and performance status scores (including pain relief) be used to describe responses until further experience with PSA in this context becomes available. Applying these guidelines, five of nine patients with metastatic, hormone-independent prostate cancer appear to have clinically benefited from phenylacetate therapy.

Phenylacetate given at a dose of 125 mg/kg BID for two consecutive weeks is well tolerated and is associated with antitumor activity in patients with high grade gliomas and advanced prostate cancer.

In vivo, PA is conjugated with glutamine to form phenylacetylglutamine (PAG). PA, however, has an unpleasant odor which limits patient acceptability. Phenylbutyrate (PB) is an odorless compound which also possesses in vitro activity (0.5 to 2 mM) and is believed to undergo rapid conversion to PA by in vivo β -oxidation.

A phase I study was undertaken to examine the pharmacokinetics (PK) of PB and characterized the disposition of the two metabolites (PA and PAG). Fourteen patients with cancer (mean age 51.8 \pm 13.8 years) received a 30 min. infusion of PB at three dose levels (600, 1200 and 2000 mg/m²). Serial blood samples and a 24 hour urine collection were obtained. Samples were assayed by HPLC (CV<10%). A model to simultaneously describe the PK of all three compounds was developed using ADAPT II. Data were modeled as molar equivalents.

The model fit the data well as demonstrated by mean (\pm S.D.) coefficients of determination (r^2) for PB, PA, and PAG which were 0.96 \pm 0.07, 0.88 \pm 0.10 and 0.92 \pm 0.06, respectively. The inpatient CV% around the parameter estimates were small (range 87.2% to 33.5%). PB achieved peak concentration in the range of in vitro tumor activity and exhibited saturable elimination (K_m =34.1 \pm 18.1 μ g/ml and V_{max} =18.1 \pm 18 mg/hr/kg). Metabolism was rapid; T_{max} for PA and PAG was 1 and 2 hours, respectively. The conversion of PB to PA was extensive (80 \pm 12.6%), but serum concentration of PA were low due to rapid, subsequent conversion to PAG. The ratio of PB AUC/PA AUC was 2.25. PB, thus, shows activity as an independent therapeutic agent, not necessarily solely as a prodrug of PA.

SECTION R: NAPA AND NAPB ALTERATIONS OF LIPID METABOLISM

NaPA has shown consistent ability to induce biochemical changes related to lipid metabolism. Such activity is of value in the treatment of lipid metabolism related disorders, including cancer and cardiovascular disease.

As discussed previously, NaPA and its derivatives activate the human peroxisomal proliferator activated receptor (PPAR). The PPAR regulates the expression of key enzymes involved in lipid metabolism such as acyl-CoA oxidase and the cytochrome P450IV family of enzymes. Other agents are capable of activating the PPAR, e.g., fatty acids and hypolipidemic drugs such as fibric acid derivatives (clofibrate). Drugs in this group have been highly effective in lowering serum triglyceride levels and therefore are widely used to reduce the level of atherogenic lipoproteins associated with elevated risk of coronary artery disease.

Glioma cells accumulate lipids following treatment with PA. Thus, cancer cells are dependent on free fatty acids for some energy metabolism. There is a 30–50% reduction in lipids in patients treated with PA and PB. The lipid-lowering capacity of PA is demonstrated in several cancer patients treated With 300–350 mg/kg/day (IV) NaPA. Table 29. These patients showed a 30–60% decline in triglycerides

(TG) levels while on the therapy. For example: Patient S.L. showed TG levels reduced from 169 \pm 5 mg/dl to 75 \pm 21 mg/dl during a course of 14 IV treatments with 350 mg/kg/day PA. Two weeks after PA treatment was discontinued, TG levels recovered and reached 185 mg/dl. Similar changes were observed in other patients. See Table 29

TABLE 29

| Serum Triglyceride Levels Upon Treatment with PA | | |
|--|-----------|----------|
| Patient | TG Before | TG After |
| 1. S. L. | 169 | 75 |
| 2. S. G. | 260 | 144 |
| 3. H. L. | 295 | 135 |
| 4. L. A. | 228 | 143 |

It thus appears that PA and PB stimulate PPAR (increase of peroxidation and decrease of DNA synthesis). In addition, because PA/PB also inhibits the mevalonate pathway (see section B. above) which decreases protein prenylation (such as ras protein, G proteins and nuclear lamins), PA acts to decrease cholesterol synthesis prior to DNA synthesis. A reduced prenylated ras fails to activate a proliferation signal. Thus, as noted above, HMG CoA reductase inhibitors, e.g. lovastatin, provide a synergistic combination with PA/PB as measured by invasiveness and metastasis.

Furthermore, the TG-reducing activity of PA is essentially free of adverse effects, and is as effective or better than that of clofibrate and its derivatives (Olsson et al., Atherosclerosis 27: 279–297, 1977).

SECTION S: MODES OF DRUG ADMINISTRATION

NaPA (or PAA derivatives) may be administered locally or systemically. Systemic administration means any mode or route of administration which results in effective levels of active ingredient appearing in the blood or at a site remote from the site of administration of said active ingredient.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for intravenous, intramuscular, subcutaneous, oral, nasal, enteral, parenteral, intravesicle or topical administration. In some cases, a combination of types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, dragees, pills, tablets (including coated tablets), elixirs, suspensions, and syrups or inhalations.

Solid dosage forms in addition to those formulated for oral administration include rectal suppositories.

The compounds of the present invention may also be administered in the form of an implant.

Suitable formulations for topical administration include creams, gels, jellies, mucilages, pastes and ointments.

Suitable injectable solutions include intravenous, subcutaneous, and intramuscular injectable solutions. The compounds of the present invention may also be administered in the form of an infusion solution or as a nasal inhalation or spray.

The compounds of the present invention may also be used concomitantly or in combination with selected biological response modifiers, e.g., interferons, interleukins, tumor

125

necrosis factor, glutamine antagonists, hormones, vitamins, as well as anti-tumor agents and hematopoietic growth factors, discussed above.

It has been observed that NaPA is somewhat malodorous. Therefore, it may be preferable to administer this compound in the presence of any of the pharmaceutically acceptable odor-masking excipients or as its precursor phenylbutyrate (or a derivative or analog thereof) which has no offensive odor.

The PAA and its pharmaceutically acceptable derivatives to be used as antitumor agents can be prepared easily using pharmaceutical materials which themselves are available in the art and can be prepared by established procedures. The following preparations are illustrative of the preparation of the dosage forms of the present invention, and are not to be construed as a limitation thereof.

EXAMPLE 30

Parenteral Solution 1

A sterile aqueous solution for parenteral administration containing 200 mg/ml of NaPA for treating a neoplastic disease is prepared by dissolving 200 g. of sterilized, micronized NaPA in sterilized Normal Saline Solution, qs to 1000 ml. The resulting sterile solution is placed into sterile vials and sealed. The above solution can be used to treat malignant conditions at a dosage range of from about 100 mg/kg/day to about 1000 mg/kg/day. Infusion can be continuous over a 24 hour period.

EXAMPLE 31

Parenteral Solution-2

A sterile aqueous solution for parenteral administration containing 50 mg/ml of NaPA is prepared as follows:

| Ingredients | Amount |
|-------------------------|------------|
| NaPA, micronized | 50 g. |
| Benzyl alcohol | 0.90% w/v |
| Sodium chloride | 0.260% w/v |
| Water for injection, qs | 1000 ml |

The above ingredients, except NaPA, are dissolved in water and sterilized. Sterilized NaPA is then added to the sterile solution and the resulting solution is placed into sterile vials and sealed. The above solution can be used to treat a malignant condition by administering the above solution intravenously at a flow rate to fall within the dosage range set forth in Example 30.

EXAMPLE 32

Parenteral Solution 3

A sterile aqueous solution for parenteral administration containing 500 mg/ml of sodium phenylbutyrate is prepared as follows:

| Ingredients | Amount |
|-------------------------|------------|
| Sodium phenylbutyrate | 500 g. |
| Dextrose | 0.45% w/v |
| Phenylmercuric nitrate | 0.002% w/v |
| Water for injection, qs | 1000 ml. |

The preparation of the above solution is similar to that described in Examples 30 and 31.

126

EXAMPLE 33

Tablet Formulation 1

A tablet for oral administration containing 300 mg of NaPA is prepared as follows:

| Ingredients | Amount |
|----------------------|----------|
| NaPA | 3000 g. |
| Polyvinylpyrrolidone | 225 g. |
| Lactose | 617.5 g |
| Stearic acid | 90 g. |
| Talc | 135 g. |
| Corn starch | 432.5 g. |
| Alcohol | 45 L |

NaPA, polyvinylpyrrolidone and lactose are blended together and passed through a 40-mesh screen. The alcohol is added slowly and the granulation is kneaded well. The wet mass is screened through a 4-mesh screen, dried overnight at 50° C. and screened through a 20-mesh screen. The stearic acid, talc and corn starch is bolted through 60-mesh screen prior to mixing by tubing with the granulation. The resulting granulation is compressed into tablets using a standard 7/16 inch concave punch.

EXAMPLE 34

Tablet Formulation 2

A tablet for oral administration containing 200 mg of sodium phenylbutyrate is prepared as follows:

| Ingredients | Amount |
|-----------------------------|---------|
| Sodium phenylbutyrate | 2240 g. |
| Compressible sugar (Di-Pac) | 934 g. |
| Sterotex | 78 g. |
| Silica gel (Syloid) | 28 g. |

The above ingredients are blended in a twin-shell blender for 15 minutes and compressed on a 13/2 inch concave punch.

EXAMPLE 35

Intranasal Suspension

A 500 ml sterile aqueous suspension is prepared for intranasal installation as follows:

| Ingredients | Amount |
|----------------------------|---------|
| NaPA, micronized | 30.0 g. |
| Polysorbate 80 | 2.5 g. |
| Methylparaben | 1.25 g. |
| Propylparaben | 0.09 g. |
| Deionized water, qs 500 ml | |

The above ingredients, with the exception of NaPA, are dissolved in water and sterilized by filtration. Sterilized NaPA is added to the sterile solution and the final suspensions are aseptically filled into sterile containers.

127

EXAMPLE 36

Ointment

An ointment is prepared from the following ingredients:

| Ingredients | Amount |
|------------------|--------|
| NaPA | 10 g. |
| Stearyl alcohol | 4 g. |
| White wax | .8 g. |
| White petrolatum | 78 g. |

The stearyl alcohol, white wax and white petrolatum are melted over a steam bath and allowed to cool. The NaPA is added slowly to the ointment base with stirring.

EXAMPLE 37

Lotion

| Ingredient | Amount |
|---------------------------------|-----------|
| Sodium phenylbutyrate | 1.00 g. |
| Stearyl methylcellulose (4,500) | 25.00 ml |
| Solution (2%) | |
| Benzalkonium chloride | 0.03 g. |
| Sterile water | 250.00 ml |

The benzalkonium chloride is dissolved in about 10 ml. of sterile water. The sodium phenylbutyrate is dispersed into methylcellulose solution by means of vigorous stirring. The methylcellulose (4,500) used is a high viscosity grade. The solution of benzalkonium chloride is then added slowly while stirring is continued. The lotion is then brought up to the desired volume with the remaining water. Preparation of the lotion is carried out under aseptic conditions.

EXAMPLE 38

Dusting Powder

| Ingredients | Amount |
|--|--------|
| NaPA | 25 g. |
| Sterilized absorbable maize starch BP dusting powder | 25 g. |

The dusting powder is formulated by gradually adding the sterilized absorbable dusting powder to NaPA to form a uniform blend. The powder is then sterilized in conventional manner.

EXAMPLE 39

Suppository, Rectal and Vaginal Pharmaceutical Preparations

Suppositories, each weighing 2.5 g. and containing 100 mg. of NaPA are prepared as follows:

| Ingredients | Amount/1000 |
|------------------------------|-------------|
| Suppositories | 100 g. |
| NaPA, micronized | |
| Propylene glycol | 150 g. |
| Polyethylene glycol 4000, qs | 2500 g. |

128

NaPA is finely divided by means of an air micronizer and added to the propylene glycol and the mixture is passed through a colloid mill until uniformly dispersed. The polyethylene glycol is melted and the propylene glycol dispersion added slowly with stirring. The suspension is poured into unchilled molds at 40° C. Composition is allowed to cool and solidify and then removed from the mold and each suppository is foil wrapped.

The foregoing suppositories are inserted rectally or vaginally for treating neoplastic disease.

EXAMPLE 40

Intravesical Treatment

Local application of PA/PB or a derivative/analog to a body surface, such as a mucosal surface, or within a body cavity, such as the bladder, kidney, uterine, vagina etc., can be used to prevent or treat a pathology involving that surface or cavity. In this manner, the PA can be targeted selectively to the particular surface or cavity so as to maintain a relatively high concentration of the PA at that site compared to elsewhere in the body.

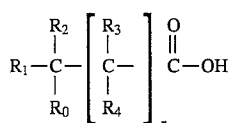
For instance, bladder cancer can be treated by an intravesical method. The bladder cancer patient can abstain from food and water for a substantial period of time prior to the treatment, such as for 8–12 hours. Following this abstinence, the patient can be catheterized. Approximately 50–150 mls of a solution of having a concentration of approximately 2.0–200.0 mM, preferably 2–20 mM, sodium phenylacetate or 1.0–100 mM, preferably 1–10 mM, sodium phenylbutyrate or other equipotent amount of a PA analog will be instilled directly into the bladder. The patient will then be requested to retain the instilled fluid for as long as possible. This treatment will be repeated, such as once daily for two-four weeks followed by two weeks of drug holiday. Cycles such as this can be repeated, such as for up to 6 months. Similarly, kidney cancers could also be similarly treated.

It is known that intracellular glutathione plays a major role in detoxification and repair of cellular injury by chemical and physical carcinogens. NaPA treatment of normal or tumor cells markedly induced the activity of intracellular glutathione approximately 2–10 fold depending on growth conditions. Nontoxic agents that can induce glutathione are highly desirable since these are likely to protect cells from damage by a variety of chemical carcinogens and ionizing radiation.

Taken together, the present invention demonstrates that NaPA, NaPB and other PAA derivatives have valuable potential in cancer prevention in case such as high risk individuals, for example, heavy smokers with familial history of lung cancer, inherited disorders of oncogene abnormalities (Li-Fraumeni syndrome), individuals exposed to radiation, and patients in remission with residual disease. Furthermore, these compounds can be used in combination with other therapeutic agents, such as chemicals and radiation, to enhance tumor responses and minimize adverse effects such as cytotoxicity and carcinogenesis. The antitumor activity, lack of toxicity, and easy administration qualify NaPA as a preferred chemopreventive drug.

What is claimed is:

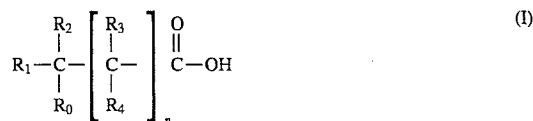
1. A method of treating a neoplastic condition in a subject comprising administering a therapeutic amount of an inhibitor of the mevalonate pathway in combination with a therapeutic amount of a compound of the formula I:



wherein

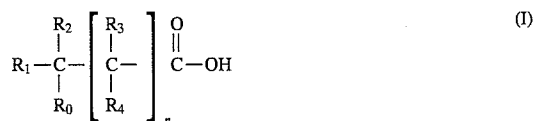
- R_0 is phenyl, naphthyl, phenoxy, substituted phenyl, substituted naphthyl, or substituted phenoxy, wherein the substitution is, independently, one or more halogen, hydroxy, or lower straight or branched chain alkyl; 10
- R_1 and R_2 are, independently, H, hydroxy, lower alkoxy, lower straight or branched chain alkyl or halogen;
- R_3 and R_4 are, independently, H, lower alkoxy, lower straight or branched chain alkyl or halogen; and 15
- n is an integer from 0 to 2;
- a pharmaceutically-acceptable salt thereof or a mixture thereof.
2. The method of claim 1, wherein the inhibitor is a vastatin. 20
3. The method of claim 2, wherein the vastatin is lovastatin.
4. The method of claim 1, wherein the inhibitor is a terpene. 25
5. The method of claim 1, wherein the terpene is limonene.
6. The method of claim 1, wherein the neoplastic condition is malignant glioma.
7. The method of claim 1, wherein the neoplastic condition is adenocarcinoma. 30
8. The method of claim 1, wherein the neoplastic condition is melanoma.
9. The method of claim 1, wherein the compound of formula I is sodium phenylacetate.
10. The method of claim 1, wherein compound of formula I is sodium phenylbutyrate. 35
11. The method of claim 1, wherein the disorder is non-malignant glioma.
12. The method of claim 1, wherein the disorder is benign prostatic hyperplasia. 40
13. The method of claim 1, wherein the disorder is papillomavirus infection.
14. The method of claim 1, further comprising the steps of: 45
- continuously monitoring the subject for rhabdomyolysis-induced myopathy; and
 - in the presence of rhabdomyolysis-induced myopathy, administering ubiquinone to the subject.
15. The method of claim 1, wherein, for R_0 , the lower straight or branched chain alkyl is of from 1 to 4 carbon atoms, for R_1 and R_2 , the lower alkoxy is of from 1 to 2 carbon atoms and the lower straight or branched chain alkyl is of from 1 to 4 carbon atoms, and for R_3 and R_4 , the lower alkoxy is of from 1 to 2 carbon atoms and the lower straight or branched chain alkyl is of from 1 to 4 carbon atoms. 50
16. The method of claim 1, wherein the compound of formula I is α -methylphenylacetic acid, α -ethylphenylacetic acid, α -hydroxyphenylacetic acid, α -methoxyphenylacetic acid, 1-naphthylacetic acid, 4-chlorophenylacetic acid, 4-iodophenylacetic acid, 4-fluorophenylacetic acid, 3-chlorophenylacetic acid, 2-chlorophenylacetic acid, 2,6-dichlorophenylacetic acid, 2-methylphenylacetic acid, 3-methylphenylacetic acid, 4-methylphenylacetic acid, phenoxypionic acid, 4-chlorophenylbutyric acid, 4-iodophenylbutyric acid, 4-fluorophenylbutyric acid, 3-chlorophenylbutyric acid, or 2-chlorophenylbutyric acid. 65

17. A product for simultaneous, separate, or sequential use in treating a neoplastic condition in a subject, comprising, in separate preparations, a therapeutic amount of a vastatin and a therapeutic amount of a compound of the formula I:



wherein

- R_0 is phenyl, naphthyl, phenoxy, substituted phenyl, substituted naphthyl, or substituted phenoxy, wherein the substitution is, independently, one or more halogen, hydroxy, or lower straight or branched chain alkyl;
- R_1 and R_2 are, independently, H, hydroxy, lower alkoxy, lower straight or branched chain alkyl or halogen;
- R_3 and R_4 are, independently, H, lower alkoxy, lower straight or branched chain alkyl or halogen; and
- n is an integer from 0 to 2;
- a pharmaceutically-acceptable salt thereof or a mixture thereof.
18. The product of claim 17, further comprising a therapeutic amount of ubiquinone.
19. The product of claim 17, wherein the compound of formula I is sodium phenylacetate.
20. The product of claim 17, wherein the compound of formula I derivative is sodium phenylbutyrate.
21. The product of claim 17, wherein, for R_0 , the lower straight or branched chain alkyl is of from 1 to 4 carbon atoms, for R_1 and R_2 , the lower alkoxy is of from 1 to 2 carbon atoms and the lower straight or branched chain alkyl is of from 1 to 4 carbon atoms, and for R_3 and R_4 , the lower alkoxy is of from 1 to 2 carbon atoms and the lower straight or branched chain alkyl is of from 1 to 4 carbon atoms.
22. The product of claim 17, wherein the compound of formula I is α -methylphenylacetic acid, α -ethylphenylacetic acid, α -hydroxyphenylacetic acid, α -methoxyphenylacetic acid, 1-naphthylacetic acid, 4-chlorophenylacetic acid, 4-iodophenylacetic acid, 4-fluorophenylacetic acid, 3-chlorophenylacetic acid, 2-chlorophenylacetic acid, 2,6-dichlorophenylacetic acid, 2-methylphenylacetic acid, 3-methylphenylacetic acid, 4-methylphenylacetic acid, phenoxypionic acid, 4-chlorophenylbutyric acid, 4-iodophenylbutyric acid, 4-fluorophenylbutyric acid, 3-chlorophenylbutyric acid, or 2-chlorophenylbutyric acid.
23. A composition, comprising a therapeutic amount of a vastatin and a therapeutic amount of a compound of the formula I:



wherein

- R_0 is phenyl, naphthyl, phenoxy, substituted phenyl, substituted naphthyl, or substituted phenoxy, wherein the substitution is, independently, one or more halogen, hydroxy, or lower straight or branched chain alkyl;
- R_1 and R_2 are, independently, H, hydroxy, lower alkoxy, lower straight or branched chain alkyl or halogen;
- R_3 and R_4 are, independently, H, lower alkoxy, lower straight or branched chain alkyl or halogen; and
- n is an integer from 0 to 2;
- a pharmaceutically-acceptable salt thereof or a mixture thereof.

131

24. The composition of claim 23, wherein, for R_0 , the lower straight or branched chain alkyl is of from 1 to 4 carbon atoms, for R_1 and R_2 , the lower alkoxy is of from 1 to 2 carbon atoms and the lower straight or branched chain alkyl is of from 1 to 4 carbon atoms, and for R_3 and R_4 , the lower alkoxy is of from 1 to 2 carbon atoms and the lower straight or branched chain alkyl is of from 1 to 4 carbon atoms.

25. The composition of claim 23, wherein the compound of formula I is α -methylphenylacetic acid, α -ethylphenylacetic acid, α -hydroxyphenylacetic acid, α -methoxyphenyl-

132

lacetic acid, 1-naphthylacetic acid, 4-chlorophenylacetic acid, 4-iodophenylacetic acid, 4-fluorophenylacetic acid, 3-chlorophenylacetic acid, 2-chlorophenylacetic acid, 2,6-dichlorophenylacetic acid, 2-methylphenylacetic acid, 3-methylphenylacetic acid, 4-methylphenylacetic acid, phenoxypionic acid, 4-chlorophenylbutyric acid, 4-iodophenylbutyric acid, 4-fluorophenylbutyric acid, 3-chlorophenylbutyric acid, or 2-chlorophenylbutyric acid.

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