Antiproliferative Effects of Inhibitors of Polyamine Synthesis in Tumors of Neural Origin

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Abstract □ The antiproliferative properties of inhibitors of polyamine synthesis were evaluated in cultured neuroblastoma and glioma cells. The diamines (1,3-propanediamine, 1,5-pentanediamine, and 1,6-hexanediamine) dramatically decreased neuroblastoma replication and inhibited the rate-limiting enzyme, ornithine decarboxylase. Glioma cells were less sensitive to the diamines in spite of significant drug-induced decreases in enzyme activity. The fact that ornithine decarboxylase was inhibited in both cell lines with different effects on proliferation suggests that the activity of other enzymes in polyamine biosynthesis may be altered selectively by these inhibitors.

Keyphrases □ Polyamines—inhibition of synthesis, effects of inhibitors on tumor cell growth, *in vitro* testing □ Ornithine carboxylase activity—inhibition of polyamine synthesis, effects of inhibitors on tumor cell growth, *in vitro* testing □ Antitumor activity—inhibition of polyamine synthesis, *in vitro* testing of ornithine carboxylase activity

Many chemotherapeutic agents that are effective in tumor treatment have had limited success in the treatment of malignant gliomas and neuroblastomas (1–4). Results have not been satisfactory due to drug toxicity, low tumor remission rates, and short durations of drug effectiveness.

Data have been obtained showing the presence of high levels of ornithine decarboxylase activity in tumor cells of neural origin (5–8). This enzyme catalyzes the rate-limiting reaction in the synthesis of putrescine, spermidine, and spermine (9); its basal activity is the lowest of the enzymes engaged in the synthesis of these polyamines (9, 10).

BACKGROUND

Several studies suggested that polyamine synthesis may be obligatory for tumor proliferation. Heby *et al.* (8) found a positive correlation between the growth rate of a rat brain tumor cell line and ornithine decarboxylase activity; the pattern of fluctuation in spermidine content correlated in a likewise manner (11). In cell-free systems, polyamines influence nearly every aspect of the formation and turnover of DNA, RNA, and protein (12–14). The relationship of polyamine synthesis to rapid growth has been reviewed extensively (10, 15–18).

Since ornithine decarboxylase activity is associated with rapid growth (16), ornithine decarboxylase inhibitors may find application as antiproliferative agents. Results obtained in these laboratories show that the cytolytic effects of bromoacetylcholine and bromoacetate in neuroblastoma are associated with decreased ornithine decarboxylase activity (5). However, recent studies suggested that a similar relationship may not be true in gliomas (20). Bromoacetylcholine and bromoacetate are potent antiproliferative agents in cultured cells of both experimental tumors (19), but ornithine decarboxylase is not inhibited in glioma cells by these agents (20). Although neuroblastoma and glioma have a common embryonic origin, these results suggest that differences may exist in the control mechanisms of polyamine synthesis.

Neuroblastoma cells are sensitive to numerous analogs of the substrate, ornithine, and of the product, putrescine (20). In this cell line, a correlation exists between the antiproliferative effects of these agents and their ability to decrease the activity of ornithine decarboxylase (20). To define further the relationship between polyamine deficiency and decreases in neuronal tumor growth rates, the effects of selective inhibitors of ornithine decarboxylase in cultured neuroblastoma and glioma cells were compared.

EXPERIMENTAL

Tumor Maintenance—Mouse neuroblastoma cells[1] were grown as described previously (5). Rat glioma cells[2] were cultivated in Ham's F-10 nutrient medium[3] supplemented with 50 units of penicillin/ml, 50 mEq of streptomycin/ml, 0.125 μg of amphotericin B/ml, 2% fetal calf serum, and 18% horse serum. Incubation was maintained at 37° with 10% CO_2–90% air and 100% humidity.

Drug Treatment—1,3-Propanediamine dihydrochloride[4], 1,6-hexanediamine[4], 1,5-pentanediamine[4], and α-methylornithine[5] were dissolved in glass-distilled water, neutralized, filter sterilized, and added in 50-μl quantities to 10 ml of the medium at time zero. For assays of ornithine decarboxylase activity, 1×10^6 cells were seeded in 100-mm tissue culture dishes; for cell counting experiments, 5×10^4 cells were seeded in 35-mm tissue culture dishes. Cells were grown for 24 hr after seeding, at which time drugs and fresh medium were added (time zero). Tumor growth was measured by counting the cells in a hemocytometer; viability was determined by trypan blue exclusion.

Ornithine Decarboxylase Assay—Enzyme assays were performed as described previously (5). After the growth medium was removed from the cells, the cells were washed with ice-cold phosphate–saline buffer (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, and 0.05% KH_2PO_4 at pH 7.2) and suspended in 1.0 ml of assay buffer (0.05 *M* sodium phosphate–potassium phosphate at pH 7.2 containing 1 m*M* 1,4-dithiothreitol, 0.25 m*M* ethylenediaminetetraacetic acid, and 3 μ*M* pyridoxal 5-phosphate). Cells then were scraped off the plate with a rubber policeman and homogenized (20 strokes) with a glass homogenizer. After centrifugation at 4500×*g*, 0.2-ml portions of the supernatant fractions were incubated for 15 min in 15 × 85-mm centrifuge tubes equipped with rubber stoppers that supported polyethylene center wells[6]. Supernatant fractions that were heated at 65° for 10 min before incubation served as controls.

The reaction was initiated when 0.05 ml of 9.5 m*M* [1-^{14}C]L-ornithine[7] (10 Ci/mole) was added. After incubation at 37° for 30 min, 0.2 ml of benzetronium[8] hydroxide was injected into each center well and the reaction was continued for 30 min. To stop the reaction, 1 *M* citric acid (0.3 ml) was injected into the incubation mixture. To release the dissolved carbon dioxide, the tubes were agitated for another 30 min. The center wells then were removed from the tubes and placed into scintillation vials, and the radioactivity was determined with a scintillation counter. Protein was determined with an assay kit[9].

RESULTS

The antiproliferative effects of the ornithine decarboxylase inhibitors are shown in Fig. 1. Neuroblastoma cell growth appeared to be more sensitive than glioma cell growth to all of the agents over the period studied. The diamine inhibitors (1,3-propanediamine, 1,5-pentanediamine, and 1,6-hexanediamine) markedly inhibited neuroblastoma replication; 1,5-pentanediamine and 1,3-propanediamine were effective as early as 24 hr after their addition. These cells were less sensitive to the competitive inhibitor α-methylornithine; a delay of one generation was required before a decrease in cell proliferation was observed using this agent.

In contrast to the neuroblastoma results, normal growth was observed in cultured glioma cells over a 48-hr period in the presence of the inhibitors. After a lag of at least two generations, a notable decrease in tumor

[1] C1300, American Type Culture Collection.
[2] Clone C6, American Type Culture Collection.
[3] Grand Island Biological Co., Grand Island, NY 14072.
[4] Aldrich Chemical Co., Milwaukee, WI 53233.
[5] Calbiochem-Behring Corp., San Diego, CA 92112.
[6] Kontes Glass Co., Vineland, NJ 08360.
[7] New England Nuclear, Boston, MA 02118.
[8] Hyamine.
[9] Bio-Rad Laboratories, Rockville Centre, NY 11570.

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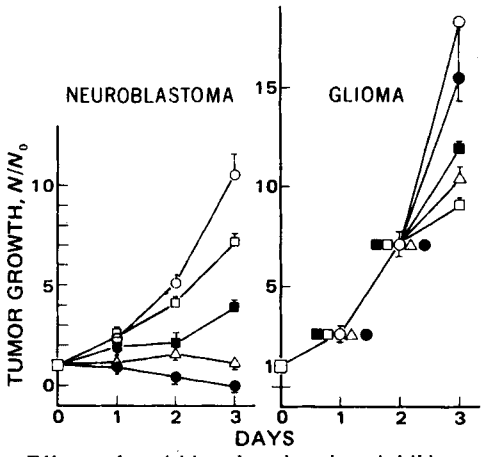

Figure 1—*Effects of ornithine decarboxylase inhibitors on neuroblastoma and glioma cell division. Key:* ○, *control;* □, *5 mM α-methylornithine;* ●, *5 mM 1,3-propanediamine;* △, *5 mM 1,5-pentanediamine; and* ■, *5 mM 1,6-hexanediamine. Results are expressed as the ratio* N/N_0, *where* N = *number of viable cells per dish at Day 1, 2, or 3 and* N_0 = *number of viable cells at time zero. Bars represent standard errors of the mean of six to 10 values.*

growth was apparent. At this time, α-methylornithine appeared to be slightly more effective than the diamines.

In experiments in which ornithine decarboxylase activity was measured, the antiproliferative effects of the diamines in neuroblastoma correlated with the decrease in enzyme activity (Fig. 2). Similarly, 1,3-propanediamine had no effect on glioma cell proliferation or on inhibition of ornithine decarboxylase activity. There were two exceptions to these parallel relationships:

1. Tumor growth was inhibited by α-methylornithine, but increases in enzyme activity were observed in both cell lines.
2. 1,5-Pentanediamine and 1,6-hexanediamine notably inhibited ornithine decarboxylase activity in glioma cells at a time when normal cell growth was observed.

DISCUSSION

An extensive amount of evidence has led to the concept that continuous polyamine synthesis is required to maintain maximum cell proliferation rates. An increase in ornithine decarboxylase activity is one of the earliest events to occur when cells are transformed from the resting to the growing state (5–7). It still is uncertain whether increases in polyamine synthesis are the cause or the consequence of rapid growth. However, a causal relationship between polyamine depletion and the inhibition of DNA synthesis has been suggested by results showing that exogenous ornithine or polyamines reversed the inhibition of DNA synthesis in tumor cells (21–24).

Most experimental efforts to deplete cellular polyamines have focused on the development of inhibitors of the rate-limiting enzyme, ornithine decarboxylase. Among these are competitive inhibitors such as α-methylornithine (22, 25, 26) and diamines such as 1,3-propanediamine, 1,5-pentanediamine, and 1,6-hexanediamine. The diamines have been shown to act by inducing the formation of macromolecular inhibitors of ornithine decarboxylase (27, 28) and by repressing the induction of the enzyme (28–32). The repression of enzyme synthesis has a relatively selective effect on ornithine decarboxylase; however, *S*-adenosylmethionine decarboxylase activity is inhibited slightly (33). A nonspecific inhibition of protein synthesis or a repression of other enzymes with short half-lives (*e.g.*, tyrosine aminotransferase) has not been demonstrated (33). The diamines are not inhibitory when added to cell-free assays (27–33).

In neuroblastoma, the diamines produced a dramatic decrease in both tumor growth and ornithine decarboxylase activity. In contrast, glioma cells continued to proliferate in the presence of the diamines. Differences in sensitivity probably are not due to drug accessibility since the enzyme was inhibited in both cell lines. These results may be explained by the interactions of the diamines with other enzymes in the polyamine biosynthetic pathway. Recent work with rat ventral prostate extracts (34) and Ehrlich ascites carcinoma cells (29) showed that diamines exert different inhibitory effects on the spermidine and spermine synthetases, regardless of their action on ornithine decarboxylase. Since a functional relationship between spermidine levels and growth rates has been confirmed in some cell lines (8, 11), these higher polyamines may be required for the expression of ornithine decarboxylase activity in proliferation (35).

In glioma cells, spermidine and spermine synthetases may be less sensitive to the diamine inhibitors and/or the half-life of endogenous spermidine may be quite long. In the latter case, the delay before the antiproliferative effects may correspond to the greater time required for spermidine depletion in this cell line. It also is possible that in glioma cells, these nonphysiological amines may be utilized to take over many of the metabolic functions of spermidine or spermine. The requirements for specific polyamines during rapid growth are being investigated.

In the present work, ornithine decarboxylase activity was increased in both neuroblastoma and glioma after treatment with α-methylornithine; competitive inhibition was observed only when the drug was added to cell-free assays (results not shown). This rise in the activity of the target enzyme in intact cells was reported for other cell lines (35) and may result either from a drug-induced increase in the apparent half-life of the enzyme (26, 36) or from a reversal of end-product inhibition of ornithine decarboxylase (24). Theoretically, an increased half-life could result from the ability of α-methylornithine to bind to the enzyme and protect it from degradation (37). However, in rat hepatoma cells, α-methylornithine increased the activities of ornithine decarboxylase as well as *S*-adenosylmethionine decarboxylase (24). This increase in an enzyme for which α-methylornithine is not a substrate is of concern. Reports that competitive ornithine decarboxylase inhibitors cause decreases in putrescine and spermidine levels (22) and that polyamines exert multiple negative feedback controls over both ornithine decarboxylase and *S*-adenosylmethionine decarboxylase (35) collectively favor the interpretation that increases in enzyme activity observed *in vivo* result from a reversal of end-product inhibition.

In summary, antiproliferative effects are achievable in tumors of neural origin using ornithine decarboxylase inhibitors. Some problems with this approach may be overcome by the design of more specific inhibitors of spermidine and spermine synthetases. The fact that ornithine decarboxylase was inhibited in both cell lines with vastly different effects on proliferation suggests that neuroblastoma and glioma cells may provide interesting model systems for drug development.

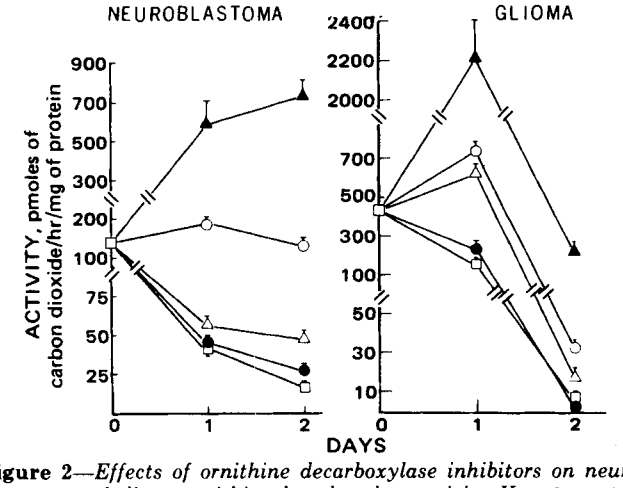

Figure 2—*Effects of ornithine decarboxylase inhibitors on neuroblastoma and glioma ornithine decarboxylase activity. Key:* ○, *control;* ▲, *0.5 mM α-methylornithine;* △, *0.5 mM 1,3-propanediamine;* ●, *0.5 mM 1,5-pentanediamine; and* □, *0.5 mM 1,6-hexanediamine. Bars represent standard errors of the mean of four to six values.*

REFERENCES

(1) D. H. James, Jr., O. Histu, E. L. Wren, Jr., and D. Pinkel, *J. Am. Med. Assoc.*, **194**, 123 (1965).

(2) A. E. Evans, R. M. Heyn, W. A. Newton, Jr., and S. L. Leiken, *ibid.*, **207**, 1325 (1967).

(3) M. P. Sullivan, A. H. Nora, P. Kulapongs, D. M. Lane, J. Windmiller, and W. G. Thurman, *Pediatrics*, **44**, 685 (1969).

(4) A. Shaw, in "Neuroblastoma," C. Pochedly, Ed., Publishing Sciences Group, Acton, Mass., 1976, p. 237.

(5) S. K. Chapman, M. Martin, M. S. Hoover, and C. Y. Chiou, *Biochem. Pharmacol.*, **27**, 717 (1978).

(6) U. Bachrach, *Proc. Natl. Acad. Sci. USA*, **72**, 3087 (1975).

(7) U. Bachrach, *FEBS Lett.*, **68**, 63 (1976).
(8) O. Heby, L. J. Marton, C. B. Wilson, and H. M. Martinez, *J. Cell Physiol.*, **86**, 511 (1975).
(9) A. Raina, R. L. Pajula, and T. Eloranta, *FEBS Lett.*, **67**, 252 (1976).
(10) J. Jänne, H. Pösö, and A. Raina, *Biochim. Biophys. Acta*, **473**, 241 (1978).
(11) O. Heby, L. J. Marton, C. B. Wilson, and H. M. Martinez, *FEBS Lett.*, **50**, 1 (1975).
(12) L. Stevens, *Biol. Rev.*, **45**, 1 (1970).
(13) A. Raina and J. Jänne, *Med. Biol.*, **53**, 121 (1975).
(14) C. M. Caldarera, C. Rossoni, and A. Casti, *Ital. J. Biochem.*, **25**, 33 (1976).
(15) U. Bachrach, "Function of Naturally Occurring Polyamines," Academic, New York, N.Y., 1973.
(16) D. H. Russell and B. G. M. Durie, *Prog. Cancer Res. Ther.*, **8**, 1 (1978).
(17) C. Clo, G. C. Orlandini, A. Casti, and C. Guarnieri, *Ital. J. Biochem.*, **25**, 94 (1976).
(18) H. G. Williams-Ashman and Z. N. Canellakis, *Perspec. Biol. Med.*, **22**, 421 (1979).
(19) C. Y. Chiou, C. J. Chu, and N. E. Liddell, *Arch. Int. Pharmacodyn. Ther.*, **235**, 35 (1978).
(20) S. K. Chapman, R. L. Hawke, S. K. Glant, S. S. Mann, K. S. Hwang, and J. W. Doyle, *Pharmacology*, **21**, 233 (1979).
(21) R. H. Fillingame, C. M. Jorstad, and D. R. Morris, *Proc. Natl. Acad. Sci. USA*, **72**, 4042 (1975).
(22) P. S. Mamont, P. Bohlen, P. McCann, P. Bey, F. Schuber, and C. Tardif, *ibid.*, **73**, 1626 (1976).
(23) J. E. Kay and A. E. Pegg, *FEBS Lett.*, **29**, 301 (1973).
(24) E. Hölttä, P. Pohjanpelto, and J. Jänne, *ibid.*, **97**, 9 (1979).
(25) M. M. Abdel-Monem, N. E. Newton, and C. E. Weeks, *J. Med. Chem.*, **17**, 447 (1974).
(26) P. P. McCann, C. Tardif, M. C. Duchesne, and P. S. Mamont, *Biochem. Biophys. Res. Commun.*, **76**, 893 (1977).
(27) W. F. Fong, J. S. Heller, and E. S. Canellakis, *Biochim. Biophys. Acta*, **428**, 456 (1976).
(28) A. Kallio, *Acta Chem. Scand.*, **32**, 759 (1978).
(29) A. Kallio, H. Pösö, G. Scalabrino, and J. Jänne, *FEBS Lett.*, **73**, 229 (1977).
(30) A. Kallio, H. Pösö, S. K. Guha, and J. Jänne, *Biochem. J.*, **166**, 89 (1977).
(31) H. Pösö, *Acta Chem. Scand. B*, **31**, 71 (1977).
(32) A. E. Pegg, C. Conover, and A. Wrona, *Biochem. J.*, **170**, 651 (1978).
(33) H. Pösö, A. Kallio, G. Scalabrino, and J. Jänne, *Biochim. Biophys. Acta*, **497**, 288 (1977).
(34) H. Hibasami and A. E. Pegg, *Biochem. Biophys. Res. Commun.*, **81**, 1398 (1978).
(35) P. S. Mamont, M. C. Duchesne, A. J. Joder-Ohlenbusch, and J. Grove, in "Enzyme-Activated Irreversible Inhibitors," Elsevier/North-Holland Biomedical Press, Amsterdam, The Netherlands, 1978, pp. 43–53.
(36) S. I. Harik, M. C. Hollenberg, and S. H. Snyder, *Mol. Pharmacol.*, **10**, 41 (1974).
(37) R. T. Schimke, *Adv. Enzymol.*, **37**, 135 (1973).

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Antineoplastic Evaluation of Pacific Basin Marine Algae

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Abstract □ Extracts of 107 marine alga specimens from Pacific islands were tested for P-388 lymphocytic leukemia and Ehrlich ascites tumor in mice. Several specimens showed high antitumor activity in both systems, with some featuring a notable lack of toxicity.

Keyphrases □ Antineoplastic activity—marine algae from Pacific islands, testing against P-388 lymphocytic leukemia and Ehrlich ascites tumor □ Marine algae—antineoplastic evaluation against P-388 lymphocytic leukemia and Ehrlich ascites tumor □ Antitumor activity—marine algae from Pacific islands, testing against P-388 lymphocytic leukemia and Ehrlich ascites tumor

There have been numerous reports of antitumor substances obtained from terrestrial plants, but no information on antitumor agents from marine plants was published prior to 1977 when Mynderse *et al.* (1) reported that chloroform extracts of some marine blue-green algae showed activity against P-388 lymphocytic leukemia in mice. A P-388 active compound was isolated from one alga, *Lyngbya majuscula* Gomont, and shown to be debromoaplysiatoxin. This finding prompted screening of other marine algae from the Pacific basin for activity against P-388 lymphocytic leukemia and Ehrlich ascites tumor in mice.

The 107 alga specimens were collected from Palau (Western Caroline Islands), Fanning Island (Line Islands), Enewetak Atoll (Marshall Islands), Johnston Island, and the Hawaiian Islands. Voucher samples were retained for most specimens, and the collection site of each sample was recorded carefully.

This paper is a preliminary report of the antitumor activity of crude extracts and some partially purified fractions from these algae.

EXPERIMENTAL

Most algal samples were refrigerated or frozen soon after collection. When refrigerator or freezer facilities were not available, the samples were air dried. The frozen samples were freed from extraneous matter and freeze dried before extraction. The dried samples were powdered in a mortar or blender[1].

There were two general extraction procedures. In Method I, the dried alga was extracted initially with a succession of organic solvents of increasing polarity followed by a final extraction with water. In Method II, the sample was subjected to extraction with 30% ethanol followed by extractions (liquid–liquid) with a succession of organic solvents of the aqueous concentrate.

In Method I, the sample was homogenized in a blender[1] for 5 min with a volume of organic solvent weighing five to 10 times the sample weight. The mixture then was allowed to stand for 24 hr or was stirred for 4–5 hr. The resultant homogenate was centrifuged, and the supernate was

[1] Waring.

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