Antitumor Activity of a Folate-cleaving Enzyme, Carboxypeptidase G₁

Bruce A. Chabner, Paul L. Chello, and Joseph R. Bertino

SUMMARY

We have studied the antitumor activity of a folate-cleaving bacterial enzyme, carboxypeptidase G₁ (CPD G₁), which hydrolyzes the C-terminal glutamate residue from folic acid analogs. CPD G₁ completely inhibited the growth of all murine leukemic cell lines tested in tissue culture at enzyme levels of 0.02 to 0.025 unit/ml. A decrease in cell number was apparent after 12 hr of exposure to the enzyme, while a decrease in cell viability, as determined by soft-agar cloning, was evident after 6 hr.

The biochemical effect of CPD G₁ in blocking incorporation of labeled deoxynucleosides into DNA was detected after 1 hr of incubation with the agent. Inhibition of DNA synthesis by CPD G₁ was characterized by a fall in the incorporation of both thymidine-³H and deoxyuridine-³H into DNA, rather than the selective depression of deoxyuridine-³H incorporation into DNA caused by methotrexate.

The folate-depleting and antitumor activity of CPD G₁ was studied in mice. CPD G₁, 800 units/kg, produced a prolonged (24-hr) fall in plasma folate to 20% of control levels and a gradual decline in hepatic folate stores. Daily doses of 800 units/kg, given for 5 to 7 days, produced a 19 to 27% prolongation of survival in mice inoculated with 10⁵ L1210 cells, while daily doses of 20 to 100 units/kg produced a 43 to 89% increase in lifespan in rats with Walker 256 carcinosarcoma.

INTRODUCTION

In 1947, Farber et al. (8) noted the acceleration of leukemic cell proliferation in children treated with polyglutamate forms of folic acid. Shortly thereafter, he demonstrated the induction of brief remissions in children with acute leukemia, using the folate analog aminopterin (9). Since that time, inhibitors of folic acid metabolism, most prominently methotrexate, have become mainstays in the treatment of certain human cancers, notably the leukemias (1) and choriocarcinoma (13).

Cell death following methotrexate treatment appears to result from inhibition of dihydrofolate reductase, with consequent depletion of intracellular stores of the reduced folate compounds required for synthesis of DNA, RNA, and certain amino acids (3). In particular, the conversion of dUMP to dTMP, a reaction requiring N⁵,N¹⁰-methylenetetrahydrofolate as substrate and cofactor, is markedly depressed in cells sensitive to the drug, presumably due to lack of the requisite reduced folate (14). Resistance to methotrexate has been associated with increased levels of the target enzyme, dihydrofolate reductase (11), and with impaired cellular uptake of the drug (16).

In a search for new antifolate chemotherapeutic agents, we have isolated and purified an enzyme, CPD G₁,¹ which hydrolyzes the peptide linkage of the C-terminal glutamate residue of both reduced and nonreduced forms of pteroylglutamate (folic acid) (18). A preliminary report described the antitumor activity of this enzyme against the L5178Y murine leukemia in tissue culture (4). The present studies were undertaken to determine the biochemical and kinetic features of tumor cell death in vitro following exposure to CPD G₁, as well as the antitumor effects of the enzyme in vivo.

MATERIALS AND METHODS

Preparation of CPD G₁. CPD G₁ was purified from Pseudomonas stutzeri according to the scheme outlined in a previous communication (18).³ Enzyme solutions used in the present studies contained 0.01 M Tris-Cl, pH 7.3, with 10⁻⁵ M ZnCl₂, and had a specific activity of 200 units/mg protein or greater. The specific activity of homogenous enzyme, as judged by disc electrophoresis, was 650 units/mg protein. Enzyme solutions were sterilized by passage through a 0.45-μm Millipore filter prior to use.

In Vitro Studies. Murine leukemia cell lines used in these studies were adapted from in vivo murine leukemia strains and maintained through serial passages as a cell suspension in the liquid media specified in Table 1. The L1210, L1210/MTX, and L5178Y cell lines have been maintained in continuous tissue culture passage for a minimum of 3 years and have produced the same murine leukemias upon periodic reinoculation into mice. The human lymphoblastoid cell line 4265 was kindly supplied by Dr. G. Moore, and the Walker 256 carcinosarcoma was a gift of Dr. F. White.

¹The abbreviations used are: CPD G₁, carboxypeptidase G₁; TdR, thymidine; UdR, deoxyuridine.
²A unit of enzyme was defined as that quantity of enzyme which hydrolyzes 1 μmole of methotrexate per min at 37°C and at pH 7.3.
Inhibition of Cell Replication in Vitro. Cultures of the various cell lines were harvested in logarithmic growth and diluted to a cell density of 8 to 40 x 10^3 cells/ml. Sterile CPD G, was prepared for filtration through a 0.45-/Lim Millipore filter, and a 0.2-ml aliquot containing the desired units of enzyme activity was added to 5-ml aliquots of the cell suspension in a series of 16- x 125-mm culture tubes, which were then incubated at 37°. At designated intervals, cultures were removed from incubation and, after appropriate dilution in 0.9% NaCl solution, cells were counted in a Model B Coulter counter.

Determination of Cell Clonability. The number of viable L5178Y cells remaining following exposure to CPD G, was further investigated by the method of soft-agar cloning as previously described by Chu and Fischer (7). Cell cultures were prepared as described for growth inhibition experiments. At designated time intervals, cultures were removed from incubation, and cells were collected by centrifugation and resuspended in 10 ml of Fischer's medium with 15% horse serum. An aliquot was removed for cell counting and an appropriate 2nd dilution was made in the same medium to give an estimated 25 to 30 viable cells/ml. Two ml of this fraction were transferred to a culture tube containing 3 ml of 0.04% Noble agar, previously softened to a liquid state at 44°. The suspension of cells in agar was cooled in an ice bath for 3 to 4 min and incubated at 37° for 14 days. Colonies were counted every 3 to 5 days until a constant number was reached. Clonability was defined as the number of clones observed divided by the number of cells added to the cloning tube. All determinations were done in quadruplicate and an average of the 4 tubes used in the calculation of clonability.

Biochemical Studies. Incorporation of TdR-3H and UdR-3H into DNA was studied in L5178Y cells according to methods previously described (14). L5178Y cells in logarithmic growth were centrifuged at 1000 rpm for 10 min and resuspended in Fischer's medium with 10% horse serum. A series of Erlenmeyer flasks (25-ml) was prepared for each enzyme level and time point, and 5 ml of cell suspension was added to each flask. CPD G, was added to each flask to give the designated level of enzyme activity. The cells were then incubated at 37° in a metabolite shaker water bath. At specified time intervals UdR-3H and TdR-3H were added to separate flasks and 1 ml of cell suspension was added to each flask. CPD G, was prepared for each enzyme level and time point, and 5 ml was added to the 4 tubes used in the calculation of clonability.

RESULTS

Inhibition of Tumor Cell Growth in Tissue Culture. CPD G, inhibited the growth of the 5 cell lines tested in tissue culture (Table 1). Complete inhibition of growth was seen with enzyme concentrations of 0.025 unit/ml or less, while the dose level which produced 50% inhibition of cell multiplication at 48 hr fell between 0.0025 and 0.025 unit/ml for each cell line tested. In the presence of 0.025 unit/ml, the depletion of folic acid from the media was complete 1 hr after the start of incubation.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Medium</th>
<th>Control doubling time (hr)</th>
<th>ED, a (units/ml X 10^-2)</th>
<th>ED, b (units/ml X 10^-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L5178Y</td>
<td>Fischer's, 10% horse serum</td>
<td>12</td>
<td>7.5</td>
<td>2.5</td>
</tr>
<tr>
<td>4265</td>
<td>RPMI 1640, 10% calf serum</td>
<td>24</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Walker 256</td>
<td>RPMI 1640, 10% calf serum</td>
<td>13</td>
<td>&gt;2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>L1210</td>
<td>RPMI 1640, 10% calf serum</td>
<td>11</td>
<td>&gt;2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>L1210/MTX</td>
<td>RPMI 1640, 10% calf serum</td>
<td>11</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

a Dose producing 50% inhibition of cell proliferation at 48 hr. Units should be multiplied by 10^-2 to obtain observed value.

b Dose producing complete inhibition of cell proliferation at 48 hr. Units should be multiplied by 10^-2 to obtain observed value.

Folic Acid Depletion in Animals. Studies of folate depletion by CPD G, were performed with BDF1 male mice, 23 to 28 g. Three groups of 9 mice were given daily i.p. injections of 0.2 ml of a solution containing 0, 400, or 800 units CPD G, per kg for 11 days. Three animals from each group were killed by cervical dislocation on Days 7, 11, and 15. Plasma was obtained by cardiac puncture for hematological and biochemical studies, which will be presented in detail elsewhere. A control necropsy examination, including histological examination of liver, spleen, kidneys, heart, lungs, thyroid, adrenals, bone marrow, and brain, was performed by the Section of Laboratory Animal Sciences of the Yale University School of Medicine.

Treatment of Transplanted Murine Leukemias. The antitumor activity of CPD G, against rodent ascitic tumors was studied in vivo. All cell lines were carried by weekly serial transplantation. L1210 lymphoma and the methotrexate-resistant subline, L1210/MTX, obtained from Dr. Dorris Hutchinson, were carried in male BDF1 mice (origin: C57BL/6J X DBA/2). The L5178Y lymphoma was carried in female AKD2F1 mice (origin: AKR X DBA/2) and the Sarcoma 180 ascites tumor in female CD1 mice. Donor animals were prepared by injection of 5 x 10^6 tumor cells i.p. Three days later, ascitic fluid was removed from the donor mice and diluted with sterile 0.9% NaCl solution; 10^5 cells were injected i.p. into each test animal.

Cr/RAR female weanling rats weighing 80 g and bearing the Walker 256 ascites carcinoma were provided by Dr. Florence White of the National Cancer Institute, Bethesda, Md. The tumor inoculum was 10^3 cells. Results of the animal experiments were analyzed for significance with Student's t test (22) and with the assistance of Dr. Richard Simon of the Medicine Branch, National Cancer Institute.

Biochemical Studies. Incorporation of TdR-3H and UdR-3H into DNA was studied in L5178Y cells according to methods previously described (14). L5178Y cells in logarithmic growth were centrifuged at 1000 rpm for 10 min and resuspended in Fischer's medium with 10% horse serum. A series of Erlenmeyer flasks (25-ml) was prepared for each enzyme level and time point, and 5 ml of cell suspension was added to each flask. CPD G, was added to each flask to give the designated level of enzyme activity. The cells were then incubated at 37° in a metabolite shaker water bath. At specified time intervals UdR-3H and TdR-3H were added to separate flasks and 1 ml of cell suspension was removed at 5, 10, 15, and 20 min for determination of the rate of incorporation of labeled deoxynucleosides into DNA (14). The rate of incorporation per 10^6 cells was calculated from the slope of a straight line through the origin fitted to the data by the method of least squares (21). The standard error of these slopes was 1 to 10% of the estimated value for all time points.

Plasma and culture medium levels of folic acid were determined by the method of Herbert (12), with Lactobacillus casei ATCC 7459 as the test organism. Liver folic acid levels were determined as described by Chanarin (2) and were expressed as μg folic acid per g, wet weight, of liver.

Folic Acid Depletion in Animals. Studies of folate depletion by CPD G, were performed with BDF1 male mice, 23 to 28 g. Three groups of 9 mice were given daily i.p. injections of 0.2 ml of a solution containing 0, 400, or 800 units CPD G, per kg for 11 days. Three animals from each group were killed by cervical dislocation on Days 7, 11, and 15. Plasma was obtained by cardiac puncture for hematological and biochemical studies, which will be presented in detail elsewhere. A complete necropsy examination, including histological examination of liver, spleen, kidneys, heart, lungs, thyroid, adrenals, bone marrow, and brain, was performed by the Section of Laboratory Animal Sciences of the Yale University School of Medicine.

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The inhibitory effect of CPD-G₁ was compared to the effect of culturing L5178Y cells in medium lacking folic acid. As shown in Chart 1, at levels of CPD-G₁ that produced less than total inhibition of growth, cells multiplied at a near normal pace for 24 hr, but plateaued prematurely in comparison to control cultures that remained in logarithmic growth for 72 hr. Cells cultured in folate-deficient medium showed a similar early plateau (Chart 2); however, this pattern differed markedly from the rapid onset of inhibition associated with levels of CPD-G₁ greater than 0.025 unit/ml.

In order to eliminate the possibility that the inhibitory effects of CPD-G₁ in vitro might be due to accumulation of the products of folate hydrolysis, we incubated L5178Y cells in Fischer's medium to which were added both pterotic and glutamic acid, 10 mg/liter. No inhibition of cell proliferation was observed.

The time course of cell death following exposure to CPD-G₁ was studied by the technique of soft-agar cloning. In the presence of 0.2 unit of CPD-G₁ L5178Y cell population remained stationary for 6 hr, following which a steady decline in number of cells was observed (Chart 3). However, a decline in clonability of cells was apparent as early as 6 hr and became more obvious with longer incubation. At 24 hr 36% of the original number of cells remained; of these, only 21.5% were viable, as judged by ability to form clones in the folate-replete cloning medium. A 10-fold higher level of CPD-G₁, 2.0 units/ml, produced an earlier fall in cell count and clonability, although the 24-hr results were similar to those observed with the lower dose.

Biochemical Effect of CPD-G₁. In order to determine whether CPD-G₁ preferentially affects the conversion of dUMP to dTMP as does methotrexate (14), we investigated the effect of CPD-G₁ on the incorporation of UdR-³H and TdR-³H into DNA of L5178Y cells in short-term tissue culture. If the thymidylate synthetase reaction were blocked preferentially due to lack of N⁵,N¹⁰-methylenetetrahydrofolic acid, a disproportionate inhibition of UdR-³H incorporation into DNA would be expected as compared to TdR-³H which bypasses the block. If incorporation of the 2 nucleosides was equally inhibited, it would be necessary to ascribe the depressed DNA synthesis to other biochemical effects of folate depletion as, for example, depressed purine synthesis. In the presence of CPD-G₁, 0.5 unit/ml, L5178Y cells incorporated both deoxynucleosides at only a fraction of the control rate (Chart 4); this fall in deoxynucleoside incorporation was evident after 1 hr of incubation and marked after 3 hr. The degree of inhibition of incorporation into DNA was similar for both TdR and UdR and did not increase with a 10-fold higher level of enzyme. The incorporation of labeled
Antitumor Activity of CPD G₁

The replication and clonability of L5178Y cells demonstrate a more rapid effect of CPD G₁ in inhibiting DNA synthesis. Culturing cells for 3 hr in medium lacking folic acid demonstrated this effect. Pharmacological Studies of CPD G₁: Studies in mice were undertaken to determine the dosage and schedule required to lower serum folates. It was found that 800 units/kg in BDF₁ male mice lowered serum folates 70 to 80% for 24 hr (Chart 5). A larger dose of 2000 units/kg produced no further drop in folate levels. Consequently, the lower dose was usually used in subsequent studies. A slow decline in the large intrahepatic stores of folates was also observed in the 24-hr period following a single i.p. injection of CPD G₁ in mice.

Toxicological studies of mice treated for 11 days with CPD G₁, 0, 400, or 800 units/kg/day, revealed no loss of weight, and no pathological changes at necropsy. There was no alteration in peripheral blood counts or in bone marrow morphology or cellularity, despite a fall in serum folate levels of approximately 80% throughout the 11-day treatment period and a gradual 40% fall in hepatic folate levels during this period.

However, occasional enzyme solutions were toxic to mice, as indicated by weight loss. Enzyme preparations displaying marked toxicity were subjected to repeat Sephadex G-200 gel filtration to remove the toxic contaminant(s).

CPD G₁ was tested against 5 types of rodent ascitic neoplasms. A standard dose of 800 units/kg/day was used in most of these trials since this was the minimum dose capable of lowering serum folates in mice for 24 hr. A 5- or 7-day course of treatment against L1210 lymphoma increased survival 19 to 27% as compared to controls (Table 2). Similar treatment also produced a brief but significant prolongation of lifespan in mice inoculated with Sarcoma 180 and L5178Y ascitic tumors. The increase in survival of mice bearing L1210/MTX was not significant. CPD G₁ was most effective in treating the Walker 256 rat carcinosarcoma; extended treatment with 20 to 100 units/kg/day produced a 43 to 89% increase in lifespan.

Chart 3. Effect of CPD G₁ on replication and clonability of L5178Y cells. See “Materials and Methods” for details. CPD G₁ concentrations are indicated in units/ml culture medium.

deoxynucleosides into DNA by L5178Y was not depressed by culturing cells for 3 hr in medium lacking folic acid, demonstrating a more rapid effect of CPD G₁ in inhibiting DNA synthesis.

Pharmacological Studies of CPD G₁: Studies in mice were undertaken to determine the toxicity, folate-depleting activity, and antitumor effects of CPD G₁. Initial studies were directed at establishing the dosage and schedule required to lower serum folates. It was found that 800 units/kg in BDF₁ male mice lowered serum folates 70 to 80% for 24 hr (Chart 5). A larger dose of 2000 units/kg produced no further drop in folate levels. Consequently, the lower dose was usually used in subsequent studies. A slow decline in the large intrahepatic stores of folates was also observed in the 24-hr period following a single i.p. injection of CPD G₁ in mice.

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Chart 4. CPD G₁ inhibition of deoxynucleoside incorporation into DNA in L5178Y cell culture. Absolute incorporation rates were calculated as described in “Materials and Methods.” The fractional incorporation rate was defined as the ratio of incorporation by cells grown in the presence of CPD G₁, 0.5 unit/ml, versus cells grown in regular Fischer’s medium. The control rate of radionucleoside incorporation varied from 2200 to 3100 cpm/min/10⁶ cells for both UdR-³H and TdR-³H. At each time point, the incorporation rate of enzyme-treated cells was significantly less than that of the control rate at the p < 0.001 level, except at the 1-hr point for UdR-³H, where p < 0.01.
DISCUSSION

CPD G₁, a 92,000 M.W. protein isolated from P. stutzeri, possesses the unique ability to hydrolyze reduced and nonreduced folic acid derivatives, as well as other glutamyl-terminal peptides (18). The present studies stem from its potential usefulness as an antifolate chemotherapeutic agent which would not require intracellular transport for activity. Such an agent might thus inhibit tumors which are resistant to methotrexate due to an inability to transport the drug, a mechanism of resistance ascribed to certain cases of human (16) and murine (17) leukemia.

CPD G₁ was found to inhibit the growth of a variety of neoplastic cell lines in tissue culture. In addition, the enzyme had marked antitumor activity in vivo against the Walker 256 carcinosarcoma and lesser although significant activity against the L1210, L5178Y, and Sarcoma 180 mouse leukemias. Several lines of evidence indicate that the antitumor activity of the enzyme is a consequence of folic acid depletion. The high degree of purity of the enzyme used in these studies, the documented depletion of folates in vitro and in vivo, and the reversal of tissue culture effects by supplementation of the medium with the end products of folate-mediated reactions (4) all support this conclusion.

Studies of L5178Y cells in tissue culture have disclosed certain differences between the lethal effect of folate deletion from the culture medium and the effect of high levels of CPD G₁. CPD G₁, 0.2 unit/ml or greater, caused a rapid inhibition of incorporation of both TdR and UdR into DNA and a decline in cell numbers and viability after 12 hr of incubation, while the effects of folate deletion on cell proliferation were not apparent for at least 2 cell divisions (24 hr). The rapid onset of action of high levels of CPD G₁ might be due to several factors, including intracellular penetration of the enzyme, an unlikely but not unprecedented phenomenon (20), or the hydrolysis of an unidentified glutamyl- or aspartyl-terminal peptide or protein required for cell replication. It is also possible that the presence of CPD G₁ in the extracellular medium produced more rapid intracellular depletion of folates by hydrolyzing the folates transported from cell to medium by an active transport mechanism (10).

These studies also indicated differences in the biochemical effects of CPD G₁ and methotrexate. In exponentially growing L5178Y cells, CPD G₁ depressed incorporation of UdR and TdR into DNA to an equal extent, while previous work from this laboratory has shown that methotrexate has a far greater inhibitory effect on UdR incorporation than on TdR incorporation, both in L5178Y and in human leukemic cells (15). Thus, CPD G₁ has no selective inhibitory effect on

Table 2

<table>
<thead>
<tr>
<th>Tumor</th>
<th>No. of animals</th>
<th>Schedule (units/kg/day)</th>
<th>Weight, Day 6 (% Day 0)</th>
<th>Survival (mean ± S.E.)</th>
<th>% increase in life span</th>
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<tr>
<td>L1210</td>
<td>8</td>
<td>Control</td>
<td>+10</td>
<td>9.6 ± 0.26</td>
<td>27 ( p &lt; 0.01 )</td>
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<tr>
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<td>8</td>
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<td>-12</td>
<td>12.25 ± 0.31</td>
<td>19 ( p &lt; 0.05 )</td>
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<tr>
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<tr>
<td>L1210/MTX</td>
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<td>-13</td>
<td>12.75 ± 0.36</td>
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<tr>
<td>L5178Y</td>
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<td>Control</td>
<td>N.D.</td>
<td>10.75 ± 0.31</td>
<td>6 ( p &gt; 0.05 )</td>
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<td>11.4 ± 0.26</td>
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<td>-3</td>
<td>13.6 ± 0.26</td>
<td>15 ( p &lt; 0.01 )</td>
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<td>6</td>
<td>Control</td>
<td>+4</td>
<td>15.7 ± 0.27</td>
<td>15 ( p &lt; 0.01 )</td>
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<tr>
<td>Walker 256</td>
<td>4</td>
<td>Control</td>
<td>20</td>
<td>17.3 ± 0.92</td>
<td>20 ( p &lt; 0.01 )</td>
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<td>3</td>
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<td>20.8 ± 0.60</td>
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<td>Control</td>
<td>+8</td>
<td>8.5 ± 0.3</td>
<td>43 ( p &lt; 0.05 )</td>
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<td>6</td>
<td>20, daily to death</td>
<td>+102</td>
<td>11.5 ± 0.3</td>
<td>61 ( p &lt; 0.01 )</td>
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<td>Control</td>
<td>+82</td>
<td>16.5 ± 1.5</td>
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Chart 5. Decline in serum and hepatic folate activity following CPD G₁ injection in mice. At time 0 mice received an i.p. injection of 800 units/kg. Serum CPD G₁ levels and serum and hepatic folate levels were measured as described in “Materials and Methods.”
thymidylate synthesis, as does methotrexate, but probably depresses DNA synthesis through its effects on purine synthesis or amino acid metabolism. The effectiveness of CPD G₁ against the ascitic form of Walker 256 carcinosarcoma, a tumor which, as a solid neoplasm, was sensitive to dietary folate depletion but resistant to methotrexate (19), suggests a possible difference in the spectrum of activity of CPD G₁ and methotrexate. This possibility was not born out in the treatment of L1210/MTX, which was resistant to CPD G₁ as well as methotrexate.

Although CPD G₁ effectively inhibited the murine leukemia cell lines tested in vitro, only the rat Walker 256 carcinosarcoma demonstrated marked sensitivity in vivo. This limited degree of success may be related to the difficulty of producing folate depletion in mice which have higher plasma levels of folic acid coenzymes (75 to 100 ng/ml) than rats (6) and humans (5 to 15 ng/ml). In other studies from this laboratory, CPD G₁, completely eliminated plasma folate activity from human plasma in vitro and canine plasma in vivo, in keeping with the low basal levels of folate coenzymes in these species (5).

The foregoing investigations have defined the time course and biochemical features of the antitumor activity of CPD G₁ in tissue culture and have presented evidence of such activity in vivo. Further studies are in progress to explore alternate doses and schedules of administration, interaction with other antineoplastic agents, and immunogenicity, all of which may influence its effectiveness as an antitumor agent.

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