Enhancement of Trimetrexate Cytotoxicity in Vitro and in Vivo by Carboxypeptidase G2

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ABSTRACT

Carboxypeptidase G2 (CPG2), an enzyme produced by Pseudomonas strain RS-16, hydrolyzes the glutamate residue from methotrexate and other folates. The possibility of enhancing trimetrexate cytotoxicity by CPG2 induced folate depletion was investigated in vitro in a human leukemia cell line, CCRF-CEM, and in three sublines of these cells each with a different methotrexate resistance phenotype. The cytotoxic effect in vitro was detected using a colorimetric assay with a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Dose-effect relationships of drugs alone and in combination were analyzed by the median effect principle and by the combination indices for quantitation of synergy or antagonism with the aid of a computer program. Trimetrexate alone was cytotoxic against the parent and all the resistant cell lines with the drug concentrations required to decrease the cell count to 50% of control in the nanomolar range (1.4, 1.6, 1.5, and 0.7 nM in CCRF-CEM, CCRF-CEM/E, CCRF-CEM/P, and CCRF-CEM/T, respectively) following 5 days of exposure. The concentration of CPG2 required to decrease the cell count to 50% control for these cell lines was 3.5, 2.6, 26.6, and 7.9 × 10⁻⁵ units/ml for CCRF-CEM, CCRF-CEM/E, CCRF-CEM/P, and CCRF-CEM/T, respectively. A synergistic cytotoxic effect of trimetrexate after simultaneous continuous exposure with CPG2 was observed with CCRF-CEM cells and with the three resistant cell lines. This drug combination given to BALB/c × DBA/2 F1 mice bearing L1210 cells also produced synergy over a narrow range of drug doses. The activity of this combination in both methotrexate sensitive and methotrexate resistant cell lines indicates that clinical trials of this combination should be undertaken.

INTRODUCTION

Folic acid is involved mainly with cell replication, and deficiencies of this vitamin result primarily in toxic effects on replicating tissues, i.e., the bone marrow and the gastrointestinal mucosa (1). Depletion of folic acid can inhibit the growth of tumors with high folic acid requirements (2). A diet deficient in folic acid can lead to folate depletion in 2 or 3 months but it is not easily accepted by patients (3). CPG2, an enzyme derived from a Pseudomonas strain, leads to a rapid depletion of intracellular folates and inhibition of cell growth (4, 5) by hydrolyzing folates to the corresponding pterate and glutamic acid (6).

The reduction of intracellular folates caused by CPG2 may increase the cytotoxic effect of TMTX or other folate antagonists, which lack a terminal glutamic acid. We therefore examined the cytotoxic effects of TMTX and CPG2 and their combination on the parental CCRF-CEM cell line and in three sublines resistant to MTX, as well as in mice bearing the L1210 tumor.

MATERIALS AND METHODS

Chemicals. CPG2 was obtained from a recombinant clone of the Pseudomonas CPG2 gene as described by Minton et al. (6) and stored in a Revo freezer at −70°C as a lypoophilized powder. The enzyme was solubilized in 10 mM Tris-Cl 0.01 mM ZnCl2 buffer, diluted, and assayed for activity before each experiment the day of use as described by McCullough et al. (7). The enzyme retained 100% of its activity for 5 days in tissue culture media at 37°C, at the concentrations utilized. It lost 20% of activity in the subsequent 2 days. Trimetrexate glucuronate was supplied by Warner-Lambert (Parke Davis, Ann Arbor, MI). MTX (M 2128) was purchased from Sigma Chemical Co. (St. Louis, MO). [3,5,7,9-H]folic acid (0.5 μCi/0.5 ml) was purchased from Moravek Biochemicals, Brea, CA. All the other chemicals were of the highest purity available. Media, sera, and antibiotics for tissue culture were purchased from Grand Island Biological Co., Grand Island, NY. Plastic ware was purchased from Corning Glass Works, Corning, NY, and Costar, Cambridge, MA.

Cell Lines. A cloned subline of the human T-lymphoblastoid cell line CCRF-CEM (8) was used as a parent cell line. The MTX resistant sublines CCRF-CEM/E, which has an 18-fold increase in the level of dihydrofolate reductase (9), CCRF-CEM/P, which has a defect in its activity to polyglutamate MTX as a result of an altered polyglutamate synthetase (10, 11), and CCRF-CEM/T, which shows an impairment in MTX uptake (12, 13), were also tested.

All the cell lines were grown in suspension in RPMI 1640 supplemented with 10% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO2 atmosphere.

Cells were treated with CPG2, TMTX, or a combination of these drugs in 15 ml tubes and then dispensed in flat-bottom 96 well microtiter plates (Costar, Cambridge, MA) using a multichannel pipet (Flow Labs. Tittertek). A total of 100 μl of cell suspension containing 1000 to 2000 cells was added to each well. The eight wells of the first row (blank) received 100 μl of culture medium.

The methodology described below represents a modification of the original MTI colorimetric assay described by Mosmann (14). In essence, the viable cell number per well is directly proportional to the production of formazan, which following solubilization can be measured spectrophotometrically.

Culture plates were incubated for 5 days prior to the addition of 60 μl of MTT solution (1.25 mg/ml). MTT stock solution was prepared as follows. Five mg MTT/ml phosphate buffered saline (Quality Biologicals, Inc.) were sterile filtered with 0.45-μm filter units (Nalgene type SCN) and stored at 4°C for a maximum of 1 month. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1:4 (v/v) in phosphate buffered saline. Cultures were incubated at 37°C for 2–4 h prior to addition of 160 μl of acid isopropl alcohol (0.04 N HCl) and thoroughly triturated to solubilize the formazan. Plates were counted after 15–20 min at room temperature at 570 nm as the test wavelength and 630 nm as the reference wavelength using a MR 580 Microelisa autorreader (Dytran Laboratories, Inc., Alexandria, VA).

Results for each assay were expressed as the treated:control ratio (mean absorbance treated wells/mean absorbance untreated control) for
each drug concentration. Dose-effect curves represent the mean of at least 3 separate experiments. All of the above doses for the given effects were calculated by the rearrangement of the median effect plot equation

\[ D = D_o \left( \frac{f_a}{1 - f_a} \right)^{\frac{1}{m}} \]

where the fraction affected \( (f_a) \) is obtained from the formula 1-(% survival/100); and \( m \) and \( D_o \) were obtained from the median effect plot (15). The CI is determined as

\[ CI = \frac{(D_1) + (D_2)}{(D_1) + (D_2) + \alpha \cdot (D_1)(D_2)/(D_1)(D_2)} \]

If the agents have similar modes of action, to be referred to as mutually exclusive, then \( \alpha = 0 \) (i.e., CI is the sum of two terms); if the agents have different modes of action, to be referred to as nonexclusive, \( \alpha = 1 \) (i.e., CI is the sum of three terms). According to this analysis a CI of 1 indicates summation or additive effects, <1 indicates synergy, and >1 indicates antagonism. Computer programs (16) based on the median effect plot and the CI equation were used for data analysis.

High Performance Liquid Chromatography. Radiolabeled folic acid (500 cpm/pmol; 40 Ci/mmol) was added to 1 ml of folic free RPMI 1640 supplemented with 10% horse serum containing 1 µg/ml of cold folic acid. The enzyme was inactivated by heating as described above. Pteroic acid and folic acid were added as tracers. An ion-exchange chromatography system was used to separate pteroic acid from folic acid using a linear gradient. The Bakerbond column (polyethyleneimine, 5 µm; 4.6 x 50 mm) was used. Solvent A was 10 mm ammonium phosphate buffer, pH 6, and solvent B was 500 mm ammonium phosphate buffer, pH 6. A Rheodyne 7125 injection valve with a 50-µl loop and a Waters Associates 990 plus photodiode array detector Spectro Physics SP 8800 ternary gradient pump were used. The retention time is 2.5 min for pteroic acid and 6.5 min for folic acid.

In Vivo Studies. L1210 cells were maintained in BALB/c x DBA/2 F₁ mice by weekly i.p. transplantation of 10⁶ cells in 0.1 ml of Fisher's medium. On day 0 of each experiment, mice were weighed, distributed into groups of at least 10 animals/group, and given injections of 10⁶ L1210 cells in 0.2 ml of Fisher's medium. Treatment with CPG₂ was started on the next day (day 1) while TMTX administration was delayed until day 3. The weights of the mice were followed during the course of the experiment and the percentage of change in body weight was used as an indication of drug toxicity. Ascorbic acid folic acid was measured by radioimmunoassay according to the method of Longo and Herbert (17). Survival was calculated from the date of inoculum of the L1210 cells using the Kaplan-Meier actuarial method (18).

RESULTS

We tested the ability of increasing concentrations of CPG₂ to deplete the medium of folic acid (RPMI containing 10% horse serum). No detectable folate remained after a 1 h exposure to CPG₂ at 10⁻⁵ unit/ml. Fig. 1 shows the HPLC pattern using [³H]folic acid added to RPMI before and after hydrolysis with CPG₂. All the [³H]folic acid added (1 µg/ml) was converted to pteroic acid after exposure to CPG₂ (10⁻⁵ unit/ml) for 1 h.

Single Agents. A dose dependent effect on the survival of the CCRF-CEM parent and MTX resistant cell lines was observed with TMTX (Fig. 2). No major difference in the TMTX \( ED_{50} \) was observed among the CCRF-CEM, CCRF-CEM/E, and CCRF-CEM/P cell lines. As reported previously (19), the \( ED_{50} \) for CCRF-CEM/T subline was approximately one-half of the value determined for the other cell lines (Table 1).

CPG₂ was effective in inhibiting the survival of all the cell lines in a dose dependent manner in a range between 10⁻⁵ and 5 x 10⁻⁴ unit/ml (Fig. 3). As shown in Table 1, the \( ED_{50} \) of CPG₂ was approximately the same in CCRF-CEM and CCRF-CEM/E cells but was 10 and 2 times higher in CCRF-CEM/P and CCRF-CEM/T, respectively.

Combinations. In parental CCRF-CEM cells the slope of the dose-response curve of TMTX was 5 times higher than for CPG₂ indicating the different nature of cell killing by the two compounds. The plot of CI with respect to the fractional kill \( (f_a) \) shows synergism at all effect levels when a 1:100 mixture (TMTX to CPG₂) is used, and higher effect levels gave more synergism than lower effect levels. Quantitative evaluation of the CI values indicates that increasing the TMTX/CPG₂ ratio maximizes synergy (Table 2).

In CCRF-CEM/E cells the slope of the dose-response curve of TMTX is 2 times higher than the one for CPG₂. The effect of 1 nm TMTX and 100 x 10⁻⁵ unit/ml of CPG₂ (1:100 ratio) gives a CI of 1.2 which indicates slight antagonism for this cell line compared to the lower CI (0.32) for the same doses in CCRF-CEM cells. Higher doses of the two compounds (e.g., 2 nm TMTX and 200 x 10⁻⁵ unit/ml of CPG₂) are required to

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TMTX (nm)</th>
<th>CPG₂ (10⁻⁵ unit/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>1.4</td>
<td>3.4</td>
</tr>
<tr>
<td>CCRF-CEM/E</td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>CCRF-CEM/P</td>
<td>1.5</td>
<td>27.6</td>
</tr>
<tr>
<td>CCRF-CEM/T</td>
<td>0.7</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Fig. 1. Folate levels in RPMI containing 10% horse serum as measured by ion exchange high performance liquid chromatography, following treatment with 10⁻⁵ unit/ml of CPG₂ for 1 h. [³H]Folic acid was added as described in "Materials and Methods."

Fig. 2. Effect of increasing doses of TMTX on survival of CCRF-CEM parent and resistant cell lines. Cell viability was measured by the MTT assay as described in "Materials and Methods."

Table 1 Effect of TMTX and CPG₂ on cell viability

<table>
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obtain a synergistic effect on this cell line (Table 2).

In the CCRF-CEM/P cells the slope of TMTX dose-response curve is also twice the value of the slope for CPG2. Marked synergism has been observed in this cell line for all doses of the two compounds tested. For CCRF-CEM/P cells, the CPG2 and TMTX combination showed a similar degree of synergism over a wide dose range (Table 2).

In CCRF-CEM/T cells the slopes of the dose-response curves of the two compounds were similar. Synergism was observed at $f_a > 0.65$ for mutually nonexclusive drugs and $f_a > 0.6$ for mutually exclusive drugs (Table 2). The higher doses (i.e., higher $f_a$ values) gave higher degrees of synergism. The mutually exclusive analysis seems to be the more conservative and more appropriate to the known mechanisms of action of TMTX and CPG2, but since the complete mechanisms of action of either drug has not yet been verified, the two methods of evaluation will be reported.

In Vivo Studies. Several preliminary studies were done to determined: (a) the dose and schedule of CPG2 and TMTX that are minimally and maximally effective when tested individually on L1210 cells in mice; (b) the effect of CPG2 on the folate level of L1210 induced ascitic fluid; and (c) the activity of the enzyme in the ascitic fluid of treated animals bearing the L1210 leukemia. Following i.p. injection of 3000 units/kg of CPG2 daily for 4 days, the folate level in the ascitic fluid was drop to nondetectable levels in medium (RPMI 1640 supplemented with 10% horse serum) with 10⁻² unit/ml of CPG2.

It was evident only when given more frequently (every 12 h) for 4 days to mice with less of a tumor inoculum (ILS 27%). The maximum ILS for CPG2 (22%) was obtained administering 3000 units/kg every 12 h for 3 days to mice inoculated with $10^5$ cells. TMTX alone, as expected, was more effective when the tumor inoculum was decreased to $10^6$ cells as compared to $10^5$ cells. The maximum ILS for TMTX given for 4 days with an inoculum of $10^5$ cells was 30%. However, the combination of twice daily CPG2 (days 1–5) together with TMTX at either 22.5 or 30 mg/kg (days 2–5) proved to be markedly toxic, and all the animals died from drug toxicity. Consequently a third survival study was performed in which the duration of treatment with CPG2 was reduced to 3 or 4 days, and injection was twice a day. In addition TMTX was administered for either 2 or 3 days, beginning 24 h after the first dose of CPG2. Under these conditions, the combination of twice a day CPG2, days 1–3, together with TMTX at 30 mg/kg, days 2 and 3, gave a significant increase in life span of the tumor bearing mice from a median survival of 8–22 days ($P < 0.001$), or 144% ILS, with 3 of 10 animals surviving longer than 30 days.

### DISCUSSION

CPG1 and CPG2 are closely related hydrolases which cleave the peptide bond in folic acid derivatives as well as other glutamyl terminal peptides (20).

The antitumor activity of CPG1, in vitro and in vivo is a consequence of folic acid depletion as was documented by measurements of folates and reversion of this effect by supplementation with the end products of folate mediated reactions (5, 21). In our study the concentration of folic acid does in fact drop to nondetectable levels in medium (RPMI 1640 supplemented with 10% horse serum) with $10^{-2}$ unit/ml of CPG2. Kalghatgi et al. (22) showed a marked depletion of intracellular folates in W256 cells after 2 days of exposure to CPG1.

TMTX is a potent inhibitor of DHFR and blocks the synthesis of tetrahydrofolate and subsequently that of other folate.

### Table 2 Comparison between fraction affected ($f_a$) and CI values* at different concentrations of TMTX and CPG2 in sensitive CCRF-CEM cells and in the three resistant sublines

<table>
<thead>
<tr>
<th>TMTX ($10^{-9}$ M)</th>
<th>CPG2 (10⁴ unit/ml)</th>
<th>CCRF-CEM</th>
<th>CCRF-CEM/E</th>
<th>CCRF-CEM/P</th>
<th>CCRF-CEM/T</th>
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<td>0.125</td>
<td>12.5</td>
<td>0.841</td>
<td>0.225</td>
<td>0.827</td>
<td>0.887</td>
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<tr>
<td></td>
<td></td>
<td>(0.215)</td>
<td>(0.318)</td>
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<td>(0.387)</td>
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<tr>
<td>0.50</td>
<td>50.0</td>
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<td>0.980</td>
<td>0.084</td>
<td>0.856</td>
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<tr>
<td></td>
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<td>(0.337)</td>
<td>(0.083)</td>
<td>(0.867)</td>
<td>(0.512)</td>
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<tr>
<td>1.00</td>
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<td>0.980</td>
<td>0.151</td>
<td>0.954</td>
<td>0.575</td>
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<tr>
<td></td>
<td></td>
<td>(0.386)</td>
<td>(0.147)</td>
<td>(0.251)</td>
<td>(0.512)</td>
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<tr>
<td>2.00</td>
<td>200.0</td>
<td>0.980</td>
<td>0.151</td>
<td>0.954</td>
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<tr>
<td>4.00</td>
<td>400.0</td>
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<td>(0.512)</td>
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* CI < 1, = 1, > 1 indicate synergy, additivity, and antagonism, respectively. CI values given are based on mutually nonexclusive assumption (i.e., totally independent modes of action of two drugs). Numbers in parentheses are obtained from mutually exclusive assumption.
CARBOXYPEPTIDASE G₂ AND TRIMETREXATE IN VITRO AND IN VIVO

To understand the basis of this synergistic combination, CPG₂ is active in reducing the concentration of folates in ascitic fluids of mice bearing L1210 to 50% of control values with 24 h. The activity of the enzyme is reduced to 10% 24 h after the i.p. injection. Thus it is necessary to repeat the injection of CPG₂ every 12 h in order to maintain low levels of folates. This could explain why no effect on the ILS was observed when CPG₂ was administered once a day. Both CPG₂ (given twice daily at 3000 units/kg) and TMTX at 22.5 and 30 mg/kg/day over 4 days base produced modest antitumor activity. When both drugs where given in full dosage, toxicity precluded evaluation of antitumor effects. Only fewer doses of these drugs produced marked antitumor effects. Since mice are difficult to render folate deficient by dietary means, and have high serum folate levels as compared to humans (4), the need for large doses of CPG₂ given at 12-h intervals may not be necessary in clinical trials. The availability of large amounts of this enzyme, now made by recombinant technology (6), should facilitate future clinical trials of this enzyme, alone or in combination with TMTX. In view of the potential toxicity of the CPG₂-TMTX combination when these drugs are used concomitantly over 4–5 days, clinical studies should be approached with caution and begin with 1–2 days of combination treatment, relating toxicity and therapeutic effects to plasma and cellular levels of folates, as well as blood levels of these drugs. Since the combination CPG₂-TMTX was less synergistic against CCRF-CEM/P cells, bone marrow cells, which lack polyglutamylation, would probably not be a target for the toxicity of these drugs, therefore providing this combination a wider therapeutically index.

REFERENCES


6022
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