Sequence and Schedule-dependent Synergy of Trimetrexate in Combination with 5-Fluorouracil in Vitro and in Mice

William L. Elliott, Curtis T. Howard, Donald J. Dykes, and Wilbur R. Leopold

ABSTRACT

The purpose of this study was to determine the conditions for optimum synergistic efficacy of the two-drug combination of trimetrexate and 5-fluorouracil. Synergistic cell killing of Chinese hamster ovary cells in these clonogenic survival assays was observed only when the cells had been exposed to trimetrexate (25 µM) for 2 to 4 h prior to 5-fluorouracil exposure (either 125 or 250 µM). The schedule dependence of the observed synergy in vitro was closely linked to trimetrexate-induced changes in cellular 5-phosphoribosyl 1-pyrophosphate (PRPP) pools. Exposure to 25 µM trimetrexate induced increases in PRPP pools to 398% and 761% of control values at 2 and 4 h, respectively. Methotrexate (20 µM) also increased Chinese hamster ovary cell PRPP content in a time-dependent fashion to values of 280 and 511% of control after 2 and 4 h of drug exposure. Previous in vivo studies demonstrated a modest degree of therapeutic synergy between trimetrexate and 5-fluorouracil against P388 leukemia. Our in vitro results suggested that the degree of synergy seen in vivo could be increased with appropriate schedule changes. Mice were implanted i.p. with 10⁶ P388 leukemia cells on Day 0 and were treated with trimetrexate (every 3 h for eight injections; Days 1, 5, and 9) and 5-fluorouracil (Days 1, 5, and 9) as single agents or in combination on one of two schedules; 5-fluorouracil was administered with either the first or the last of the eight trimetrexate doses on Days 1, 5, and 9. Both treatment regimens demonstrated therapeutic synergy but, as predicted from the in vitro data, the “5-fluorouracil last” was superior to the “5-fluorouracil first” sequence. Treatment with the optimal doses on the “5-fluorouracil last” sequence (trimetrexate, 31; 5-fluorouracil, 33 mg/kg/injection) produced an increased life span of 183% and a net reduction in tumor cell burden of 6.7 logs compared with a 111% increased life span (net reduction in tumor burden of 2.6 logs) produced by the most active of the single agents, 5-fluorouracil. Thus the efficacy of the combination of trimetrexate with 5-fluorouracil was sequence and time dependent both in vitro and in vivo. The synergy, observed in vitro and probably in vivo, was linked to a trimetrexate-induced elevation of intracellular PRPP, thus facilitating the production of 5-fluoropyrimidine nucleotides. These data are similar to the sequence and schedule dependency of the methotrexate/5-fluorouracil combination with important differences. In particular, the rapid loss of synergy observed upon removal of trimetrexate from the culture medium and the associated decrease in intracellular PRPP levels are not observed with methotrexate treatment and indicate that the combination of trimetrexate and 5-fluorouracil cannot be equated with the clinical combination of methotrexate and 5-fluorouracil.

INTRODUCTION

Trimetrexate is a nonclassical, lipophilic, folate antagonist which has been shown to have a spectrum of activity superior to methotrexate in preclinical models (1–4). The cellular target of trimetrexate is dihydrofolate reductase to which it tightly binds (3, 4). Trimetrexate is thought to enter cells via passive diffusion (5, 6), although a role for facilitated transport has also been proposed (7, 8). Clearly, trimetrexate enters cells by a mechanism different from methotrexate, as evidenced by its activity against cell lines made resistant to methotrexate by virtue of a methotrexate transport defect (3, 9, 10). The turnover of trimetrexate in cells is rapid, and the half-life in dogs is approximately 3.5 h (11). Hence optimal trimetrexate schedules use highly divided dose regimens that can better maintain cytotoxic plasma levels for sustained periods of time (12). Trimetrexate is currently in Phase II clinical trial.

The success or failure of a new anticancer agent in the clinic depends not only on its utility as a single agent but also on its ability to interact positively with other anticancer drugs in combination chemotherapy protocols. In earlier in vivo studies we demonstrated strong therapeutic synergy between trimetrexate and Cytoxan, doxorubicin, or 6-thioguanine and weaker but still significant synergy for combinations of trimetrexate with 5-fluorouracil, vincristine, and cis-platinum (13). Since those protocols were not optimized with respect to treatment schedule and since the efficacies of antimetabolite combinations are often highly schedule dependent, we have evaluated in more detail the two-drug combination of trimetrexate and 5-FU in vitro and in vivo.

The synergistic combination of methotrexate and 5-FU has been the subject of detailed experimental investigation (14–19), but key differences between methotrexate and trimetrexate with respect to both metabolism and pharmacokinetics suggested that the parameters for optimal synergy would not necessarily be identical for the trimetrexate/5-FU and methotrexate/5-FU combinations. Based upon in vitro data obtained with methotrexate and 5-FU, the degree of synergy observed is a function of the relative timing of 5-fluorouracil and methotrexate administration (14, 16, 17, 20). Preincubation with methotrexate was required to produce synergy. This allowed a methotrexate-induced increase in the intracellular concentration of PRPP, which then facilitates the production of fluorinated pyrimidines (16, 20–23). In this study we used in vitro modelling of the sequence and schedule dependence of the synergy between trimetrexate and 5-FU to predict an optimal in vivo protocol. In addition, intracellular PRPP levels following in vitro exposure to trimetrexate were measured in order to ascertain their role in the observed synergy.

MATERIALS AND METHODS

Materials. Trimetrexate glucuronate was obtained from Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co., Ann Arbor, MI. Methotrexate, 5-FU, and PRPP were purchased from Sigma Chemical Co., St. Louis, MO. PEI-impregnated cellulose thin-layer chromatography plates were obtained from Brinkmann Instruments, Westbury, NY, and J. T. Baker Chemical Co., Phillipsburg, NJ. [8-³¹C]-Hypoxanthine (54.4 mCi/mmol) was purchased from Du Pont-New England Nuclear, Boston, MA. Cell culture medium, serum, trypsin, and balanced salt solutions were obtained from Gibco Laboratories, Grand Island, NY. All other chemicals were of the highest purity available.

1 The abbreviations used are: 5-FU, 5-fluorouracil; PEI, polyethylenimine; PRPP, 5-phosphoribosyl 1-pyrophosphate; ILS, increased life span; CHO, Chinese hamster ovary; FUMP, 5-fluorouridine monophosphate.
Cell Culture. CHO-K1 cells were obtained from the American Type Culture Collection, Rockville, MD. Cells were grown in e-medium essential medium without nucleosides supplemented with 10% dialyzed fetal bovine serum and gentamicin sulfate (equivalent to 50 µg/ml). For cell survival assays, CHO cells were grown in 25-cm² tissue culture flasks seeded at a density of 10⁶ cells/flask in 5 ml of complete medium. Flasks were incubated 48 h at 37°C (5% CO₂ in air) prior to addition of 50 µl of drug solution. Flasks, in duplicate, were exposed to either 125 or 250 µM 5-FU for 2 h at various times (−2, 0, 2, 4, 6, and 8 h) relative to the beginning of a 6-h 25 µM trimetrexate exposure. Drug concentrations were selected based on the concentration of drug alone yielding an expected survival of 40 to 70%. Following drug treatment, cell monolayers were rinsed with Hanks’ balanced salt solution containing 0.5 mM EDTA. Cells were trypsinized, counted (Model ZM Coulter Counter), seeded in 5 ml of medium into triplicate 60-mm culture dishes, and incubated at 37°C for 7 days, at which time colonies were stained with crystal violet and counted (12).

For measurement of intracellular PRPP levels, 2 × 10⁶ CHO cells were seeded into 150-cm² flasks with 50 ml of medium and incubated for 48 h prior to drug treatment. Duplicate flasks were treated with either trimetrexate (25 µM) or methotrexate (20 µM) for 0, 2, 4, and 6 h. Following drug exposure, cells were harvested as described below. In some experiments additional flasks were incubated with either trimetrexate or methotrexate for 6 h. The drug-containing medium was removed, replaced with 50 ml of fresh drug-free medium (50% conditioned), and incubated at 37°C for 2 additional h (6 plus 2) to examine the effect of drug removal on PRPP levels.

PRPP Determination. Assay of PRPP concentration was based on the conversion of the PRPP in cell extracts to [8-³¹⁴C]IMP using [8-¹⁴C]hypoxanthine as substrate with the reaction being catalyzed by yeast hypoxanthine-guanine phosphoribosyl transferase (EC 2.4.2.8; Sigma Chemical Co., St. Louis, MO). Following the detachment of cell monolayers, trypsin was quenched with complete drug-containing medium such that the final drug concentration equalled that present during the incubation. Control flasks were quenched with drug-free medium. Cell suspensions in duplicate flasks were pooled and counted, and a volume equivalent to 1 × 10⁶ cells was centrifuged at 4°C at 700 × g for 5 min. At each time point 2 or 3 sets of duplicate flasks were harvested for each drug. Viability of cells was determined by trypan blue dye exclusion. After centrifugation, supernatants were removed, and the inside of each tube was wiped dry. Then 0.5 ml of ice-cold extraction medium (0.25 M sucrose-0.2 mM EDTA-50 mM potassium phosphate buffer-10 mM NaF-4.5 mM 2,3-diphosphoglycerate, final pH 7.4) were added per tube. Cell pellets were resuspended by mild vortexing and immediately frozen in a dry ice-alcohol bath. Tubes were kept in dry ice until extract preparation. Tubes, 3 at a time, were thawed at 37°C for 1 min, plunged into boiling water for 1 min, and then chilled on ice for 2 min. The lyed cell preparations were transferred to microcentrifuge tubes and centrifuged 5 min at 5°C in an Eppendorf microcentrifuge. Extracts (200 µl) were assayed immediately for PRPP content. The final reaction volume of 300 µl contained 200 µl of extract, 120 mM triethanolamine-HCl, 3 mM MgCl₂, 2 nmol of [8-¹⁴C]hypoxanthine, and 2 units of enzyme at a final pH of 7.4. Tubes were incubated at 37°C for 10 min. Reactions were terminated by spotting 25 µl of reaction mixture onto a 2 × 2 cm² NaCl-activated PEI cellulose thin-layer chromatography sheet. Squares were washed 3 times in 1 mM sodium formate (pH 6–7) for 5 min and finally washed with 95% ethanol. Squares were cut out and placed into scintillation vials, 10 ml of scintillation fluid (Ready-Gel; Beckman Instruments) were added, and radioactivity was determined in a Beckman LS-9000 liquid scintillation counter. A PRPP standard curve was run in each experiment. PRPP recovery was based upon the standard addition of PRPP to cell pellets just prior to the addition of extraction medium. Statistical significance was determined with Student’s t test.

Chemotherapy Studies. P388 leukemia was passaged weekly as an i.p. implant of 10⁶ cells in DBA/2 mice. Anticancer activity was evaluated in BALB/c × DBA/2 F₁ (hereafter called CDF₂/F₁) hybrid mice. For all tests the mice (18 to 22 g) were randomized and then inoculated i.p. with 1 × 10⁶ tumor cells on Day 0. The mice were then rerandomized for distribution into treatment cages. Each group of 10 mice was treated on the basis of average group weight. Trimetrexate was given i.p. every 3 h for 8 injections on Days 1, 5, and 9 while 5-FU was given i.p. once daily on Days 1, 5, and 9 simultaneously with either the first or last trimetrexate injection of the day. The vehicles for trimetrexate and 5-FU were distilled water and saline, respectively. Average group weights were determined on each day of treatment. Weight change was calculated as the difference in mean group weight on the first and last days of treatment. Calculation of the median day of death, percentage of host ILS, and logarithm of net tumor cell kill (change in tumor burden at last treatment) used standard methods (13, 24). Briefly, the difference in the median day of death between the treated (T) and the control groups (C) was used to calculate net logs of tumor cell kill as follows:

\[
\text{Net } \log_{10} \text{ cell kill} = \frac{(T - C) - (\text{duration of treatment in days})}{3.32 \times T_d}
\]

where \(T_d\) is the mean tumor doubling time (days) calculated from a log-linear least-squares fit of the titration groups and their respective median days of death. A positive net \(\log_{10}\) cell kill indicates that the tumor burden at the end of therapy was smaller than that at the beginning of treatment. A negative net \(\log_{10}\) cell kill indicates that the tumor burden increased during therapy. Tumor-free survivors (cures) at the experiment end were excluded from these calculations.

Therapeutic synergism for these studies is defined as follows. Two drugs are therapeutically synergistic if a combination regimen produces a significantly better tumor cell kill than either of the single agents alone at optimal or maximum tolerated doses (13). Since necropsy data were not obtained, deaths were assumed to be due to drug toxicity if, in an otherwise active treatment regimen, an animal died at a significantly earlier date than its cage mates that received similar treatment.

RESULTS

Effect of Trimetrexate and 5-Fluorouracil on CHO Cells in Vitro. Logarithmically growing CHO cells were exposed to trimetrexate and 5-FU alone and in combination. The total exposure of cells to trimetrexate and 5-FU was always 6 and 2 h, respectively. As a single agent at 25 µM, trimetrexate gave a 65% survival while 5-FU at concentrations of 250 and 125 µM gave survivals of 48 and 72% of control, respectively. For additive cytotoxicity of the two-drug combination, survivals of 31 and 47% would be expected (Fig. 1). When a 2-h exposure to 5-FU either immediately preceded (Fig. 1, −2 h) or followed a 6-h trimetrexate exposure (Fig. 1, 6 h) at best only additive cytotoxicity was observed. The simultaneous addition of tri-
TRIMETREXATE SYNERGY WITH 5-FLUOROURACIL

metrexate and 5-FU to cell cultures (Fig. 1, 0 h) provided some degree of synergy at both 5-FU concentrations. However, when a 2-h 5-FU exposure was initiated at 2 or 4 h after the beginning of a 6-h trimetrexate exposure, the degree of cytotoxicity was significantly greater than additive at both 5-FU concentrations (Fig. 1, 2 h, 4 h), indicating a synergistic interaction. Thus, the synergy observed between 5-FU and trimetrexate was both sequence and schedule dependent. Optimal synergy was obtained when initial exposure to trimetrexate preceded exposure to 5-FU by 2 to 4 h. Synergy was not observed when a 6-h exposure to trimetrexate followed a 2-h 5-FU exposure (Fig. 1, −2 h). Synergy was rapidly lost when trimetrexate was removed from the culture medium prior to 5-FU exposure (Fig. 1, 6 h).

PRPP Content after Trimetrexate Treatment. Under the standard conditions described in “Materials and Methods,” the assay for the determination of PRPP was linear in the range of 0.1 to 1.7 nmol of pure PRPP (data not shown). Also, under the standard reaction conditions, 1.2 nmol of PRPP was quantitatively converted to [8-14C]IMP in the presence of 2 nmol of [8-14C]hypoxanthine within 20 min at 37°C (data not shown). Blank values were always less than 2% of the total recovered counts per minute. Cell viability was always greater than 95% just prior to extraction.

Intracellular PRPP levels following treatment of CHO cells for various times with either trimetrexate or methotrexate are shown in Tables 1 and 2. The recovery of standard PRPP (0.5 nmol) added to cell pellets just prior to the addition of extraction medium was 82 to 95%. PRPP levels in control cultures were approximately 50 nmol/10⁹ cells (range of all experiments, 39 to 76 nmol/10⁹ cells). PRPP levels in cultures treated with trimetrexate increased in a time-dependent fashion to 818% of the control value after 6 h. PRPP levels dropped almost 2-fold within 2 h after removal of trimetrexate from the medium. As expected, PRPP levels also increased in methotrexate-treated cultures in a time-dependent fashion (Table 2). However, unlike trimetrexate, the removal of methotrexate from cultures resulted in only a slight decrease in the intracellular PRPP concentration within 2 h.

In Vivo Chemotherapy. Based upon the sequence dependence observed in vitro for synergy between trimetrexate and 5-FU, an experiment was designed to determine if the synergy was also sequence dependent in vivo. Two treatment sequences were evaluated (Table 3). 5-FU was given either with the first or last of 8 trimetrexate injections on each treatment day. For either regimen, combination therapy was synergistic at several dose levels, as it was clearly superior to the best response obtained with either of the single agents alone (Table 3; Fig. 2). When 5-FU was given with the first trimetrexate injection, the optimal combination regimen (31 mg/kg/injection of trimetrexate and 54 mg/kg/injection of 5-FU) resulted in an ILS of 150% and 5.4 net log reduction in tumor cell burden (Table 3). When 5-FU was given with the last trimetrexate dose the best combination regimen (31 mg/kg/injection of trimetrexate and 33 mg/kg/injection of 5-FU) produced a 183% ILS (a 6.7-log reduction in tumor cell burden). In addition, the “5-FU last” regimen produced greater synergy over a wider range of doses than the “5-FU first” protocol, further strengthening the conclusion that the “5-FU last” regimen was superior. Based on the number of leukemia cells present at first treatment, a 6.7-log reduction in tumor cell burden was the maximum attainable, approaching cure. Thus, as predicted from the in vitro studies, the regimen that allowed the highest degree of trimetrexate exposure prior to treatment with fluorouracil was superior.

DISCUSSION

These results confirmed the previously reported (13) in vivo synergy between trimetrexate and 5-FU and demonstrated a similar effect in vitro (Fig. 1; Table 3). The synergy produced by this two-drug combination was sequence dependent both in vitro and in vivo. Based on the work of others with the combination of methotrexate and 5-FU (14, 16, 17, 21), we expected that the synergy observed between trimetrexate and 5-FU would be both sequence and schedule dependent. Thus it was not surprising that no synergy was observed in vitro when exposure to 5-FU preceded trimetrexate exposure. We were surprised, however, to find that all synergy was lost if the 2-h 5-FU exposure was delayed until after a 6-h exposure to trimetrexate (Fig. 1, 6 h). The lack of a glutamate residue and, therefore, polyglutamylation of trimetrexate (2, 7) as a mechanism for retention within the cell may be the source of this key difference between methotrexate (18) and trimetrexate.

The time dependence of synergy in vitro on preincubation with trimetrexate before 5-FU exposure closely paralleled the time course of trimetrexate-induced elevations in PRPP pools of the treated cells. This suggested that the trimetrexate-induced elevations in PRPP levels could account for at least a fraction of the observed synergy by facilitating the conversion of 5-FU to FUMP by orotate phosphoribosyltransferase. This concept was further supported by the observation that removal of trimetrexate from the medium prior to the 2-h 5-FU exposure resulted in a complete loss of synergy that corresponded to a 1.7-fold decrease in PRPP levels in the target cells.

These observations on the somewhat transient effect of trimetrexate exposure on PRPP pools and the associated implication that trimetrexate is not retained for long periods within the tumor cell correlate with the previously observed schedule dependence of the activity of trimetrexate against P388 leuke-

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Table 1 PRPP content of CHO cells after exposure to trimetrexate

<table>
<thead>
<tr>
<th>Treatment time (h)</th>
<th>PRPP content</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49 ± 4</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>195 ± 20</td>
<td>398*</td>
</tr>
<tr>
<td>4</td>
<td>373 ± 17</td>
<td>761*</td>
</tr>
<tr>
<td>6</td>
<td>401 ± 21</td>
<td>818</td>
</tr>
<tr>
<td>6 + 2'</td>
<td>237 ± 10</td>
<td>448*</td>
</tr>
</tbody>
</table>

* Cultures were treated for the indicated time with 25 μM trimetrexate. Cells were harvested, and pellets (triplicates) containing known numbers of cells were extracted and PRPP quantitated as described in “Materials and Methods.”

Table 2 PRPP content of CHO cells after exposure to methotrexate treatment

<table>
<thead>
<tr>
<th>Treatment time (h)</th>
<th>PRPP content</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56 ± 7</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>157 ± 2</td>
<td>280*</td>
</tr>
<tr>
<td>4</td>
<td>286 ± 9</td>
<td>511*</td>
</tr>
<tr>
<td>6</td>
<td>309 ± 4</td>
<td>552*</td>
</tr>
<tr>
<td>6 + 2'</td>
<td>291 ± 3</td>
<td>519*</td>
</tr>
</tbody>
</table>

* Cultures were treated for the indicated time with 20 μM methotrexate. Cells were harvested, and pellets containing known numbers of cells (triplicates) were extracted and PRPP quantitated as described in “Materials and Methods.”

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that maximized tumor cell exposure to trimetrexate prior to injection of 5-FU was superior. Thus the optimum sequence of trimetrexate and 5-FU in vivo was similar to schedules used with methotrexate and 5-FU in other preclinical models except for two key differences: (a) trimetrexate is administered more frequently than methotrexate; and (b) the therapeutic window after which 5-FU follows trimetrexate to obtain optimal synergy is narrower than that for the methotrexate/5-FU combination due to differences in the transport and retention of these two folate antagonists within cells (3, 5, 6, 9, 10, 18).

Trimetrexate is now in Phase II clinical trials. The results presented here suggest that, pending the results of the Phase II trials, trials of trimetrexate and 5-FU in combination may be warranted. Should such trials be undertaken, careful consideration of pharmacokinetics and the sequence and timing of drug administration is imperative.

**REFERENCES**


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