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Cell cycle effects of trimetrexate (CI-898)

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Summary. The cell cycle phase specificity of trimetrexate (CI-898) was examined. CHO cells synchronized by mitotic selection were exposed to 50 μ*M* trimetrexate for 2 h at various time points after release from Colcemid block. Only S phase cells were sensitive to trimetrexate when survival was measured by a cloning assay. Comparison of plateau phase and log phase cultures indicated that plateau phase CHO cells were relatively insensitive to 5 μ*M* trimetrexate. Exponentially growing L1210 cells were continuously exposed to either 30 n*M* or 3 n*M* trimetrexate and analyzed by DNA flow cytometry. Incubation with 30 n*M* trimetrexate produced cell cycle arrest in late G_1 or early S phase, while exposure to 3 n*M* trimetrexate caused only a delay in progression through S phase. In an in vivo schedule dependence study with mice bearing approximately 3×10^6 P388 leukemia cells, trimetrexate was most effective with frequent administration. Mice treated on the optimal schedule, every 3 h ×8 on days 1, 5, and 9 after tumor implant, had life-span increases in excess of 100%.

Introduction

Trimetrexate is a lipophilic folate antagonist that has shown considerable activity in experimental tumor models [2, 3, 14]. The biochemical effects of trimetrexate are similar to those of methotrexate in many respects (for reviews see [12–14]): both drugs are potent inhibitors of dihydrofolate reductase [3] and bind tightly to the enzyme [15]. The cytotoxicity of each can be reversed by leucovorin or thymidine plus a purine [12–14]. They each exert both antithymidylate and antipurine effects (the latter is cell line- and concentration-dependent), and it appears that the antithymidylate effect is the primary cytotoxic mechanism for both drugs [12, 13, 14]. Despite these similarities between the two agents, trimetrexate's lipophilic nature allows for higher intracellular concentrations [15], and it demonstrates uncompromised in vitro cytotoxicity to cells possessing transport-mediated methotrexate resistance [6, 14, 15, 18]. Because of its potent antitumor activity in experimental tumor models, this promising 'nonclassical' antifolate has entered clinical trials.

Known folate antagonists are primarily S phase-specific agents [12]. Differences between trimetrexate and methotrexate with respect to spectrum of activity, membrane transport, cross-resistance profiles, and pharmacology leave open the possibility that trimetrexate might possess one or more mechanisms of cytotoxicity differing from that of methotrexate. To further define the mechanism of anticancer activity of trimetrexate we have explored the cell cycle effects of trimetrexate as demonstrated by its cell cycle phase-specific toxicity, inhibition of cell cycle progression, and the dependence of its in vivo activity on treatment schedule.

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Materials

Trimetrexate glucuronate monohydrate was synthesized at the Warner-Lambert/Parke-Davis Pharmaceutical Research Division Laboratories in Ann Arbor, Mich. Propidium iodide, methotrexate, RNase, sodium citrate, and gentamicin were purchased from Sigma Chemical Co., St. Louis, Mo. Colcemid was obtained from Ciba Pharmaceutical Co., Summit, NJ. Cell culture medium, serum, trypsin, and balanced salt solutions were obtained from Gibco Laboratories, Grand Island, NY. Lysing reagent was purchased from American Scientific Products, McGaw Park, Ill. Tetrasodium EDTA and HEPES buffer were purchased from Calbiochem-Behring, La Jolla, Calif. ^{3}H-Thymidine was obtained from Amersham Corporation, Arlington Heights, Ill.

Cell culture. CHO-K1 cells were obtained from American Type Culture Collection, Rockville, Md. They were grown in MEM Alpha medium without nucleosides (α-MEM) [19], supplemented with 10% dialyzed fetal bovine serum and gentamicin sulfate (equivalent to 50 μg/ml gentamicin). For growth inhibition studies, cells were seeded into 24-well Linbro plates at a concentration of 5×10^3 cells/well in 1 ml α-MEM. After 30 h incubation at 37 °C in 5% CO_2 in air, 10 μl aqueous solution of trimetrexate or methotrexate was added to duplicate wells. Control wells were left untreated. The plates were then incubated in the presence of drug for an additional 66 h. Cells were prepared for counting by a modification of the technique described by van der Bosch et al. [22]. The medium was removed and the cells were allowed to swell in 0.4 ml of an aqueous solution containing 2 m*M* EDTA and 1 m*M* HEPES, pH 7.5. After 2–3 min nuclei were released by addition of an equal volume of an aqueous solution containing 20% lysing reagent and 4 m*M* EDTA. The nuclei were fixed with 0.7 ml 10% formalin – 10% methanol in H_20 and were counted on a Coulter Counter Model ZBI. The IC_{50}, the

drug concentration at which growth over the entire growth period (96 h) is reduced to 50% of the control growth, was calculated for both trimetrexate and methotrexate.

For cell survival assays, CHO cells were grown in 25-cm^2 tissue culture flasks seeded at 10^5 cells/flask in 5 ml. Each was incubated at 37 °C for 48 h prior to addition of 50 µl of one of various dilutions of trimetrexate. Duplicate flasks were exposed to trimetrexate for 3, 8, 12, 18, or 26 h. After drug treatment the cells were rinsed with Hank's balanced salt solution with 0.5 m*M* EDTA and trypsinized. The cells were counted and triplicate 60-mm petri dishes were seeded with 300 cells/dish in 5 ml α-MEM. The dishes were incubated at 37 °C in 5% CO_2 in air for 7 days. Colonies were fixed in ethanol and stained with a saturated solution of crystal violet dissolved in phosphate-buffered saline. Colonies larger than approximately 75 cells were counted.

CHO cells growing in 25-cm^2 tissue flasks in α-MEM were allowed to reach plateau phase before treatment with 5 µ*M* trimetrexate. After exposure to trimetrexate for 2, 4, 8, or 24 h, duplicate flasks were trypsinized and assayed for cell survival as described above. Similarly treated log phase cultures were included in this experiment.

CHO cells were synchronized by mechanical selection of cells arrested in mitosis with Colcemid by a method similar to that described by Bhuyan [4]. Cells were inoculated into 150-cm^2 tissue culture flasks at 4×10^6 cells/flask and grown for 2 days prior to mitotic arrest. The medium was removed and replaced with 25 ml warm, conditioned α-MEM containing 0.033 µg/ml Colcemid. The flasks were returned to the incubator for 1.5 h. Mitotic cells were removed by shaking the flasks horizontally ten times, followed by two taps on the side and five more horizontal shakes. This "shake-off" contained considerable G_1 and S phase contamination and was discarded. Cells in the decanted medium from the second 1.5-h arrest and shake-off were centrifuged, resuspended in ice-cold α-MEM, and used for subsequent drug treatment. The cell yield was typically 10^6 cells/flask (~2.5%), of which approximately 95% were mitotic. The synchronized cells, released from Colcemid block by resuspension in medium without Colcemid, were seeded into 25-cm^2 tissue culture flasks at 5×10^5 cells/flask in 5 ml. Cells in duplicate flasks were exposed to 50 µ*M* trimetrexate for 2-h periods beginning immediately after release from Colcemid block and starting every 2 h thereafter through 20 h after Colcemid release. At the end of a 2-h trimetrexate exposure, cells were trypsinized and assayed for survival as described above.

Using a procedure similar to that of Fry et al. [7], the timing of DNA synthesis was determined in synchronized CHO cells by pulse labelling with tritiated thymidine (^{3}H-TdR). Cells in triplicate 25-cm^2 flasks were exposed to 1 µCi/ml ^{3}H-TdR for 20 min at various times after release from Colcemid block. The radioactivity of TCA-precipitable material was determined in a Beckman LS 6000 liquid scintillation counter.

For cell cycle arrest experiments, L1210 leukemia cells in exponential growth were suspended in RPMI 1640 medium supplemented with 5% fetal bovine serum and gentamicin sulfate (equivalent to 50 µg/ml gentamicin). Spinner cultures containing 200 ml cell suspensions were treated continuously with trimetrexate at 30 n*M*, 3 n*M*, and 0.3 n*M*. One flask was left untreated. At 1-h intervals, aliquots were removed for flow cytometric analysis.

Flow cytometry. CHO cells were stained with propidium iodide (PI) by the method of Tate [20]. L1210 cells were stained with PI by the method of Taylor [21]. Chicken erythrocytes were included as internal standards, and all samples were analyzed on a Becton Dickinson FACS Analyzer.

DNA histograms of synchronized CHO cells were analyzed by a method, similar to that of Barfod [1], that examines narrow bands of channels in G_1, S, and G_2M phases. When the windows are set to the left of the G_1 peak, in mid-S, and to the right of the G_2M peak, respectively, it is assumed that the contribution to these counts from adjacent cell cycle phases is minimal. The 'waves' of synchronized cells passing through these windows provided an approximation of the midpoints of the cell cycle phases. This, along with previous population doubling time experiments provided an estimate of the duration of the cell cycle phases.

Chemotherapy. An in vivo schedule dependence experiment was carried out with trimetrexate, by methods similar to those previously described [8, 16]. Test mice were inoculated IP with approximately 10^6 P388 cells. Treatment schedules were as follows: day 1 only; days 1, 5, and 9; days 1 through 9; every 3 h × 8 on days 1, 5, and 9. All mice were weighed on days 0, 6, 10, and 12. Control mice were treated on days 1–9 with 0.5 ml of the vehicle, water. All treatments were given IP.

Results

Trimetrexate and methotrexate demonstrated comparable potency ($IC_{50} = 10$ n*M* and 9 n*M*, respectively) in the inhibition of CHO cell growth (Table 1). These results were highly dependent on the medium used (data not shown), probably because of varying amounts of thymidine, purines, and reduced folates present in some tissue culture media and nondialyzed serum. Ham's F-12 medium, for example, contains thymidine and hypoxanthine, which can protect cells from the toxicity of antifolates [12–14]. In preliminary experiments to determine an appropriate growth medium, CHO cells grown in Ham's F-12 medium were relatively insensitive to trimetrexate, compared with cells grown in α-MEM (data not shown).

CHO cell killing by trimetrexate was highly dependent on the length of exposure to the drug (Fig. 1). Lengthening the exposure time approximately 9-fold (from 3 to 26 h) produced nearly a 1000-fold difference in the LC_{50} (50% le-

Table 1. Growth inhibition of CHO cells by continuous (66 h) exposure to timetrexate and methotrexate

Drug concentration (nM)	Percentage of control growth	
	Trimetrexate-treated	Methotrexate-treated
64	4	6
32	12	9
16	29	22
8	63	54
4	95	92
2	100	104
	IC_{50} = 10 nM	IC_{50} = 9 nM

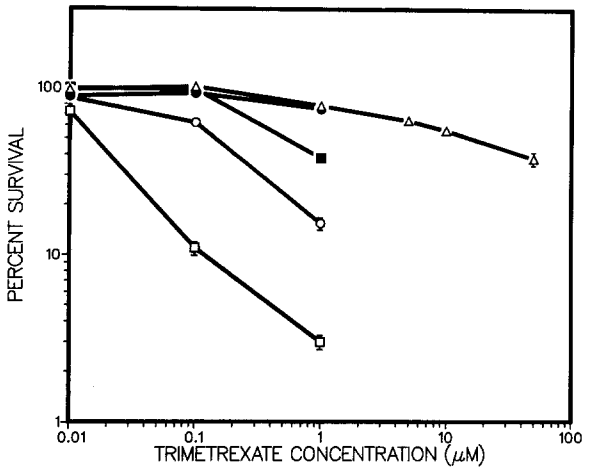

Fig. 1. Survival of asynchronous CHO cells following exposure to trimetrexate. △, 3-h drug exposure; ●, 8-h exposure; ■, 12-h exposure; ○, 18-h exposure; □, 26-h exposure. *Error bars*, ±1 SE. Colony counts of untreated control cells were normalized to 100%

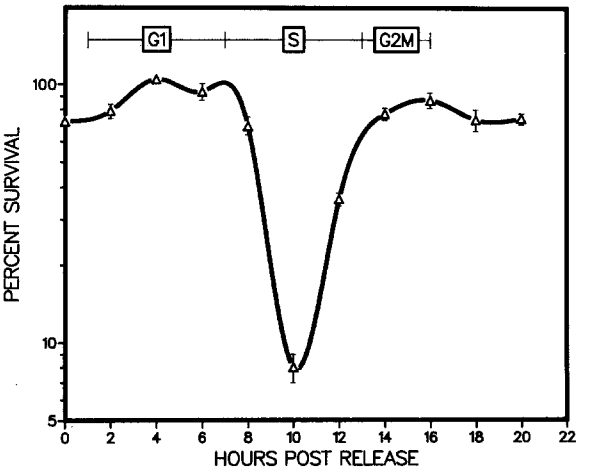

Fig. 2. Survival of synchronized CHO cells exposed to 50 μ*M* trimetrexate for 2 h. *Error bars*, ±1 SE. Colony counts of untreated control cells were normalized to 100%

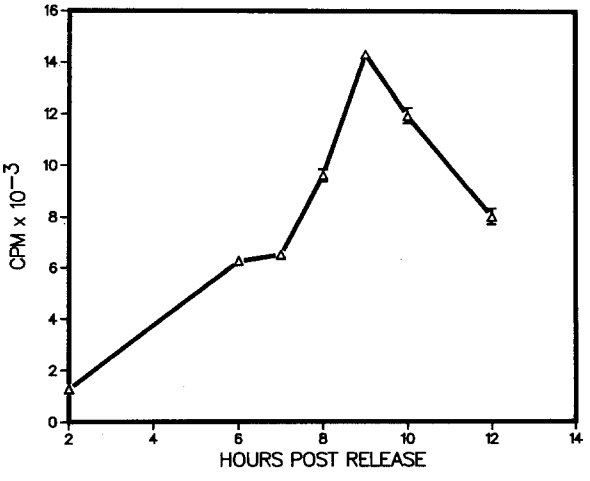

Fig. 3. Uptake of ^{3}H-thymidine by CHO cells at various times after release from Colcemid block. Twenty minute labelling period began at the points indicated. *Error bars*, ±1 SE

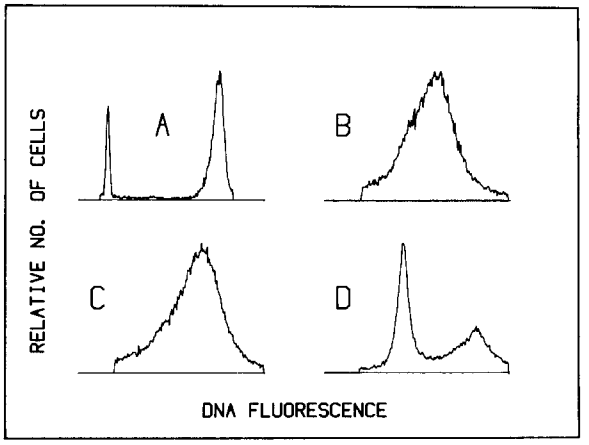

Fig. 4 A–D. DNA histograms of untreated CHO cells at various times after release from Colcemid block. **A** 0 h after release; **B** 8 h; **C** 10 h; **D** 16 h. The histograms are shifted so that the chicken erythrocyte peak **(A)** occupies the same channel in all histograms. CHO cells are isolated in **B** through **D** and the same channel range is displayed in all histograms

thal concentration). For a 3-h drug exposure, increasing the trimetrexate concentration 1000-fold (from 0.01 to 10 μ*M*) produced a decrease of only about 50% in survival. The plating efficiency of CHO cells in this experiment was 50%–80%.

Figure 2 clearly shows the S phase specificity of trimetrexate. CHO cell survival at 10 h after release from Colcemid block is markedly lower than any other time point tested. This time point corresponds to the peak (~9 h) of DNA synthetic activity as measured by ^{3}H-TdR incorporation (Fig. 3) and to the point at which the S phase fraction in Fig. 4 is largest (8–10 h). The plating efficiency of synchronized CHO cells was 15%–20%.

Table 2 shows the relative insensitivity of plateau phase CHO cells to trimetrexate. The survival of plateau phase cells exposed to 5 μ*M* trimetrexate for 24 h was about 10-fold higher than that of similarly treated log phase cells.

Figure 5 depicts the cell cycle arrest of asynchronous L1210 cells continuously exposed to either 30 n*M* or 3 n*M* trimetrexate. A 3-h exposure to 30 n*M* trimetrexate produced a marked depletion of G_2M phase cells. At 10 h the broadening of the G_1 peak probably represents cells arrested in late G_1 or early S phase. Trimetrexate exposure at 3 n*M* caused a delay in progression through S phase, as evidenced by the histograms from 6, 10, and 12 h after addition of trimetrexate. Depletion of G_2M phase cells in the 6- and 10-h histograms shows that S phase cells were prevented from entering G_2M phase. A G_2M peak reappeared at 12 h as the first of the delayed cells completed S phase. Exposure to 0.3 n*M* trimetrexate had no apparent effect (data not shown).

In the in vivo schedule dependence study, trimetrexate was most effective with frequent administration (Table 3). Maximum percent T/C and net log cell kill [8, 16] values were obtained with a treatment schedule of every 3 h × 8 on days 1, 5, and 9. Doses of 45 and 30 mg/kg/injection on that schedule produced percent T/C values of 212 and 203, and net log cell kill values of 2.1 and 1.6, respectively. None of the treatment regimens tested produced cures.

Table 2. Percentage survival of CHO cells exposed to 5 μM trimetrexate

Length of drug exposure	Percentage survival	
	Plateau phase cells	Log phase cells
2	111	88
4	96	96
8	89	37
24	70	5

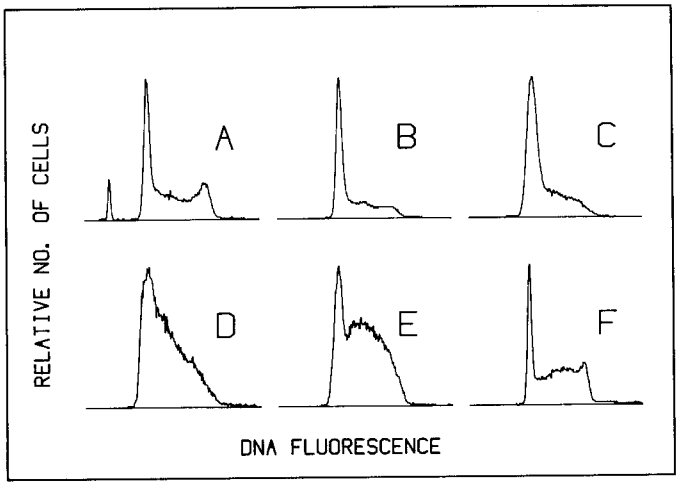

Fig. 5A–F. DNA histograms of asynchronous CHO cells continuously exposed to trimetrexate for various time periods. **A** untreated controls; **B** 30 n*M* trimetrexate exposure for 3 h; **C** 30 n*M* trimetrexate for 10 h; **D** 3 n*M* trimetrexate for 6 h; **E** 3 n*M* trimetrexate for 10 h; **F** 3 n*M* trimetrexate for 12 h. Histograms are presented as in Fig. 4

Trimetrexate clearly showed schedule-dependent toxicity, as smaller, more frequent doses were much less toxic than larger single doses. Given as a single bolus injection, the maximum total nontoxic dose was 120 mg/kg. A single dose of 180 mg/kg produced death within 24 h in 100% of the animals (data not shown).

Weight loss data for the schedules with administration on days 1–9 and every 3 h × 8 on days 1, 5, and 9 show that the top doses (75 and 45 mg/kg/injection, respectively) were near the maximum tolerated doses for those schedules.

The total maximum nontoxic dose was 9-fold higher for the every 3 h × 8 on days 1, 5, and 9 schedule than it was with the day 1 only schedule. Toxicity in the day 1 only and days 1, 5, and 9 schedules is clearly seen in the percent T/C values. In the every 3 h × 8 on days 1, 5, and 9 schedule the mice tolerated a total daily dose of 360 mg/kg as against 120 mg/kg with a single daily injection. This suggests the possibility that for the day 1 only and the days 1, 5, and 9 treatment schedules, the peak plasma concentrations of trimetrexate exceeded some threshold value for lethal CNS, cardiac, or other toxicity [5]. Administration of smaller, more frequent doses probably allowed the peak plasma concentration to remain below this threshold value.

Discussion

The data presented are consistent with the hypothesis that the lethal and growth-inhibitory effects of trimetrexate are limited to cells in S phase. There is a sharp increase in sensitivity to trimetrexate as synchronized CHO cells enter S phase. G_1 and G_2M phase cells are not killed efficiently at the doses tested. Cells from plateau phase cultures, consisting mainly of cells in G_0/G_1 phase, are similarly insensitive when compared with cells from log phase cultures. The lethality of trimetrexate is more dependent on length of exposure than on dose. These phenomena have been demonstrated with other folate antagonists, such as metoprine [11].

The short drug exposure periods needed to demonstrate toxicity in narrow cell cycle positions create the necessity of very high drug concentrations with some antimetabolite drugs. It has been shown that the different mechanisms (e.g., antipurine effects) involved in trimetrexate toxicity are concentration dependent [14]. It is possible that the high drug concentration used in our experiments (50 μ*M*) produced effects that are not relevant to the in vivo situation. However, we have seen no evidence that this would alter the S phase specificity.

Table 3. Schedule dependence of trimetrexate activity against P388 murine leukemia

Dose (mg/kg/injection)	Schedule	Wt. change (g)	Net log kill	% T/C
180	Day 1 only			12
120			0.7	113
80			0.2	104
53			0.0	100
180	Days 1, 5, and 9			9
120		−2.5	−0.9	154
80		1.3	−1.9	119
53		2.9	−2.0	116
75	Days 1–9	−2.6	0.5	181
50		0.3	−0.2	166
33		0.8	−0.1	169
22		1.8	−0.4	164
45	Every 3 h × 8, days 1, 5, and 9	−1.7	2.1	212
30		0.6	1.6	203
20		−0.2	1.2	194
13		1.0	0.7	185

Achievement of cytotoxic concentrations of trimetrexate for the requisite length of time for demonstration of anticancer activity requires much more frequent administration than that required for methotrexate or metoprine. Both methotrexate and metoprine are most effective when administered intermittently [9, 10], while trimetrexate, in our tests, was both most effective and least toxic on a treatment schedule of every 3 h × 8 on days 1, 5, and 9. The difference in schedule dependence between trimetrexate and methotrexate and metoprine might be expected from known differences in their pharmacokinetics and metabolism. Methotrexate is retained within cells longer than trimetrexate, because of the intracellular formation of polyglutamate forms of the drug [12]. Metoprine has an unusually long plasma half-life [10]. Frequent dosing of either drug increases the toxicity to normal cells to unacceptable levels. In contrast, trimetrexate has a much higher turnover rate in both plasma [17, 23] and individual cells [14]. Frequent dosing ensures adequate intracellular drug concentrations for a long enough period of time to effect significant cell killing.

Because of its ability to enter cells that are resistant to methotrexate by virtue of impaired drug transport [6, 15, 18], trimetrexate may find a place in the treatment of methotrexate-resistant tumors. Its interesting biochemical and antitumor properties warrant further work toward exploiting its advantages over other antifolates, and clinical trials are in progress.

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