Critical Modulation by Thymidine and Hypoxanthine of Sequential Methotrexate–5-Fluorouracil Synergism in Murine L1210 Cells

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ABSTRACT

Treatment of murine L1210 cells with methotrexate (MTX) followed by 5-fluorouracil (FUra) produced synergistic cytotoxicity, but only in media containing serum with low concentrations of hypoxanthine, such as horse serum and dialyzed fetal calf serum. Addition of hypoxanthine (1 to 10 μM) during drug exposure reduced the synergism of sequential MTX (1 to 100 μM)-FUra (30 to 300 μM) treatment. The reduction of synergy by hypoxanthine varied with the MTX concentration, higher hypoxanthine concentrations being required to prevent synergy at higher MTX concentrations. The cytotoxicity produced by sequential MTX (10 μM)-FUra (30 to 300 μM) treatment was also reduced if thymidine was added to the regrowth media following drug exposure. The rescue by thymidine was concentration dependent, but as little as 0.5 μM thymidine was sufficient to substantially reduce the synergistic cytotoxicity. These results indicate that both hypoxanthine and thymidine are critical determinants of sequential MTX-FUra synergy and call into question the relevance of experiments in low-thymidine- and low-hypoxanthine-containing media to the clinical situation, where plasma hypoxanthine and thymidine concentrations are often greater than 1 and 0.5 μM, respectively.

INTRODUCTION

Combination chemotherapy regimens containing MTX2 and FUra have been in clinical use, particularly in the management of advanced breast cancer, for many years. Concern about the optimum method of combining MTX and FUra in cancer treatment has arisen from reports that the combination may show antagonistic or synergistic cytotoxicity, depending upon the sequence of drug administration. Synergistic effects observed when MTX precedes FUra have been ascribed either to enhanced binding of 5-fluorodeoxyuridine monophosphate to thymidylate synthetase in the presence of increased levels of dihydropteridinopropaglates (5) or to enhanced FUra-nucleotide formation with resultant increased incorporation of FUra into RNA (4). The latter mechanism is dependent upon accumulation of phosphoribosyl pyrophosphate in cells exposed to MTX (11). Antagonistic effects of sequential MTX-FUra have been attributed to increased levels of dUMP in cells pretreated with MTX (11). Antagonistic effects observed when FUra precedes MTX have been ascribed to sparing effects of thymidylate synthetase inhibition on the utilization of reduced folates for purine biosynthesis (11).

Studies of combined MTX and FUra treatments in vivo are not numerous. Bertino et al. (2) noted that pretreatment of Sarcoma 180-bearing mice with MTX significantly enhanced the antitumor activity of the MTX-FUra combination relative to simultaneous treatment or to treatment with MTX following FUra. Similar results were reported by Mulder et al. (10) for L1210 leukemia and osteosarcoma C22LR-bearing mice, although they noted that MTX followed by FUra also gave substantially greater host toxicity than obtained with simultaneous treatment or when MTX followed FUra. Clinical studies in general have not included treatment groups in which the drug scheduling differs. The efficacy of MTX-FUra combinations in vivo may depend not only on the individual drug doses used and the timing of administration but also on the regional concentrations of thymidine and purines. In this paper, we report studies of the effects of thymidine and hypoxanthine on the synergistic growth inhibition obtained with sequential MTX-FUra treatment of L1210 cells cultured in media containing dialyzed FCS.

MATERIALS AND METHODS

Murine leukemia L1210 cells were grown in suspension culture in Roswell Park Memorial Institute Medium 1640 supplemented with 10% of either horse serum, FCS, or dialyzed FCS (FCS was dialyzed against 5 changes in 10 volumes of Hanks’ balanced salt solution over 3 days). Cultures of log-phase cells (1 × 10⁵ cells/ml) were exposed to either MTX (1, 10, or 100 μM for 4 hr), FUra (30 or 300 μM for 1 hr), or MTX-FUra (MTX for 4 hr; FUra added for last hr; concentrations as above). The cells were then washed 3 times and resuspended (1 × 10⁶ cells/ml) in appropriate drug-free medium, and the changes in numbers of live cells (measured by trypan blue exclusion) were monitored at various times during a period of at least 7 days. Growing cultures were diluted into fresh medium as required. In some experiments, hypoxanthine (1 to 10 μM) or thymidine (0.5, 1.5, or 15 μM) was present in dialyzed FCS-containing medium both during and after drug exposure. In others, thymidine was added to the drug-free regrowth medium only. FUra, thymidine, and hypoxanthine were obtained from Sigma Chemical Co., St. Louis, Mo., and methotrexate was from Lederle Laboratories, Pearl River, N. Y. All cell culture media components were obtained from Grand Island Biological Co., Grand Island, N. Y.

RESULTS

Pretreatment with MTX (3 hr at 10 μM) markedly potentiated the growth-inhibitory effects of a 1-hr exposure to 30 μM FUra on L1210 cells cultured in medium containing 10% horse serum (Chart 1A) but had no such effect on cells growing in medium containing 10% FCS (Chart 1B). The subsequent growth-inhibitory effect of a 1-hr exposure to 30 μM FUra alone was similar in both horse serum- and FCS-containing media (Chart 1). (The growth inhibition during a 48-hr exposure to FUra was also similar in both media, the 50% inhibitory dose in either medium being approximately 0.8 μM.) The growth inhibition caused by 4 hr exposure to 10 μM MTX was less in FCS-containing medium than in horse serum-containing medium, but it was still slightly greater than that caused by 1 μM MTX in horse serum-containing medium.
Chan 1 Effect of serum source on sequential MTX-FUra cytotoxicity L1210 cells were exposed to either FUra (30 μM, 1 hr) (•); MTX (1 μM, 4 hr) (•); MTX (10 μM, 4 hr) (○); MTX (1 μM, 3 hr) followed by FUra (30 μM, 1 hr) (□); or MTX (10 μM, 3 hr) followed by FUra (30 μM, 1 hr) (△); in medium containing either 10% horse serum (A) or 10% FCS (B). Cells were then washed and suspended in appropriate drug-free medium, and subsequent growth was compared to that of non-drug-treated cells (□).

Since the combination of 1 μM MTX followed by FUra was also markedly synergistic in horse serum-containing medium (Chart 1A), we conclude that the lack of synergy observed in FCS-containing media is due not to a lack of efficacy of MTX inhibition but to the composition of the serum itself.

Synergistic cytotoxicity produced by MTX-FUra could, however, be demonstrated with FCS if the serum was dialyzed before use. Thus, exposure of cells to 10 μM MTX followed by 30 μM FUra in 10% dialyzed FCS-containing medium produced substantially greater cytotoxicity than did either drug alone (Chart 2). Nondialyzed FCS contains approximately 100 μM hypoxanthine (12), compared to <1 μM in horse serum (12); thus, we tested the effect of added hypoxanthine on the synergy produced by MTX-FUra in dialyzed FCS-containing medium. In the experiment shown in Chart 2, addition of 1 μM hypoxanthine during and after MTX-FUra exposure did not reduce cytotoxicity within the first 4 days after drug exposure but enabled the MTX-FUra treated cells to grow thereafter. However, addition of 3 μM hypoxanthine almost completely abolished the sequential MTX-FUra synergy, the growth of the latter cells approaching that of cells exposed to MTX (10 μM) alone in the presence of 3 μM hypoxanthine (Chart 2).

The growth inhibition resulting from exposure to 10 μM MTX alone in dialyzed FCS-containing medium was extremely variable, the number of cells present 96 hr after drug exposure varying from 0.3 to 53% of control cells in 12 experiments (average, 13.7% of control). We believe that this is because the dose-response curve of a 4-hr exposure to MTX changes rapidly around 10 μM MTX, so that small changes in MTX concentrations, in time of exposure, and in media components or immediately previous growth conditions give rise to large differences in subsequent growth inhibition. In the experiment shown in Chart 2 (and in 2 other similar experiments), 3 to 10 μM hypoxanthine was required to reduce substantially MTX-FUra synergy; but in

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Chart 1. Effect of serum source on sequential MTX-FUra cytotoxicity. L1210 cells were exposed to either FUra (30 μM, 1 hr) (○); MTX (1 μM, 4 hr) (□); MTX (10 μM, 4 hr) (△); MTX (1 μM, 3 hr) followed by FUra (30 μM, 1 hr) (□); or MTX (10 μM, 3 hr) followed by FUra (30 μM, 1 hr) (○); in medium containing either 10% horse serum (A) or 10% FCS (B). Cells were then washed and suspended in appropriate drug-free medium, and subsequent growth was compared to that of non-drug-treated cells (□).

Chart 2. Prevention by hypoxanthine of synergistic MTX-FUra cytotoxicity in medium containing 10% dialyzed FCS. L1210 cells cultured in medium containing 10% dialyzed FCS were supplemented with either 0, 1, or 3 μM hypoxanthine (0 Hx, 1 Hx, 3 Hx) and then exposed to either FUra (30 μM, 1 hr), MTX (10 μM, 4 hr), or MTX (3 hr) followed by FUra (1 hr). Growth in the appropriate hypoxanthine-containing drug-free medium was then determined. Controls are similar at all 3 hypoxanthine concentrations. Growth of FUra-1 μM hypoxanthine was between that of FUra-0 μM hypoxanthine and FUra-3 μM hypoxanthine; data not shown.

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3 other similar experiments where the growth inhibition by 10 μΜ MTX alone was much less than in Chart 2, 1 to 3 μΜ hypoxanthine reduced substantially the MTX-FUra synergy.

The possibility that the efficacy of hypoxanthine in preventing MTX-FUra synergy depended on the degree of growth inhibition caused by MTX was tested by determining the effect of hypoxanthine on MTX-FUra (30 μΜ) synergy at 1, 10, and 100 μΜ MTX. As shown in Table 1, the MTX-FUra synergy obtained at 1 μΜ MTX was substantially reduced by 1 μΜ hypoxanthine, the cell numbers in 1 μΜ MTX-FUra-treated cells increasing from 0.5 to 47.6% of that in MTX-treated cells 96 hr after drug exposure (Table 1). However, at 10 μΜ MTX, 1 μΜ hypoxanthine increased cell numbers in MTX-FUra-treated cells to a lesser extent, from 1.2 to only 9.6% of that in MTX-treated cells. At 100 μΜ MTX, 1 μΜ hypoxanthine had no significant effect on MTX-FUra synergy. Similarly, while 3 μΜ hypoxanthine completely prevented MTX-FUra synergy at 1 μΜ MTX, it only partially prevented synergy at 10 and 100 μΜ MTX. As has been reported previously for L1210 cells (9), hypoxanthine also reduced the cytotoxicity of MTX alone; but as shown in Table 1, this reduction by hypoxanthine also depended on the MTX concentration used. For example, while 3 μΜ hypoxanthine completely prevented the cytotoxicity caused by a 4-hr exposure to 10 μΜ MTX, it had no effect on the cytotoxicity produced by 100 μΜ MTX (Table 1). Cytotoxicity at 100 μΜ MTX was, however, substantially reduced in the presence of 10 μΜ hypoxanthine, which increased MTX-treated cell numbers to 60% of control (results not shown). The data shown in Table 1 are from one experiment, but similar findings were obtained in 2 other such experiments. Thus, in all 3 experiments, the reduction of MTX-FUra synergy caused by hypoxanthine decreased as the MTX concentration increased.

As shown in Chart 3, thymidine is also a critical determinant of the outcome of sequential MTX-FUra treatment. Addition of thymidine to the regrowth media following drug exposure results in a marked decrease in the cytotoxicity produced by 10 μΜ MTX-30 μΜ FUra treatment. Rescue was maximal at 15 μΜ thymidine, but at little as 0.5 μΜ was sufficient to substantially reduce MTX-FUra cytotoxicity. Similar results were obtained at all 3 thymidine concentrations when thymidine was present both during and after drug exposure (results not shown). Average results of 4 separate experiments investigating thymidine rescue from MTX-FUra cytotoxicity are shown in Table 2. Consistent with the results shown in Chart 3, addition of 15 μΜ thymidine to the regrowth media increased the cell numbers present, 96 hr following drug exposure, of FUra-treated cells 2.5-fold, of MTX-treated cells 4.4-fold, but of MTX-FUra-treated cells 58-fold (Table 2). Similar results were obtained when thymidine was present both during and after drug exposure (Table 2).

At a 10-fold higher (300 μΜ) FUra concentration, cells exposed to FUra alone died almost as rapidly as did cells exposed to sequential MTX (10 μΜ)-FUra (300 μΜ), apparently indicating that no synergy occurred at this FUra concentration (Chart 4A). Addition of 15 μΜ thymidine to the regrowth phase of 300 μΜ FUra-treated cells resulted in a substantial increase (66-fold) in cell numbers, suggesting that the majority of the cytotoxicity was due to inhibition of thymidylate synthetase. However, some growth inhibition due to impairment of RNA metabolism may also have been produced since the subsequent growth of FUra-treated cells in the presence of thymidine was slower than that of control cells (Chart 4A). Addition of thymidine to the regrowth media of MTX-FUra (300 μΜ)-treated cells also resulted in an...
The ability of hypoxanthine to reduce MTX-FUra synergy is dependent to some degree on the MTX concentration; the lower the MTX concentration, the lower is the hypoxanthine concentration required to reduce synergy.

It has been proposed previously (4) that MTX-FUra synergy results from inhibition by MTX of de novo purine synthesis, which causes elevation of phosphoribosyl pyrophosphate levels (3, 4, 8), thereby stimulating FUra anabolism via orotate phosphoribosyltransferase (4, 8). Since hypoxanthine can reduce both the MTX-induced elevation of phosphoribosyl pyrophosphate levels (via its conversion to IMP) (3, 4) and the accompanying enhancement of FUra-nucleotide synthesis (4), our observation that hypoxanthine prevents MTX-FUra synergistic cytotoxicity in L1210 is consistent with this biochemical explanation of synergy (4). Although MTX pretreatment has been reported to stimulate both 5-fluorodeoxyuridine monophosphate and fluorodeoxyuridine triphosphate synthesis to a similar degree (4), our finding that addition of thymidine to the regrowth media following drug exposure substantially reduced MTX-FUra cytotoxicity suggests that enhanced inhibition of thymidylate synthetase is the main cause of the synergistic cytotoxicity. Enhanced impairment of RNA metabolism was found to influence MTX-FUra cytotoxicity only in the presence of thymidine, at high (300 μM) FUra doses.

These results call into question the relevance of in vitro studies in nonphysiological culture conditions to in vivo situations. Plasma hypoxanthine levels in humans vary between 0.2 and 2.6 μM (in cancer patients, the mean is 0.9 μM) (7) and thus may reduce the sequential MTX-FUra synergy occurring in vivo. Similarly, serum thymidine levels in humans vary between 0.1 and 7.4 μM (in cancer patients, the mean is 0.61 μM) (6) and therefore may rescue from the DNA-mediated cytotoxic effects of MTX-FUra. Regional variations in both hypoxanthine and thymidine levels may result in differential cytotoxicity in different tissues; e.g., bone marrow may be protected from the synergistic cytotoxicity of MTX-FUra since hypoxanthine levels in bone marrow are at least 10-fold higher (12) than in plasma (7). In L1210 cells, hypoxanthine reduction of MTX-FUra synergistic cytotoxicity is lessened at higher MTX concentrations, supporting the use of relatively high-dose MTX in clinical studies of MTX-FUra treatment.

Further studies of MTX-FUra treatment in vivo should include treatment arms in which the drugs are given simultaneously to explore whether or not the observations made in vitro have any clinical relevance. It is even possible that optimal antitumor effects may be observed when the drugs are given in an antagonistic regimen, such that the antagonism is manifest in normal cells but not in tumor cells with their variable biochemical mechanisms and high mutation probabilities.

**REFERENCES**


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