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Combined Effects of Methotrexate and 5-Fluoropyrimidine on Human Breast Cancer Cells in Serum-free Culture

ROSS C. DONEHOWER, JOSEPH C. ALLEGRA, MARC E. LIPPMAN and BRUCE A. CHABNER

Clinical Pharmacology Branch and Medicine Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20205, *U.S.A.*

Abstract—*The combined effects of methotrexate and 5-fluoropyrimidines on the growth of a human breast cancer cell line in serum-free hormone-supplemented medium, lacking nucleosides, have been studied. All combinations of methotrexate* (10–50 nM) *and 5-fluorodeoxyuridine* (10–100 nM) *resulted in at least additive inhibition of cell growth and when analyzed by* 50% *response isobologram demonstrated synergism. Combinations of methotrexate* (10–50 nM) *and 5-fluorouracil* (0.04–0.4 μM) *gave enhanced inhibition as compared to single agents, but* 50% *response isobologram revealed only additive effects. The combination was most effective when methotrexate was added to the cultures prior to the 5-fluoropyrimidines or when the drugs were added simultaneously. The addition of 5-fluoropyrimidine prior to methotrexate appears to decrease the effect of the antifol on purine synthesis and lessen the cytotoxicity of the combination in this cell line.*

INTRODUCTION

METHOTREXATE* (MTX) and 5-fluorouracil (5-FU) are commonly used in combination chemotherapy regimens for the treatment of human breast cancer. These two drugs have been used in the adjuvant therapy of operable breast cancer [1], as well as for the treatment of advanced disease [2–4]. When MTX and 5-FU are used in combination the results are difficult to anticipate since the drugs have several theoretical mechanisms of interaction, some antagonistic and others synergistic. For example, the use of MTX with 5-FU may either deplete the reduced folate cofactors necessary for optimal 5-fluorodeoxyuridylate (5-FdUMP) inhibition of thymidylate synthetase [5] or may augment the intracellular accumulation of 5-FU derived nucleotides and increase the efficacy of the combination [6]. These and other proposed mechanisms of interaction have been the subject of a great deal of investigative interest and have been studied in detail by a variety of *in vitro* techniques in rodent tumor lines. However, the applicability of this work to human malignancies has not been established. We have investigated the effects of MTX and 5-FU or 5-fluorodeoxyuridine (5-FUdR) on the growth of the ZR-75-1 human breast cancer cell line in serum-free hormone-supplemented medium lacking nucleosides. This cell line can be propagated under serum-free conditions at a rate equivalent to that with optimal serum supplementation [7]. The use of this system avoids the difficulties in interpretation of experiments with cells cultured in serum-supplemented medium containing high concentrations of nucleosides known to modify the toxicity of these agents [8–11].

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*Abbreviations used: methotrexate, MTX; 5-fluorouracil, 5-FU; 5-fluorodeoxyuridine, 5-FUdR; thymidine, TdR; 5-fluorodeoxyuridylate, 5-FdUMP; 5-fluorouridylate, 5-FUMP; 5-phosphoribosyl-1-pyrophosphate, PRPP; improved minimal essential medium, IMEM; improved minimal essential medium, hormone supplemented, IMEM-HS; ethylene diaminetetra-acetate, EDTA.

MATERIALS AND METHODS

Chemicals

The following compounds used in these experiments were purchased from Sigma Chemical Company, St. Louis, Missouri: 5-

FU, 5-FUdR, thymidine (TdR), deoxyinosine, deoxyadenosine, deoxyguanosine, transferrin, L-tri-iodothyronine, dexamethasone, and 17-β-estradiol. MTX was obtained from the Pharmaceutical Resources Branch of the National Cancer Institute. Insulin U-100 was purchased from Eli Lilly and Company, Indianapolis, Indiana.

Cells

The ZR-75-1 cell line was derived from malignant ascites in a patient with infiltrating ductal carcinoma of the breast [12]. The cells contain cytoplasmic receptors for estrogen, progesterone, glucocorticoid, and androgen and membrane receptors for insulin [12, 13]. The morphology and karyotype of the cells in culture are consistent with the original malignant cells in the ascitic fluid [12]. The cell line has been in continuous culture for more than 3 yr.

Medium

Improved minimal essential medium, hormone supplemented (IMEM-HS), was used for all culture experiments [7]. Improved minimal essential medium (IMEM) [14] was prepared by the National Institutes of Health Media Unit. It was supplemented with L-glutamine (0.6 g/l), penicillin (62 mg/l), streptomycin (135 mg/l), as well as transferrin (10^{-11}M), L-tri-iodothyronine (10^{-8}M), 17-β-estradiol (10^{-8} M), dexamethasone (10^{-8} M) and insulin (5×10^{-7} M).

Cell culture experiments

Cells growing exponentially in IMEM plus 5% fetal calf serum were suspended with trypsin (0.05%) and ethylenediaminetetra-acetate (EDTA) (0.02%) and were plated in IMEM plus 5% charcoal-treated calf serum [15] in sterile 6 well (35 mm) plastic tissue culture dishes (Linbro Scientific Inc., Hamden, Connecticut). After the cells became adherent, the medium was changed to IMEM-HS. The cells were then allowed to grow for 48 hr, with fresh medium change of identical composition added at 24 hr. It has been shown previously that the ZR-75-1 cell line grows as well in IMEM-HS as in IMEM plus 5% fetal calf serum [7]. Experiments were begun by the addition of IMEM-HS containing the chemotherapeutic agents and nucleosides in the concentrations specified. After 48 hr, the cell layer was washed once with Dulbecco's phosphate-buffered saline (KCl, 0.20 g/l; KH_2PO_4, 0.20 g/l; NaCl, 8.0 g/l; $Na_2HPO_4 \cdot 7\ H_2O$, 2.16 g/l, pH 7.4), harvested by suspension with 0.02% EDTA in Dulbecco's phosphate-buffered saline, and counted in a hemocytometer. Results are expressed as a percentage of control cell count at 48 hr. Some data are plotted as 50% response isobolograms [16]. A 50% response isobologram demonstrates graphically the fractional inhibitory concentrations of the two drugs in combination which have resulted in a 50% response, in this case a 50% decrease in cell count at 48 hr. Statistical significance was determined by the use of Student's *t*-test.

RESULTS

The individual effects of MTX, 5-FU, and 5-FUdR are shown in Fig. 1. The dose-response curves shown represent the mean of three determinations. The concentrations of drug which decreased cell count at 48 hr to 50% of control (ID_{50}) were as follows: MTX, 42 nM; 5-FU, 0.3 μM; and 5-FUdR, 80 nM.

The effects of MTX in combination with 5-FU or with 5-FUdR are shown in Fig. 2 with

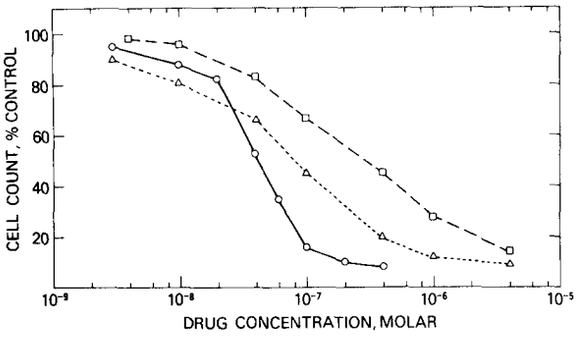

Fig. 1. Dose response curves in the ZR-75-1 breast cancer cell line in culture. Cells were cultured as described in the "Materials and Methods". Shown are effects of MTX (○—○), 5-FU (□– –□) and 5-FUdR (△----△) on cell number at 48 hr, as a percentage of control. The dose-response curves shown represent the mean of three determinations. Standard errors of the triplicate experiments were less than 10%.

the results plotted as a 50% response isobologram. The dashed lines in Figures 2A and 2B represent the theoretical line on which experimental points would fall if the drugs exerted additive effects. Figure 2A demonstrates that the combined fractional doses of MTX and 5-FUdR required to decrease cell number to 50% of control were smaller than expected. Thus the effects of this combination were synergistic under these conditions. Figure 2B examines the combined effects of MTX and 5-FU in the same manner. The experimental data closely follow the theoretical line for

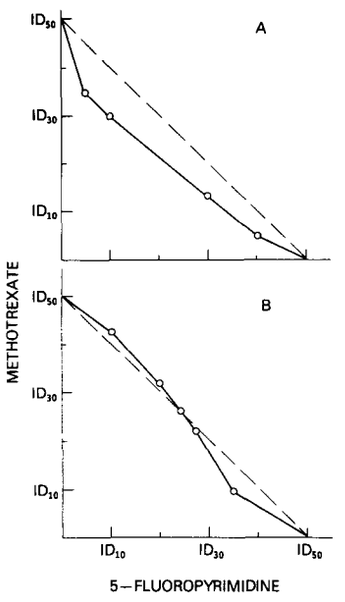

Fig. 2. Combined effects of MTX and 5-fluoropyrimidines plotted as a 50% response isobologram. A 50% response isobologram is a diagram which demonstrates which fractional doses (ID values) when combined have resulted in a 50% response, in this case, a 50% reduction in cell count at 48 hr. The dashed lines represent the theoretical plot if the drugs exerted additive effects. The results shown are (A) for (MTX) and 5-FUdR and (B) for MTX and 5-FU.

additivity of drug effects. At all the concentrations studied, the combination was more effective than either single agent, but no clear synergism was observed.

In vivo [17–19] and *in vitro* [6, 20–22] experimental chemotherapy studies have consistently demonstrated that combinations of these drugs are most effective when MTX precedes the 5-fluoropyrimidine by at least 1 hr. Therefore, differences in the combined drug effects resulting from changes in schedule of administration were studied in this system. Initial studies were done with concentrations of MTX, 5-FU, and 5-FUdR at which the combined inhibition of cell growth was completely reversible by the addition of 10 μM TdR. An example of the effects of schedule administration at these drug concentrations is shown in Table 1. There was no significant difference in the cytotoxicity of the combination of MTX and 5-FU when the drugs were added to the culture simultaneously or when MTX addition precedes 5-FU by 2 hr, as shown by lines 5 and 6 ($P>0.05$). This was also the case with combinations of MTX and 5-FUdR as shown by lines 8 and 9 ($P>0.05$). However, the addition of 5-FU 2 hr prior to MTX (line 7) resulted in significantly less toxicity than either of the other schedules studied ($P<0.02$ in both cases). This was also true for the addition of 5-FUdR 2 hr prior to MTX (line 10) when compared to either of the other schedules ($P<0.01$). These results indicate that the combined effect of MTX and either 5-FU or 5-FUdR on the synthesis of thymidylate was decreased if the addition of MTX was delayed 2 hr following 5-fluoropyrimidine.

The decrease in cell number at 48 hr resulting from MTX concentrations greater than 90 nM could not be reversed by 10 μM TdR alone. Complete reversal of effect was accomplished only when 10 μM deoxyinosine or other purine nucleoside was added to the culture in addition to 10 μM TdR, indicating that at higher drug concentrations MTX has an effect on both thymidylate and purine synthesis in this human cell line. The degree to which the effect of 100 nM MTX was reversed by 10 μM TdR or deoxyinosine can be seen in lines 1 and 2 of Table 2. The antipurine effect of MTX was also affected by the sequence of MTX and 5-fluoropyrimidine addition as shown in Table 2. No difference was observed between the schedule in which MTX, TdR, and 5-FU were added together and the schedule in which MTX and TdR addition preceded 5-FU by 4 hr, as shown in lines 5 and 6 ($P>0.05$); or between identical schedules substituting 5-FUdR for 5-FU, as shown in lines 8 and 9 ($P>0.05$). These data, when compared to the toxicity of MTX and TdR alone (line 2), suggested that the effect of MTX on purine synthesis was preserved when the drugs were added by these schedules. However, the antipurine effect of the antifol was compromised significantly when either 5-FU ($P<0.02$) or 5-FUdR ($P<0.05$) were added to the cultures 4 hr before MTX.

DISCUSSION

Combination chemotherapy regimens employing MTX and 5-FU have been used effectively in the treatment of a variety of human malignancies, including breast cancer [1–4, 23, 24]. However, it has been questioned whether these drugs are rationally used in combination since several proposed mechanisms by which antagonism may occur have sound theoretical bases. A major mode of action of the 5-fluoropyrimidines involves the inhibition of thymidylate synthetase by 5-fluorodeoxyuridylate (5-FdUMP) [25]. Since the formation of a covalently bound enzyme-inhibitor complex has been shown to depend on the presence of 5,10-methylenetetrahydro-

Table 1. Effect of schedule on combined effects reversible by thymidine

Group	Drugs added	Cell No. $\times 10^{-5}$ ($\pm$S.E.M.)	% of control	% Control expected*	%Control with TdR added†
1	60 nM MTX	3.61 $\pm$ 0.15	38%	—	96%
2	20 nM MTX	7.60 $\pm$ 0.38	80%	—	101%
3	100 nM 5-FU	5.70 $\pm$ 0.35	60%	—	100%
4	10 nM 5-FUdR	6.93 $\pm$ 0.23	73%	—	103%
5	60 nM MTX + 100 nM 5-FU	2.01 $\pm$ 0.15	21%	23%	98%
6	60 nM MTX $\xrightarrow{2hr}$ 100 nM 5-FU	2.09 $\pm$ 0.12	22%	23%	100%
7	100 nM 5-FU $\xrightarrow{2hr}$ 60 nM MTX	3.04 $\pm$ 0.26	32%	23%	102%
8	20 nM MTX + 10 nM 5-FUdR	2.85 $\pm$ 0.19	30%	58%	102%
9	20 nM MTX $\xrightarrow{2hr}$ 10 nM 5-FUdR	2.47 $\pm$ 0.19	26%	58%	101%
10	10 nM 5-FUdR $\xrightarrow{2hr}$ 20 nM MTX	4.56 $\pm$ 0.28	48%	58%	101%

*Expected results if combination was additive, calculated by multiplying fractional remaining cell counts for the single agents.
†10 μM thymidine.

Table 2. Schedule dependency of MTX *antipurine effects*

Group	Drugs added*	Cell No. $\times 10^{-5}$ ($\pm$1 S.D.)	% of control	% Control expected†	%Control with purine added‡
1	MTX	1.32 $\pm$ 0.11	12%	—	50%
2	MTX + TdR	7.15 $\pm$ 0.61	65%	—	101%
3	5-Fu $\xrightarrow{4hr}$ TdR	10.56 $\pm$ 0.91	101%	—	—
4	5-FUdR $\xrightarrow{4hr}$ TdR	10.80 $\pm$ 0.85	99%	—	—
5	MTX + 5-FU + TdR	6.60 $\pm$ 0.61	60%	65%	96%
6	MTX + TdR $\xrightarrow{4hr}$ 5-FU	6.93 $\pm$ 0.58	63%	65%	102%
7	5-FU $\xrightarrow{4hr}$ MTX + TdR	10.67 $\pm$ 0.98	97%	65%	—
8	MTX + 5-FUdR + TdR	6.70 $\pm$ 0.58	61%	65%	100%
9	MTX + TdR $\xrightarrow{4hr}$ 5-FUdR	6.38 $\pm$ 0.41	58%	65%	101%
10	5-FUdR $\xrightarrow{4hr}$ MTX + TdR	10.23 $\pm$ 0.95	93%	65%	—

*Concentrations of compounds used: MTX, 100 nM; TdR, 10 μM; 5-FUdR, 20 nM; 5-FU, 100 nM.
†Percentage of control cell No. remaining in experiment with MTX + TdR.
‡10 μM deoxyinosine.

folate [5], the availability of intracellular reduced folate may be a critical determinant of 5-fluoropyrimidine cytotoxicity. Ullman *et al.* have demonstrated that L1210 murine leukemia cells grown in folate-free medium form only 6% of the ternary thymidylate synthetase complex with 5-FdUMP when exposed to [^{3}H]5-FUdR as compared to control cells grown in the presence of folates [26]. Whether MTX treatment alone is sufficient to decrease intracellular reduced folate to levels that limit complex formation has not been established. While the biochemical pharmacology of the drug interaction has not been detailed in the present work, it is apparent that under conditions where their toxicity is limited to effects on thymidylate synthesis (Table 1) MTX and either 5-FU or 5-FUdR are not antagonistic in this human cell line. Other authors have proposed explanations for the greater efficacy of schedules in which MTX is added before 5-FU or 5-FUdR as compared to prior treatment with 5-fluoropyrimidine. Bertino and co-workers have suggested that initial treatment with MTX might increase the amount of 5-FdUMP bound to thymidylate synthetase in an inhibited complex by serving as a folate cofactor for this binding [17]. However, the addition of MTX to an incubation mixture containing 5-FdUMP, excess 5,10-methylenetetrahydrofolate and thymidylate synthetase purified from human myeloblasts decreased the degree of inactivation of the enzyme by 50% at 60 min

[27]. Furthermore, studies with a purified bacterial enzyme have demonstrated decreased total binding [28] and greatly decreased affinity of 5-FdUMP for thymidylate synthetase when MTX replaces the physiologic cofactor [29]. An alternative kinetic explanation has been proposed by Bowen *et al.* [30]. They demonstrated that as the basal rate of thymidylate synthesis was decreased in Ehrlich ascites tumor by treatment with 5-FUdR, the fractional additional inhibition of DNA synthesis by a given dose of MTX was decreased. These authors reasoned that the decreased rate of reduced folate consumption in the 5-fluoropyrimidine-treated cells should make it possible for a smaller fraction of cellular dihydrofolate reductase activity to maintain reduced folate levels, and the intracellular level of MTX necessary to produce the same degree of inhibition of a smaller critical fraction of reductase activity should increase. A third possible explanation comes from the work of Cadman *et al.* [6, 22]. These workers have shown that pretreatment of murine L1210 leukemia with MTX resulted in augmented intracellular levels of [^{3}H]5-FU nucleotides. This accumulation was dependent on the presence of free intracellular MTX and increased as the concentration of MTX increased. The increase in intracellular radioactivity was distributed between 5-FdUMP and the 5-FU ribonucleotides. Their data suggested that the mechanism was increased availability of 5-phosphoribosyl-1-pyrophosphate (PRPP) for the conversion of 5-FU to 5-fluorouridylate (5-FUMP) by the enzyme phosphoribosyltransferase in the MTX-treated cells as a result of the inhibition of *de novo* purine synthesis. This sequence of drug administration also resulted in synergistic cell kill as determined by soft agar cloning techniques. However, the precise mechanisms involved in the ZR-75-1 cell line and other human tumors remain to be elucidated.

The importance of the antipurine effects of MTX in the cytotoxicity of the drug has been well recognized [31–36]. By inhibiting thymidylate synthetase, 5-fluoropyrimidines may prevent the conversion of available reduced folates to dihydrofolate and may thus preserve this pool for *de novo* purine biosynthesis. This has been proposed as a possible mechanism by which the 5-fluoropyrimidines antagonized MTX action in murine L5178Y cells in culture [20]. The data in Table 2 suggest that the antipurine effects of MTX were undiminished by therapy with either 5-FU or 5-FUdR provided that MTX administration precedes the 5-fluoropyrimidine or the drugs were added simultaneously. However, when 5-FU or 5-FUdR were added 4 hr before MTX the antipurine effects were significantly reduced. These findings in a human tumor cell line confirm the schedule dependency noted in similar experiments with the murine L5178Y cell line [20] and the Novikoff hepatoma [21]. In those cell lines, the greatest antagonism of the MTX antipurine effect was noted when 5-FU or 5-FUdR preceded MTX but lesser degrees of antagonism remained even when MTX was added to the culture medium first.

In vivo and *in vitro* investigations of a wide variety of mammalian tumors have led to the recognition that the biological differences in these tumor types may result in varying responses to combinations of MTX and 5-fluoropyrimidines. Before conclusions can be reached concerning the biochemical interactions of these drugs and their optimal sequence in man, further studies of human tumor lines must be carried out. In the present work, additive or synergistic chemotherapeutic interaction of these drugs in a human cell line has been reported. A schedule of administration in which 5-fluoropyrimidine preceded MTX was less efficacious than the alternatives studied, confirming the consistent finding in rodent tumors. The study of this and other human tumor cell lines may yield valuable information concerning the optimization of the use of these drugs in man, since the tumor type used in this study is commonly treated with this combination. In addition, the line can be grown in a serum-free defined medium, lacking the nucleosides which are known to modify the toxicity of these agents.

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