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13. ABSTRACT (Maximum 200) High-dose (10 μ M) methotrexate (MTX) cytotoxicity is maintained in MCF-7 and MDA-MB-436 human breast cancer cells but reduced in Hs824.T human bone marrow by a priming-and nontoxic 5-fluorouracil (5-FU) dose (10 μ M). The growth rates of MCF-7 and MDA-MB-436 cells in the presence of 5-FU, respectively are 97.59 $\pm$ 0.97 % and 94.89 $\pm$ 1.35 % of control rates; and the growth rate of bone marrow cells is 90.61 $\pm$ 3.71 %. The combinations of 5-FU 2h prior to MTX or MTX 2h prior to 5-FU followed by a 48h incubation, respectively, gave growth rates of 1) 20.96 $\pm$ 2.44 % and 19.86 $\pm$ 2.56 % in MCF-7 cells, 2) 25.60 $\pm$ 1.28 % and 25.17 $\pm$ 1.23 % in MDA-MB-436 cells, and 3) 79.66 $\pm$ 7.41 % (a protective effect of 5-FU) and 31.39 $\pm$ 1.77 % in bone marrow. The % of control rates of MTX in MCF-7, MDA-MB-436, and bone marrow cells, respectively, are 21.81 $\pm$ 3.33 %, 22.54 $\pm$ 1.56 %, and 29.58 $\pm$ 2.99 %. A MTX level, at least 1 order of magnitude above 1 μ M, is necessary for the cytotoxicity of 5-FU and MTX to be independent of sequence of administration. At equiconcentrations of trimetrexate (TMQ) and MTX, the nonpolyglutamated antifolate TMQ or TMQ-5-FU combinations inhibited the growth of breast cancer cells less than MTX and MTX-5-FU combinations. However, in bone marrow, TMQ and MTX effects alone or with 5-FU are identical.				
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INTRODUCTION

Utilizing the fluoropyrimidine 5-fluorouracil (5-FU) and the classical and nonclassical antifolates methotrexate (MTX) and trimetrexate (TMQ), respectively, the goal of this research project is to illustrate how these agents may improve the quality of life by: exploiting differences in the biochemical pharmacology of MTX in human MCF-7 and MDA-MB-436 breast cancer cells and human bone marrow cells and providing a clear basis for the rescue or protection of normal host cells, such as bone marrow, from MTX toxicity when high-dose MTX is used in combination with 5-FU. The aim of this work is to provide support for the hypothesis that breast cancer cells tend to synthesize significant higher levels of MTX-polyglutamates (MTXPGs) than normal cells. A priming-and nontoxic dose of 5-FU by conserving cellular reduced-folates protects against the effects of MTX but not MTXPGs and, therefore, should provide a greater protective effect to normal cells than to cancer cells.

Preclinical studies from this laboratory showed that high-dose MTX (245 mg/kg given by i.p. injection) toxicity is reduced by a priming-and nontoxic dose of 5-FU (25 mg/kg administered by i.p. injection). Changes in the hematopoietic system (platelets, erythrocytes, leukocytes, hemoglobin, and hematocrit), ileal tissue, body weight, and mean survival were used as parameters to assess toxicity. For all parameters studied, there were no significant differences between the scheduling of MTX after a priming dose of 5-FU, 5-FU alone, and control. However, sequential treatment with MTX followed by 5-FU, and MTX alone resulted in: (a) a marked decrease in the hematopoietic parameters; (b) significant morphological changes in ileal tissue; (c) a reduction of body weight; and (d) increase in mortality of animals.

BODY

• Methods

MCF-7 and MDA-MB-436 breast cancer and Hs824.T bone marrow cells were grown in monolayer culture in Dulbecco's modified Eagles medium (DMEM) or Leibovitz's L-15 medium. MCF-7 breast cancer cells and bone marrow cells were grown in DMEM containing 10% fetal bovine calf serum, 100 units/ml of penicillin, 100 mg of streptomycin, and 10 μ g/ml of insulin. MDA-MB-436 breast cancer cells were grown in Leibovitz's L-15 medium containing 10 μ g/ml insulin, 16 μ g/ml glutathione, 10% fetal bovine serum. Stock cultures were maintained in 75-cm² flasks and incubated at 37°C in the presence and absence of CO₂, respectively, for bone marrow, MCF-7 and MDA-MB-436 breast cancer cells. Cell populations were serially passed every 3-5 days.

For each experiment, 1 X 10⁴ MCF-7 breast cancer and human bone marrow cells, respectively, were passed into T-25 flasks containing: MTX, 5-FU, 5-FU 2 hours (2h) prior to MTX exposure [5-FU (2h) + MTX], MTX (2h) + 5-FU, and no drugs (control). The doses were 10 μ M 5-FU and 1-10 μ M MTX. After a 48h incubation in a humidified atmosphere of 5% CO₂, the monolayers were washed with phosphate buffered saline (PBS), and cells were separated from the monolayer with 2 ml of 0.25% trypsin-EDTA. The density of cells were determined by

microscopic counting of trypan blue treated cells in a hemocytometer. Cell number also were determined electronically using a Coulter Counter. Doubling times were calculated using the formula: Doubling time = $T_{\text{final}} - T_{\text{initial}} / 3.32 (\log \text{ cell no. } T_{\text{final}} - \log \text{ cell no. } T_{\text{initial}})$.

Human MDA-MB-436 breast cancer cells were harvested and 1×10^4 cells passed in 1 ml of Leibovitz's L-15 medium to T-25 flasks containing : MTX, 5-FU, 5-FU (2h) + MTX, MTX (2h) + 5-FU, and no drugs (control). The concentrations of drugs were $10 \mu\text{M}$ 5-FU and $10 \mu\text{M}$ MTX. After a 48h incubation in a humidified atmosphere, the density of cells were determined by microscopic counting of trypan blue treated cells in a hemocytometer. Doubling times were determined as described above.

Studies to assess the roles of 5-FU and polyglutamation in selectivity entailed an evaluation of the non-polyglutamyl antifolate trimetrexate (TMQ) in combination with 5-FU. Similar studies to those above with MTX were done with TMQ. The concentrations of 5-FU and TMQ were $10 \mu\text{M}$, respectively.

Thermodynamic evaluations of TMQ, MTX, and MTX-polyglutamates interaction with human dihydrofolate reductase utilized the molecular dynamics program CHARMM.

Note: An approved revised statement of work was given for the above studies.

● Results and Discussion

Selective Effects of a Priming-and Nontoxic Dose of 5-FU on High-Dose MTX Cytotoxicity:

Logarithmically growing MCF-7 and MDA-MB-436 breast cancer and Hs 824.T bone marrow cells, respectively, were exposed to 5-FU and MTX alone and in combination. The total time of exposure to MTX and 5-FU was 48h. Figures 1 and 2, respectively, illustrate the effects of 1) high-dose MTX and the independence of MTX and 5-FU sequence of administration on the growth of MCF-7 and MDA-MB-436 breast cancer cells (Figures 1 and 2) and 2) high-dose MTX, the dependence of MTX and 5-FU sequence of administration on bone marrow growth, and the protective effect of a priming-and nontoxic 5-FU dose on bone marrow (Figure 3). In breast cancer cells, similar inhibitory effects of MTX, 5-FU (2h) + MTX (at the arrow), and MTX (2h) + 5-FU exist on cell number, but a dissimilar (protective) effect occurs with 5-FU (2h) + MTX (at the arrow). Panel B of Figure 1 (MCF-7 cells) shows that MTX as a single agent gave a growth rate of $21.81 \pm 3.33\%$ of the control rate. The combinations of 5-FU (2h) + MTX and MTX (2h) + 5-FU, respectively, gave growth rates of $20.96 \pm 2.44 \%$ and $19.86 \pm 2.56 \%$ of the control rates. (A priming-and nontoxic dose of 5-FU has no effect on cell growth; it's rate is $97.59 \pm 0.97\%$ of the control.) Similarly in MDA-MB-436 breast cells, Figure 2B, MTX alone gave a growth rate of $22.54 \pm 1.56 \%$; combinations of 5-FU (2h) + MTX and MTX (2h) + 5-FU, respectively, were $25.60 \pm 1.28 \%$ and $25.17 \pm 1.23 \%$ of control rates. The priming dose of 5-FU has no effect on the growth of MDA-MB-436 cells (5-FU growth rate is $94.89 \pm 1.35 \%$ of the control rate). In bone marrow (Figure 3A), similar inhibitory effects of MTX and MTX (2h) + 5-FU exist on cell number, but a dissimilar (protective) effect occurs with 5-FU (2h) + MTX

(at the arrow). Panel B of Figure 3 shows that the growth rate of MTX and MTX (2h) + 5-FU are 29.58 ± 2.99 % and 31.39 ± 1.77 % of control rates, respectively; while 5-FU (2h) + MTX rate is 79.66 ± 7.41 % of the control (a protective effect of a priming-and nontoxic dose of 5-FU).

These results suggest that the incidence and severity of MTX (2h) + 5-FU (2h) + MTX cytotoxicity in breast cancer cells are best related to MTX rather than 5-FU (since 5-FU had no effect which differed from MTX alone). However, 5-FU administered prior to MTX modulated MTX toxicity in bone marrow. The selective cytotoxic effect of MTX in breast cancer may result from the formation of MTX-polyglutamates (MTXPGs) (1) and the inability of 5-FU to prevent the inhibitory effects of MTX and MTXPGs. MTXPGs synthesis increases with increases in drug concentration. In human breast cancer cells, formation of MTXPGs occurs at a concentration of $2 \mu\text{M}$ MTX (1) -- a concentration 1/5 th of that used in this study. The formation of MTXPGs allows for the inhibition of dihydrofolate reductase, thymidylate synthase, and inhibition of other folate-requiring enzymes not affected by MTX (such as aminoimidazolecarboxamide ribonucleotide transformylases (2)). Whereas, bone marrow form little or no MTXPGs when exposed to MTX (3,4); and, therefore, certain folate-requiring enzymes will not be inhibited due to the absence or very low levels of MTXPGs. Hence, sequence dependency in bone marrow and platelets may best be related to 5-FU conserving reduced-folates to protect against the direct effects of MTX.

Assessment of the Nonpolyglutamylated Antifolate Trimetrexate (TMQ) and MTX in Combination with 5-FU in Breast Cancer and Bone Marrow Cells: To assess the importance of the role of polyglutamation in antifolate chemotherapy with 5-FU, a comparison of the nonpolyglutamated antifolate trimetrexate and polyglutamated antifolate MTX was made on the growth of MCF-7 and MDA-MB-436 human breast cancer cells and Hs 824.T bone marrow cells. Figures 4 and 5 illustrate the differential inhibitory effects of TMQ and MTX in the absence and presence of 5-FU on MCF-7 and MDA-MB-436 cells, respectively. In breast cancer cells, similar inhibitory effects of TMQ, 5-FU (2h) + TMQ, and TMQ (2h) + 5-FU exist on cell number; and a pattern with MTX, 5-FU (2h) + MTX, and MTX (2h) + 5-FU was similar to TMQ and TMQ and 5-FU combinations. (However, in all cases, the inhibitory effects of MTX and MTX and 5-FU were greater than TMQ and TMQ and 5-FU combinations.) In bone marrow (Figure 6), the inhibitory effects of TMQ and MTX, and TMQ and MTX plus 5-FU combinations were very similar.

The growth rate of MCF-7 cells incubated with TMQ and TMQ and 5-FU combinations was 46.31 ± 1.01 %; whereas, the growth rate of MTX and MTX and 5-FU combinations was 20.88 ± 1.43 % (Figure 7). The growth rate of MDA-MB-436 breast cells incubated with TMQ and TMQ and 5-FU combinations was 50.02 ± 1.24 %; and the growth rate of cells exposed to MTX and MTX and 5-FU combinations was 24.40 ± 0.63 % (Figure 8). Hence, the degree of inhibition of TMQ exposed MCF-7 and MDA-MB-436 breast cancer cells was similar (50 % and 54 %); whereas, the inhibitory effects in MTX exposed breast cancer cells were also similar (79 % and 76 %). Figure 9, illustrates the % of control rate of TMQ, TMQ and combinations of 5-FU, MTX, and MTX and combinations of 5-FU in bone marrow. Note that in all cases 1) TMQ

and MTX, 2) TMQ (2h) + 5-FU and MTX (2h) + 5-FU, and 3) 5-FU (2h) + TMQ and 5-FU (2h) + MTX, respectively, are very similar. The percentage of the control growth rate of 1) TMQ and MTX is $30.12 \pm 4.77\%$ and $30.71 \pm 2.39\%$; 2) TMQ (2h) + 5-FU and MTX (2h) + 5-FU is $26.86 \pm 5.03\%$ and $30.59 \pm 1.49\%$; and 3) 5-FU (2h) + TMQ and 5-FU (2h) + MTX is $63.17 \pm 1.23\%$ and $77.93 \pm 5.51\%$. The identical effects of TMQ and MTX, TMQ + 5-FU and MTX + 5-FU, and 5-FU + TMQ and 5-FU + MTX (protective effects) suggest that TMQ and MTX are acting on a common site and that activity at this common site does not require polyglutamation. The established site in which TMQ and MTX interact is dihydrofolate reductase (DHFR).

TMQ, MTX, and MTXPGs Binding to Human Dihydrofolate Reductase (DHFR): A comparison of the stability of the interaction among TMQ, MTX, and MTXPGs (triglutamylMTX) to human DHFR is shown in Table 1. Using DHFR from x ray crystallography, the electrostatic energy (kcal/mol) from three versions of CHARMM software indicate that TMQ binding is greater than MTX and MTXPGs. The stability of binding is TMQ > MTX > MTXPGs.

The electrostatic energy of TMQ and MTX binding to human DHFR coupled to the identical effects of TMQ and MTX alone and in combinations with 5-FU suggest that these agents are affecting a common site in bone marrow that does not require polyglutamation. However, in MCF-7 and MDA-MB-436 human breast cancer cells, the greater inhibitory effect of MTX alone and in combinations with 5-FU, when compared to the nonpolyglutamyl antifolate TMQ, supports the view that polyglutamation may be an important determinant in MTX and 5-FU selectivity.

Dose Response of MTX in MCF-7 and MDA-MB-436 Cells: Figure 10 shows the responses of MTX doses and a priming-and nontoxic dose of 5-FU. The inhibition of MTX alone and in combinations with 5-FU increases when the concentrations of MTX are 1, 10, and 100 μM . A priming-and nontoxic dose of 5-FU protects cells when the concentration of MTX is 1 μM . However, when the doses of MTX are 10 and 100 μM , a priming-and nontoxic dose of 5-FU do not protect MCF-7 cells. The degree of inhibition of MTX, MTX (2h) + 5-FU, and 5-FU (2h) + MTX on cell number are the same.

Figure 11 shows the response of MTX doses and a priming-and nontoxic dose of 5-FU in MDA-MB-436 breast cancer cells. As the dose of MTX increases from 10 μM to 100 μM , there is a concomitant increase in the inhibitory effect of MTX. The degree of inhibition of MTX, MTX (2h) + 5-FU, and 5-FU (2h) + MTX are the same.

Comparison of Optimal Doses of MTX and TMQ in Combinations with 5-FU: To determine if a differential effect between MTX and TMQ in combinations with 5-FU exist in breast cancer cells, MDA-MB-436 cells were incubated with 100 μM of MTX, 100 μM TMQ, and 10 μM 5-FU (Figure 12). The % of control growth rates were: 1) $93.82 \pm 1.69\%$, 5-FU; 2) $16.20 \pm 0.74\%$, MTX; 3) $15.19 \pm 0.62\%$, MTX (2h) + 5-FU; 4) $16.53 \pm 0.85\%$, 5-FU (2h) + MTX; 5) $28.39 \pm 0.94\%$, TMQ; 6) $29.01 \pm 1.83\%$, TMQ (2h) + 5-FU; and 7) $30.05 \pm 0.68\%$.

The % of control growth rates for MTX and MTX in combinations with 5-FU are very similar -- so are TMQ and TMQ in combinations with 5-FU. The mean % of control growth rates for 1) MTX and 5-FU-MTX combinations is 15.98 ± 0.42 % and 2) TMQ and 5-FU-TMQ combinations is 29.15 ± 0.67 %. As with 10 μ M MTX and 10 μ M TMQ, 100 μ M MTX inhibitory effect also exceeds 100 μ M of the nonpolyglutamated antifolate TMQ.

MCF-7 cells exposed to 100 μ M MTX and 100 μ M TMQ (Figure 13) yielded patterns similar to those of MDA-MB-436 cells. In MCF-7 cells, the mean % of control growth rates for 1) MTX and 5-FU-MTX combinations is 7.91 ± 0.29 % and 2) TMQ and 5-FU-TMQ combinations is 20.88 ± 0.82 %.

Doubling Times of MCF-7 and MDA-MB-436 Breast Cancer Cells 48h after MTX, TMQ, and 5-FU Combinations: Tables 1 and 2, respectively, are representative studies in which the doubling times were determined after incubating MCF-7 and MDA-MB-436 cells for 48h with 10 μ M MTX, TMQ, and 5-FU. Regardless of cell type, the doubling times for MTX exposed cells are similar; and TMQ doubling times in the presence and absence of 5-FU are also similar. In all cases, TMQ exposed cells doubled at a greater rate than cells incubated with MTX.

CONCLUSIONS

High-dose MTX cytotoxicity is maintained in MCF-7 and MDA-MB-436 human breast cancer cells but reduced in Hs824.T human bone marrow by a priming-and nontoxic 5-FU dose. These studies suggest that: 1) MTX and 5-FU combinations on the growth of human MCF-7 and MDA-MB-436 breast cancer cells are independent of sequence; 2) the severity and incidence of MTX (2h) + 5-FU and 5-FU (2h) + MTX cytotoxicity in breast cancer cells are best related to MTX rather than 5-FU (since 5-FU had no effect which differed from control and sequential MTX and 5-FU had no effect which differed from MTX alone); and 3) a priming-and nontoxic dose of 5-FU will protect bone marrow from MTX cytotoxicity but not breast cancer cells. Therefore, a priming-and nontoxic dose of 5-FU and MTX may have maximum antineoplastic activity while at the same time provide protection to the hematopoietic system.

Modulation of MTX cytotoxicity by 5-FU will only be of clinical importance if it (MTX) is more selective against breast cancer cells than hematopoietic cells. Preclinical studies demonstrate that synergistic cytotoxicity occurs when MTX administration precedes 5-FU; however, it may not result in an increase in the therapeutic index since toxicity to normal cells may occur in a similar synergistic manner.

A MTX level (10 μ M) at least one order of magnitude above the concentration (1 μ M) required for leucovorin rescue is necessary for the cytotoxic effect of 5-FU and MTX to be independent of sequence of administration. When the concentrations of MTX are 10 and 100 μ M, a priming-dose of 5-FU will not protect cancer cells. The selective toxic effect of MTX in MCF-7 and MDA-MB-436 cells may result from the formation of MTXPGs and the inability of 5-FU to prevent the inhibitory effects of MTX and MTXPGs. In MCF-7 cells, formation of MTXPGs

occurs at a concentration of 2 μ M MTX (4) -- a concentration 1/5 th of that used in this study. (Growth of cancer cells by a MTX concentration one half of that needed for MTXPGs formation is antagonized by a priming-dose of 5-FU).

At equiconcentrations (10 and 100 μ M) of TMQ and MTX, the nonpolyglutamated antifolate TMQ inhibited the growth of breast cancer cells less than MTX. In MCF-7 and MDA-MB-436 cells, respectively, growth inhibition of TMQ (2h) + 5-FU and 5-FU (2h) + TMQ is identical to TMQ; while, MTX (2h) + 5-FU and 5-FU (2h) + MTX is identical to MTX. In bone marrow, the effects of 1) TMQ, MTX, TMQ (2h) + 5-FU and MTX (2h) + 5-FU, and 2) 5-FU (2h) + TMQ and 5-FU (2h) + MTX are very similar.

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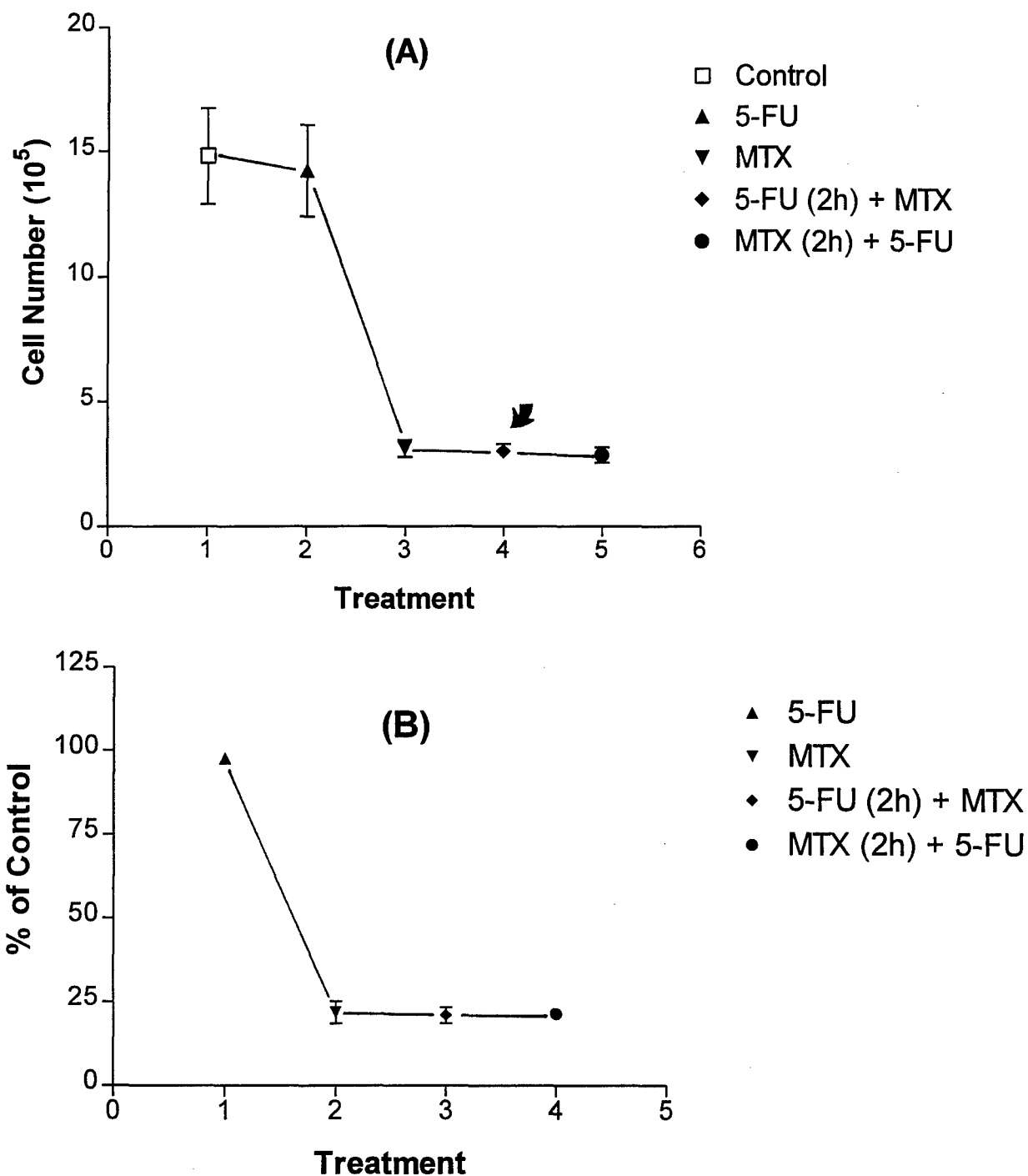


Figure 1. Sequence independence of methotrexate (MTX) and 5-fluorouracil (5-FU) administration on the proliferation of human MCF-7 breast cancer cells (Panel A). MCF-7 cells were exposed to 10 μ M MTX and 5-FU alone, MTX 2h prior to 5-FU [MTX (2h) + 5-FU], 5-FU 2h prior to MTX [5-FU (2h) + MTX] (at the arrow), and no drugs (control). Cells were then incubated for 48h, harvested, and counted. The symbols represent the mean $\pm$ the standard error of three different experiments and panel B represents the percentage of control growth rates for each drug treatment.

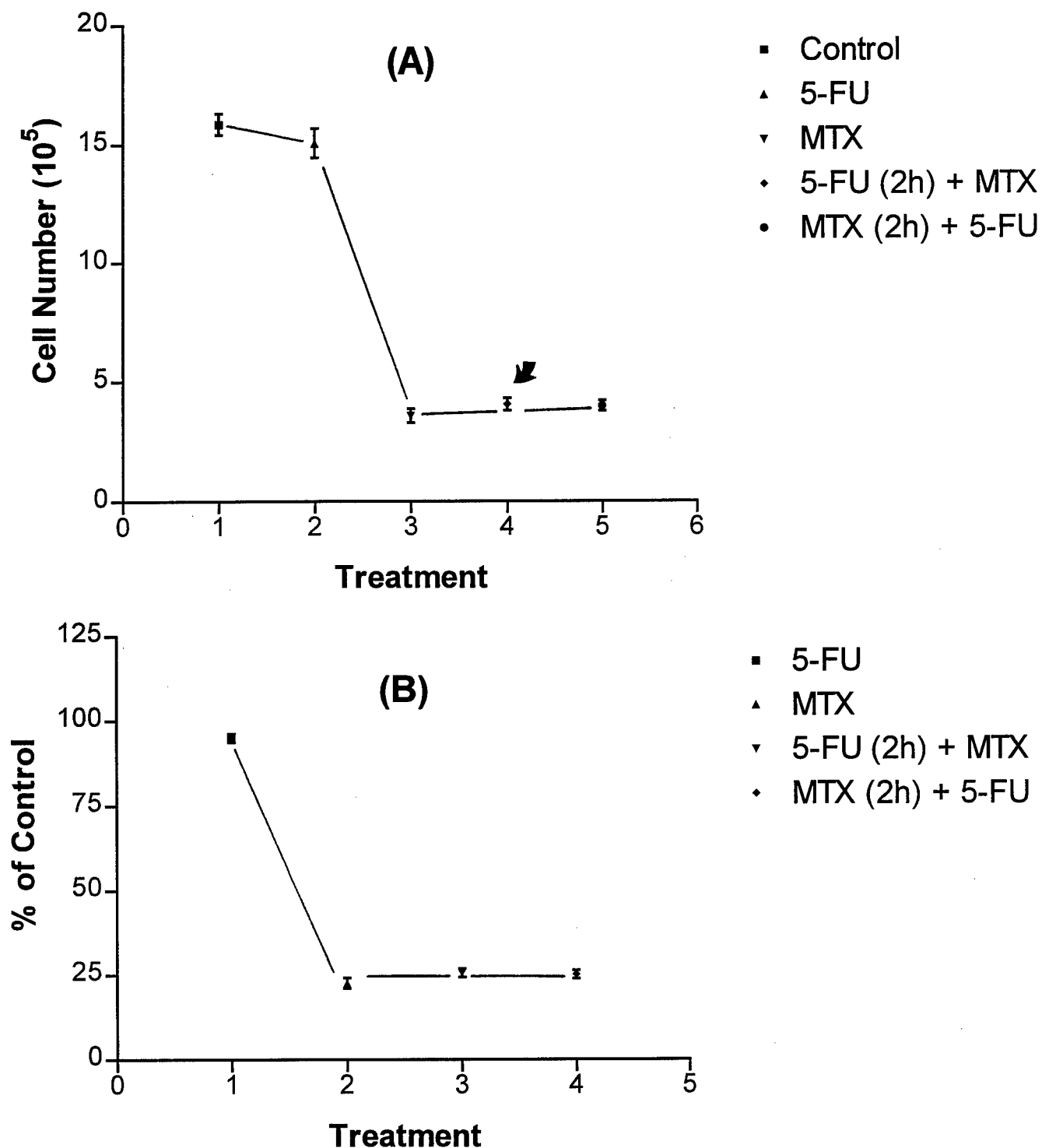


Figure 2. Sequence independence of methotrexate (MTX) and 5-fluorouracil (5-FU) administration on the proliferation of human MDA-MD-436 breast cancer cells (Panel A). MDA-MB-436 cells were exposed to 10 μ M MTX and 5-FU alone, MTX 2h prior to 5-FU [MTX (2h) + 5-FU], 5-FU 2h prior to MTX [5-FU (2h) + MTX] (at the arrow), and no drugs (control). Cells were then incubated for 48h, harvested, and counted. The symbols represent the mean $\pm$ the standard error of three different experiments and panel B represents the percentage of control growth rates for each drug treatment.

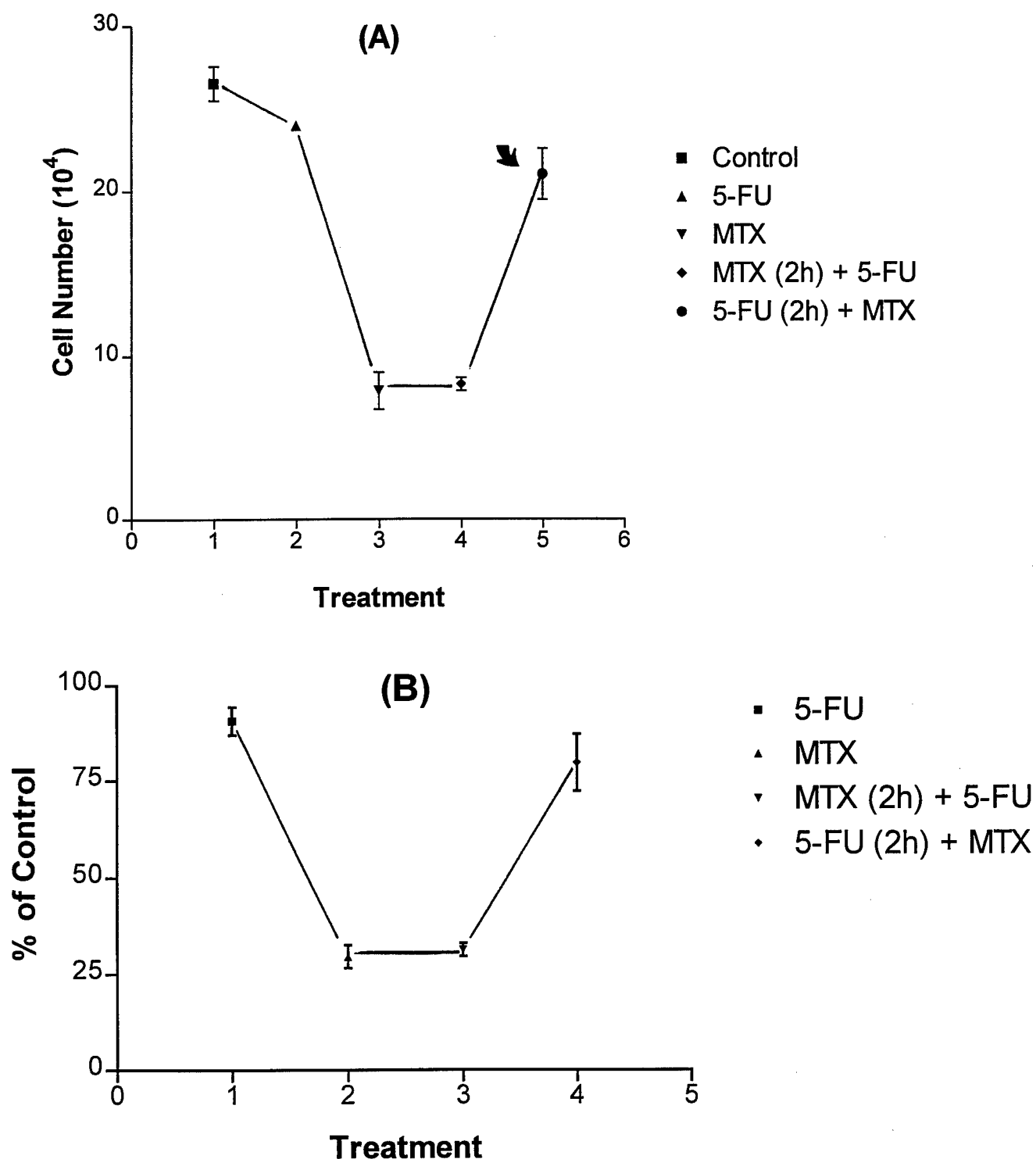


Figure 3. The effect of methotrexate (MTX) and 5-fluorouracil (5-FU) alone and in combination on the proliferation of female human bone marrow (Panel A). Hs824.T human bone marrow cells were incubated with $10 \mu\text{M}$ MTX or $10 \mu\text{M}$ 5-FU alone or in combinations (5-FU 2h prior to MTX and MTX 2h prior to 5-FU) for 48h. Similar inhibitory effects of MTX alone and MTX (2h) + 5-FU exist on cell number, but a dissimilar (protective) effect occurs with 5-FU (2h) + MTX (at the arrow). The symbols represent the mean $\pm$ the standard error of three different experiments and panel B represents the percentage of the control growth rates for each drug treatment.

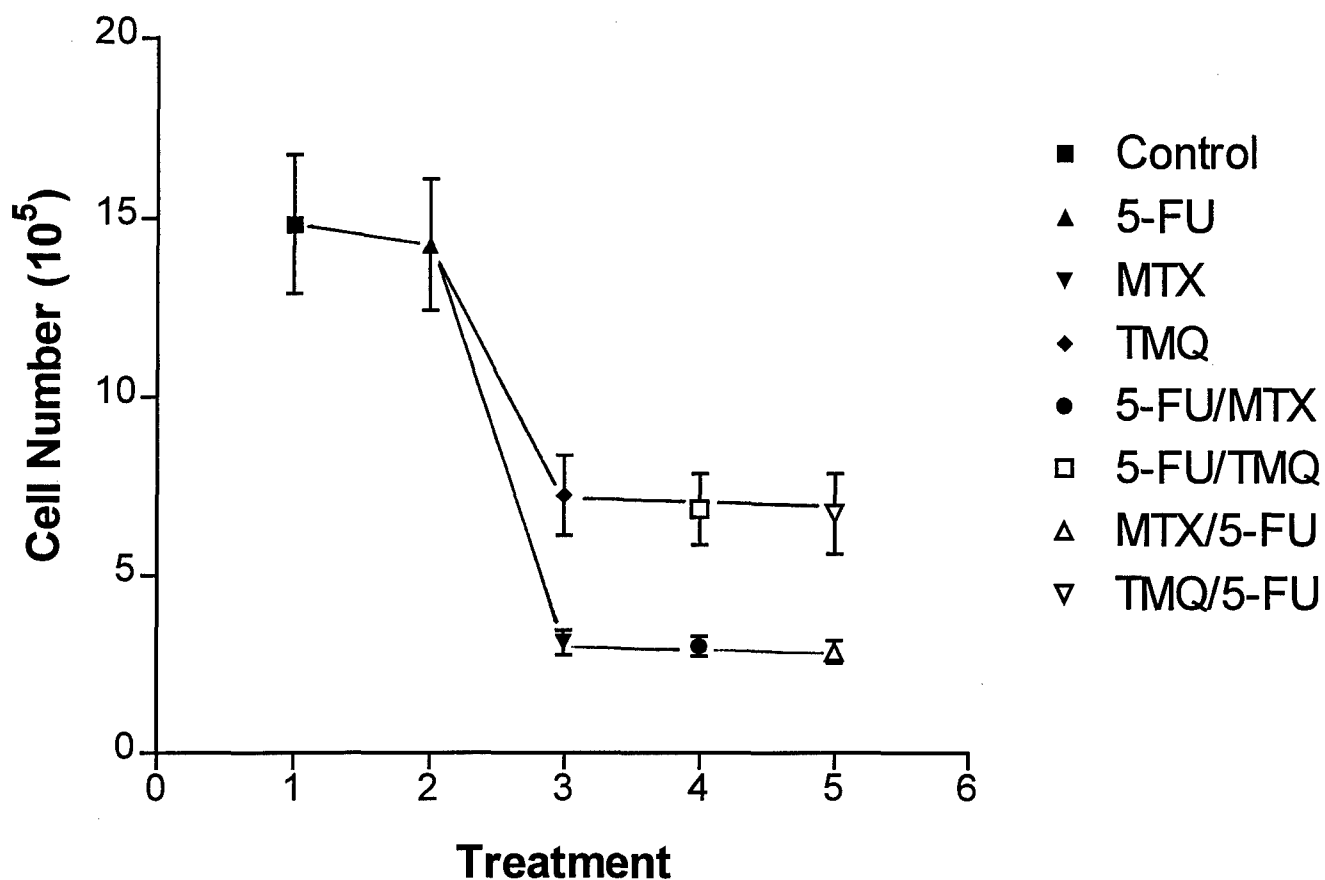


Figure 4. Differential effects of 10 μ M trimetrexate (TMQ) and 10 μ M methotrexate (MTX) on the proliferation of MCF-7 breast cancer cells in the presence and absence of 10 μ M 5-FU. The maximum inhibitory effects of TMQ, TMQ (2h) + 5-FU (TMQ/5-FU), and 5-FU (2h) + TMQ (5-FU/TMQ), respectively, are approximately the same. The maximum inhibitory effects of MTX, MTX (2h) + 5-FU (MTX/5-FU), and 5-FU (2h) + MTX (5-FU/MTX), respectively, are very similar. Note that MTX or MTX in combinations with 5-FU affect cell proliferation greater than TMQ or TMQ in combinations with 5-FU. The symbols represent the mean $\pm$ the standard error of four different experiments.

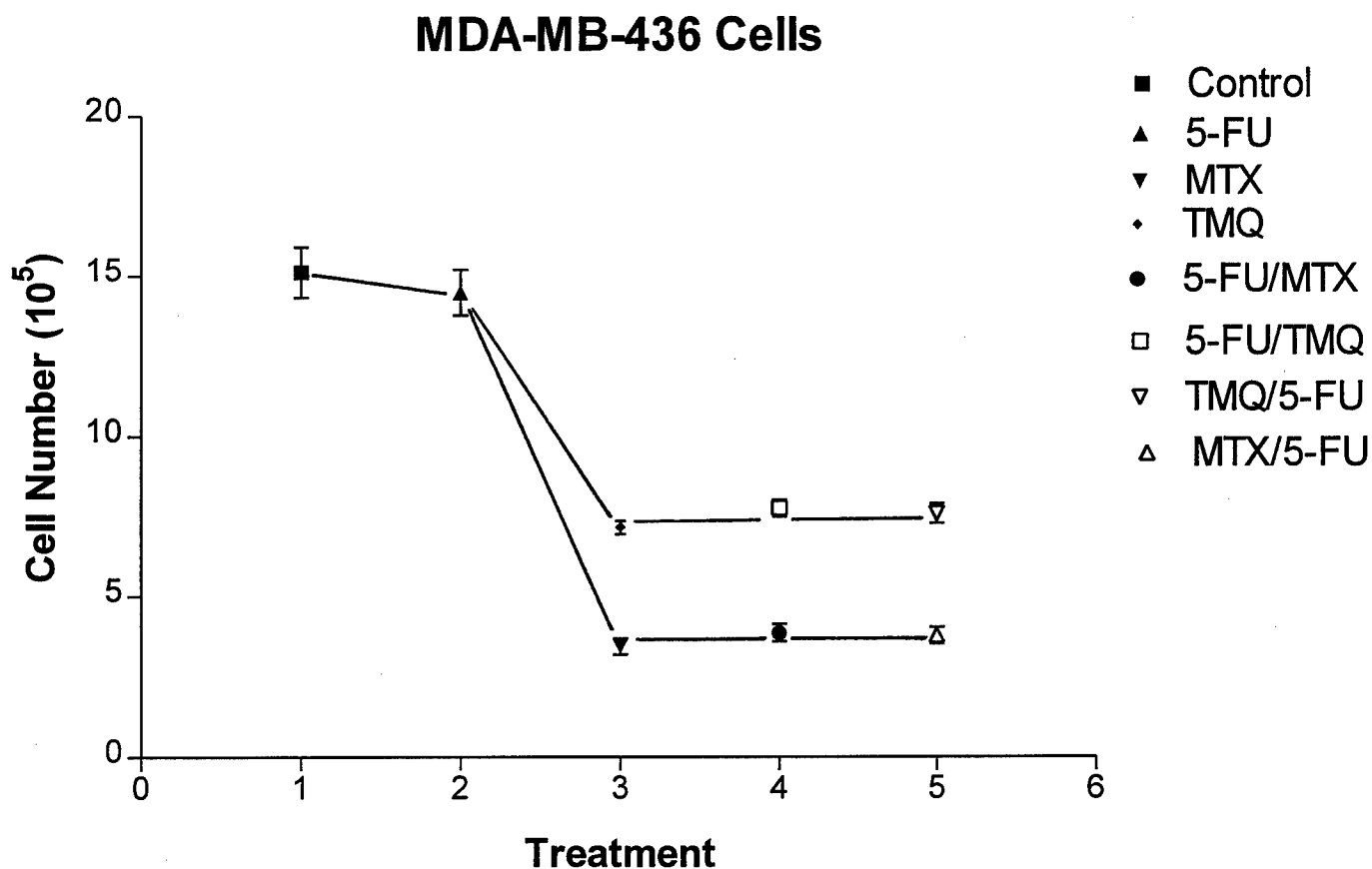


Figure 5. Differential effects of 10 μ M trimetrexate (TMQ) and 10 μ M methotrexate (MTX) on the proliferation of MDA-MB-436 breast cancer cells in the presence and absence of 10 μ M 5-FU. The reduction in cell proliferation of TMQ, TMQ (2h) + 5-FU (TMQ/5-FU), and 5-FU (2h) + TMQ (5-FU/TMQ), respectively, are very similar. There's no difference in the reduction of MTX, MTX (2h) + 5-FU (MTX/5-FU), and 5-FU (2h) + MTX (5-FU/MTX), respectively, on cell proliferation. MTX or MTX in combinations with 5-FU affects cell proliferation greater than TMQ or TMQ in combinations in combinations with 5-FU. The symbols represent the mean $\pm$ the standard error of four different experiments.

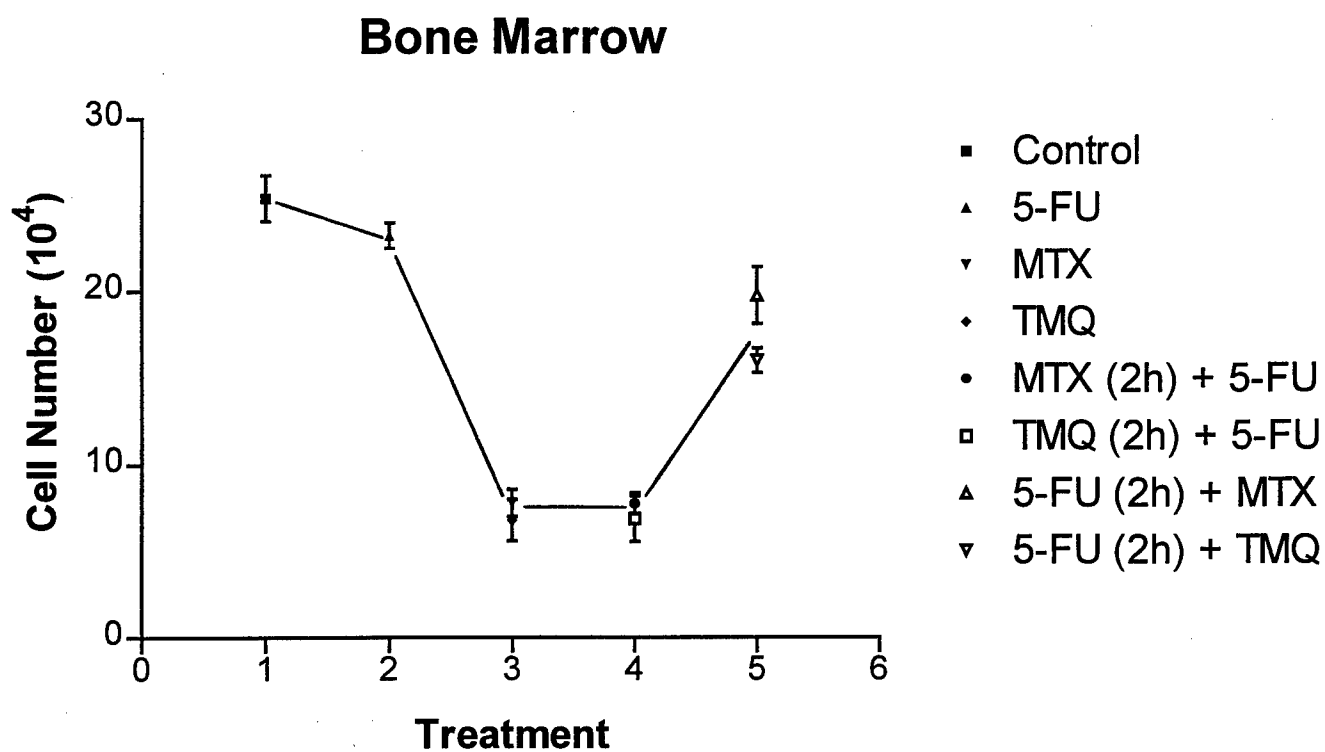


Figure 6. Comparative effects of trimetrexate (TMQ) and methotrexate (MTX) on the proliferation of Hs824.T bone marrow cells in the presence and absence of 5-fluorouracil (5-FU). All drug concentrations were $10 \mu\text{M}$. There's no difference in the reduction of 1) TMQ and MTX. 2) TMQ (2h) + 5-FU and MTX (2h) + 5-FU, and 3) 5-FU (2h) + TMQ and 5-FU (2h) + MTX. The different symbols represent the mean $\pm$ the standard error of three experiments.

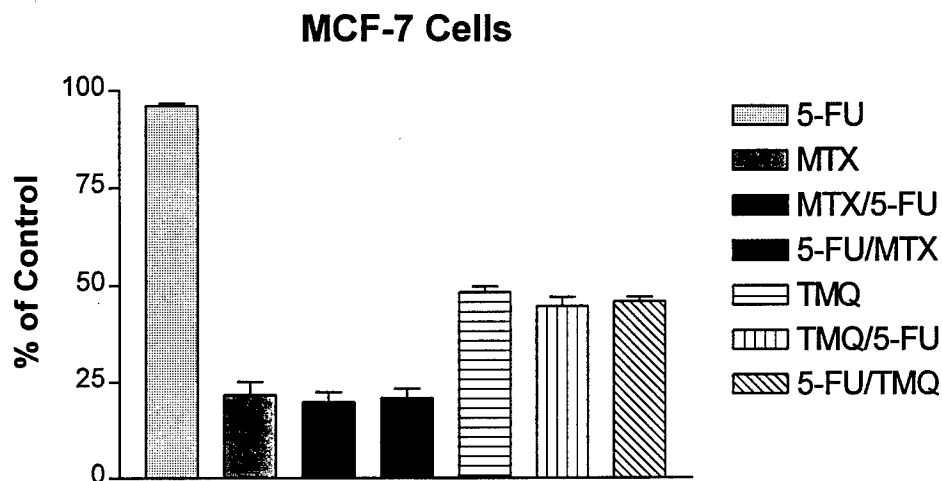


Figure 7. The percentage of the control growth rates of trimetrexate (TMQ) and methotrexate (MTX) alone and in combinations with 5-FU in MCF-7 cells after 48h. The drug concentrations were 10 μ M, respectively. The bars represent the mean $\pm$ standard error of three determinations. MTX/5-FU (MTX given 2h prior to 5-FU); 5-FU/MTX (5-FU given 2h prior to MTX); TMQ/5-FU (TMQ given 2h prior to 5-FU); and 5-FU/TMQ (5-FU given 2h prior to MTX)

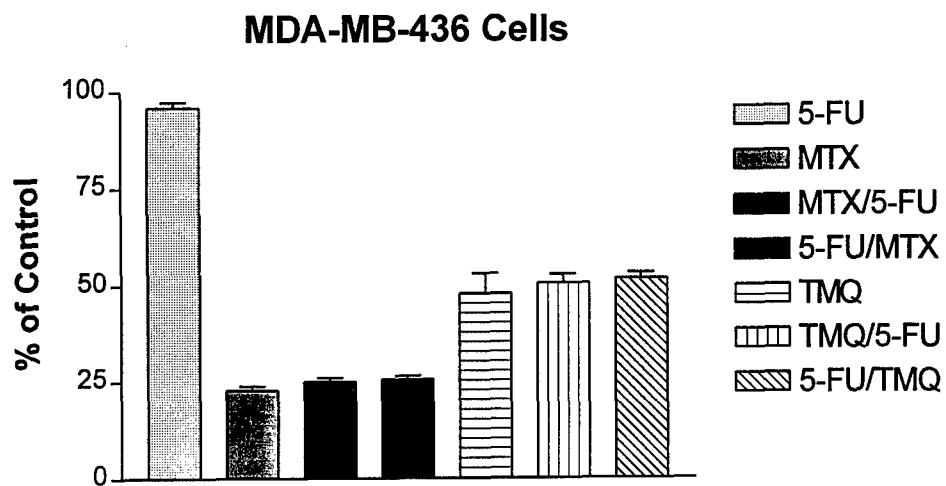


Figure 8. The percentage of the control growth rates of trimetrexate (TMQ) and methotrexate (MTX) alone and in combinations with 5-FU in MDA-MB-436 breast cancer cells after 48h. The drug concentrations were 10 μ M, respectively. The bars represent the mean $\pm$ standard error of four determinations. MTX/5-FU (MTX given 2h prior to 5-FU); 5-FU/MTX (5-FU given 2h prior to MTX); TMQ/5-FU (TMQ given 2h prior to 5-FU); and 5-FU/TMQ (5-FU given 2h prior to TMQ)

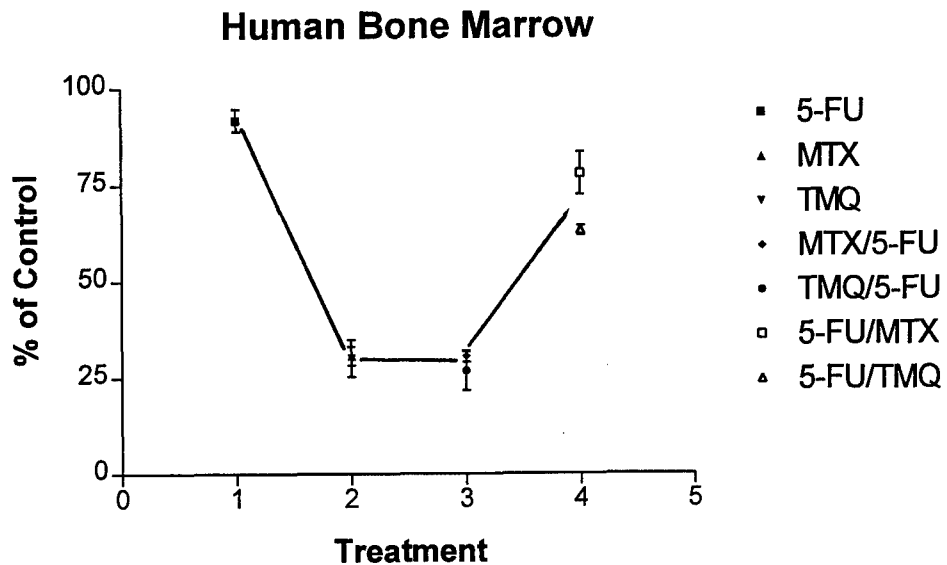


Figure 9. The percentage of the control growth rates of trimetrexate (TMQ) and methotrexate (MTX) alone and in combinations with 5-FU in human bone marrow (Hs824.T) cells after 48h. The drug concentrations were 10 μ M, respectively. The symbols represent the mean $\pm$ standard of four determinations. MTX/5-FU (MTX given 2h prior to 5-FU); TMQ/5-FU (TMQ given 2h prior to 5-FU); 5-FU/MTX (5-FU given 2h prior to MTX); and 5-FU/TMQ (5-FU given 2h prior to TMQ)

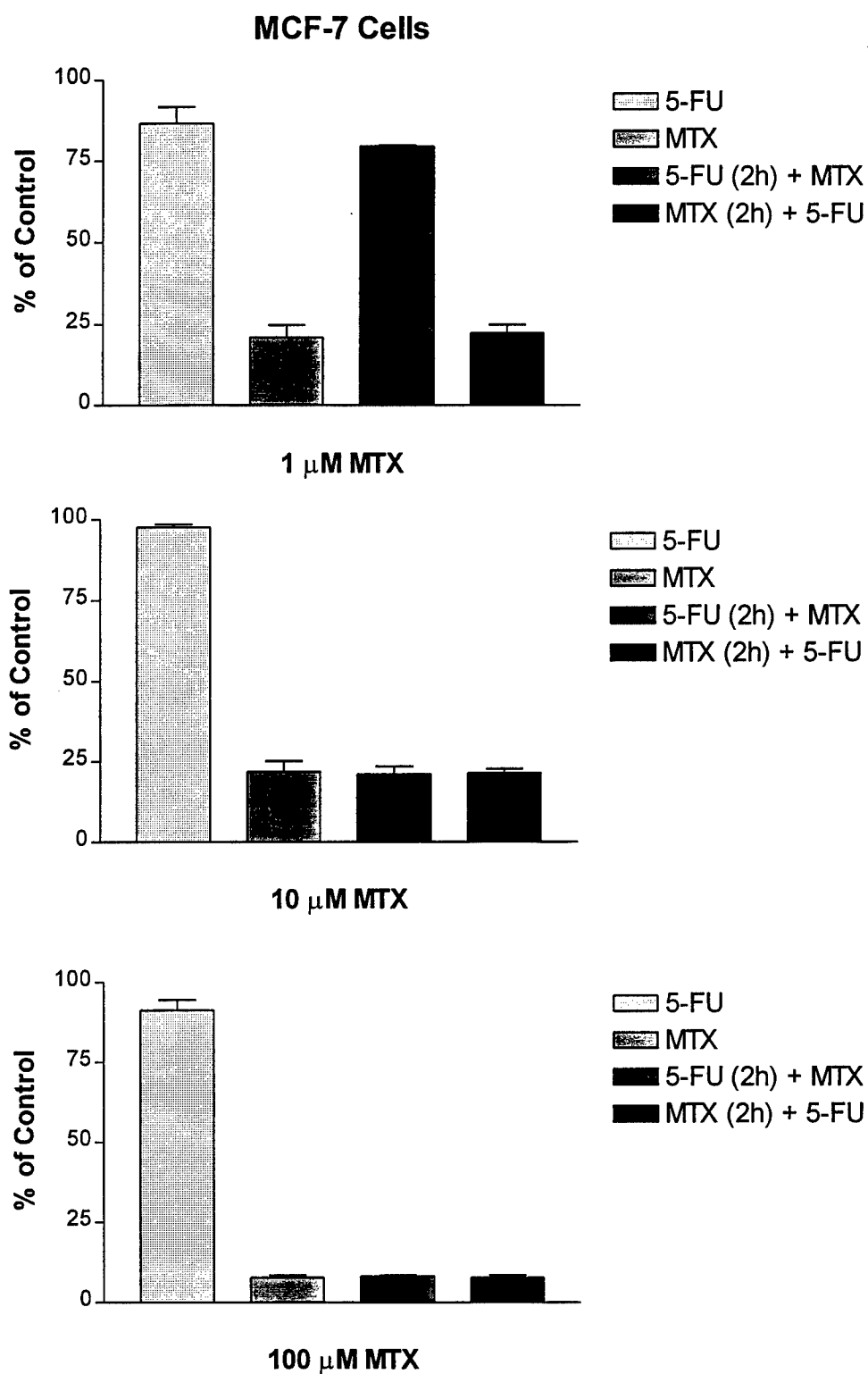


Figure 10. Dose response of MTX to a priming-and nontoxic 5-FU dose in MCF-7 cells. The doses of MTX are 1, 10, and 100 μ M; and the dose of 5-FU is 10 μ M. The bars represent the mean $\pm$ standard errors of three experiments.

MDA-MB-436 Cells

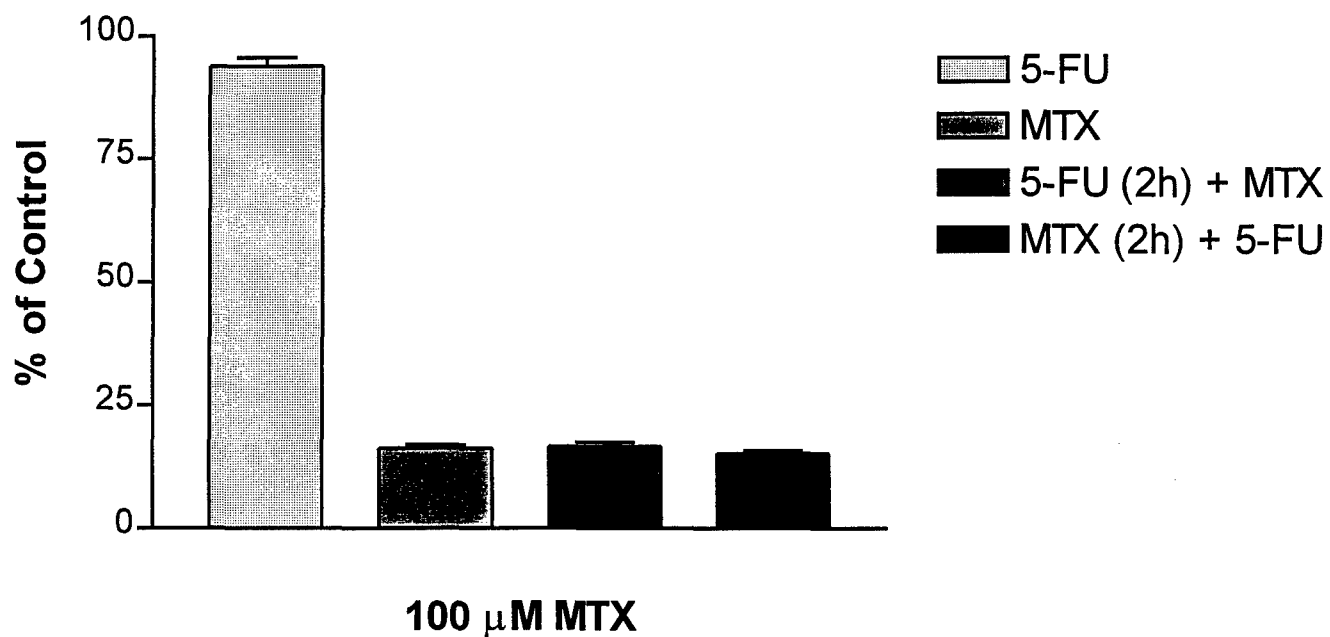
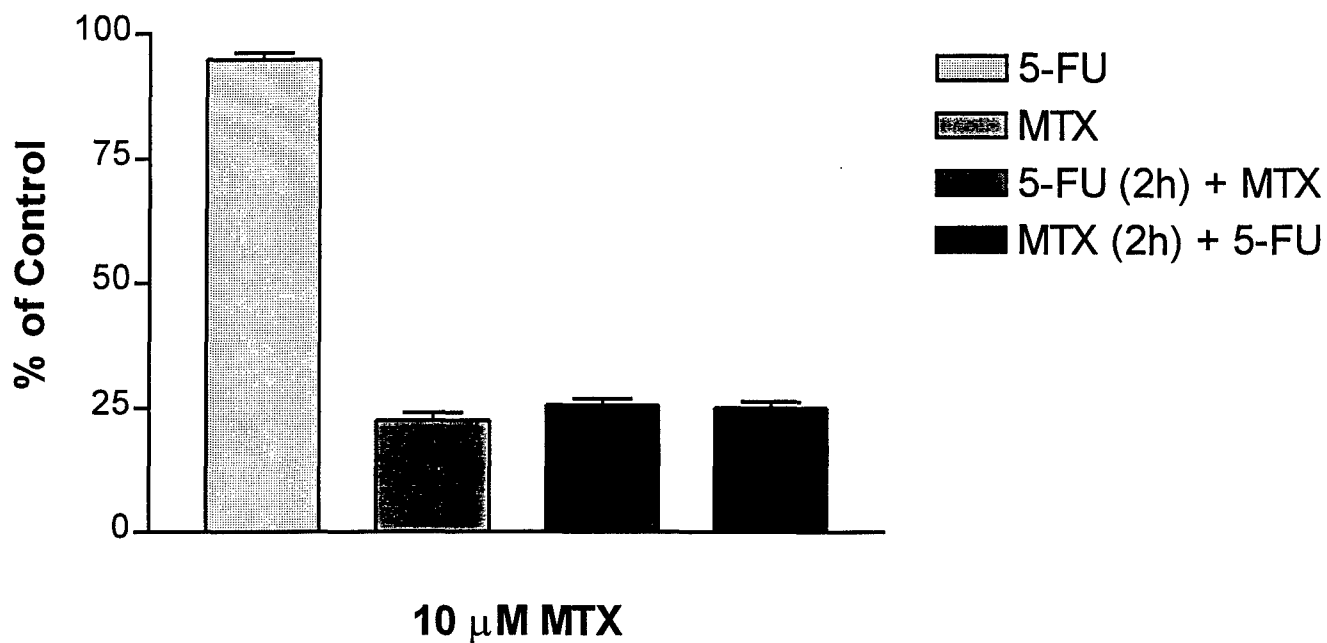


Figure 11. Dose response of MTX to a priming-and nontoxic 5-FU dose in MDA-MB-436 cells. The doses of MTX are 10 and 100 μM ; and the 5-FU dose is 10 μM . The bars represent the mean $\pm$ standard errors of three experiments.

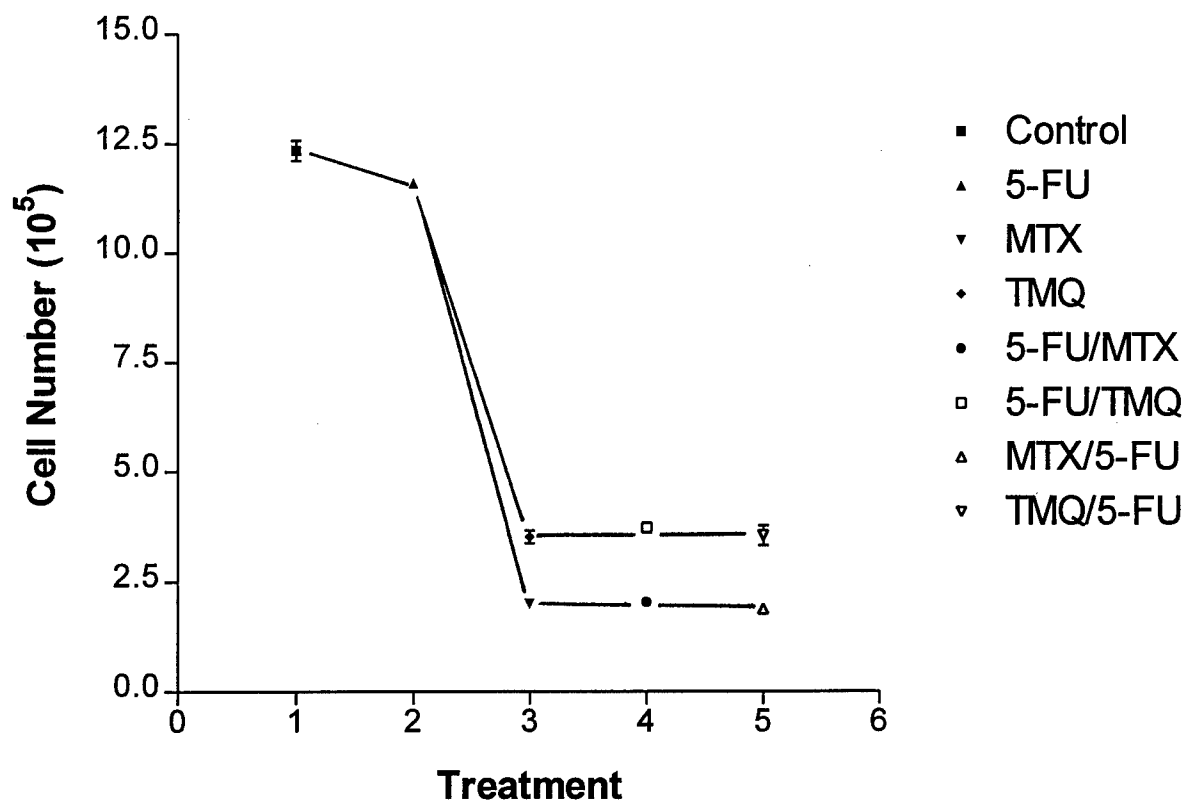


Figure 12. Comparison of optimal doses of TMQ and MTX alone and in combinations with 5-FU in MDA-MB-436 cells. The concentrations of TMQ and MTX are $100 \mu\text{M}$, respectively; and the concentration of 5-FU is $10 \mu\text{M}$. The symbols represent the mean $\pm$ standard errors of three different experiments.

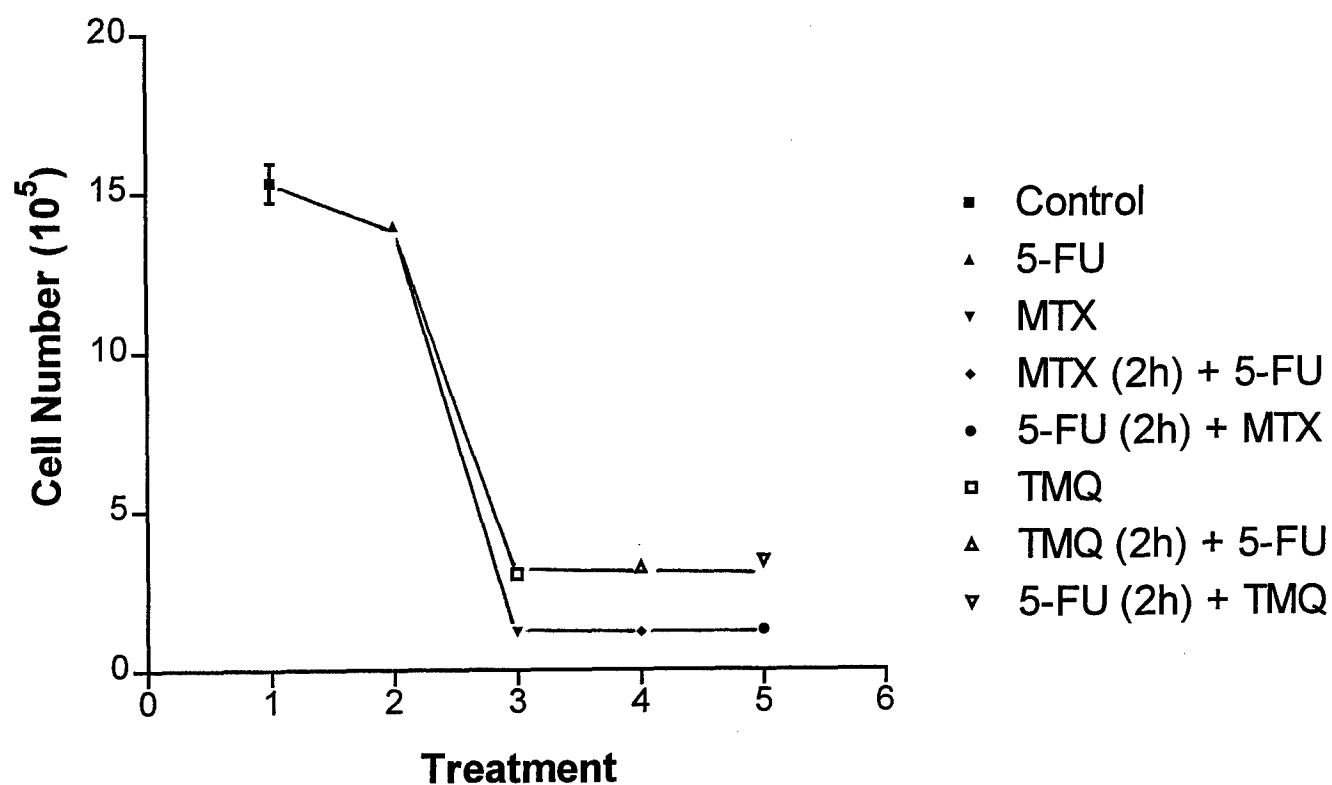


Figure 13. Comparison of optimal doses of TMQ and MTX alone and in combinations with 5-FU in MCF-7 cells. The concentrations of TMQ and MTX are $100 \mu\text{M}$, respectively; and the concentration of 5-FU is $10 \mu\text{M}$. The symbols represent the mean $\pm$ standard errors of two experiments.

Table 1. Interaction and Stability of Antifolate Binding to Human Dihydrofolate Reductase

Agent	Versions of CHARMM Software		
	Commercial Software	HP Software	Silicon Graphics Academic
	Electrostatic Energy (kcal/mol)		
TMQ	-87.5428	-105.0509	-82.1581
MTX	34.6394	14.1762	14.9173
MTXPG ₃	165.7223	25.6043	80.6234

TMQ (Trimetrexate); MTX (Methotrexate); MTXPG₃ (MTX-triglutamate)

Table 2. Doubling Time of MCF-7 Cells 48 h after Drug Treatment

Agents	Concentration (μ M)	Viable Cell Number	Time (h)
Control	0	1.66×10^6	6.51
5-FU	10	1.61×10^6	6.54
MTX	10	2.62×10^5	10.19
MTX/5-FU	10	2.50×10^5	10.32
5-FU/MTX	10	2.75×10^5	10.04
TMQ	10	8.13×10^5	7.57
TMQ/5-FU	10	8.12×10^5	7.57
5-FU/TMQ	10	7.99×10^5	7.61

5-FU (5-Fluorouracil); MTX (Methotrexate); MTX/5-FU (MTX given 2h prior to 5-FU); 5-FU/MTX (5-FU given 2h before MTX); TMQ (Trimetrexate); TMQ/5-FU (TMQ given 2h prior to 5-FU); 5-FU/TMQ (5-FU given 2h prior to TMQ)

Table 3. Doubling Time of MDA-MB-436 Cells 48 h after Drug Treatment

Agents	Concentration (μ M)	Viable Cell Number	Time (h)
Control	0	1.36×10^6	6.79
5-FU	10	1.15×10^6	7.02
MTX	10	2.50×10^5	10.32
MTX/5-FU	10	2.62×10^5	10.19
5-FU/MTX	10	2.87×10^5	9.90
TMQ	10	5.62×10^5	8.26
TMQ/5-FU	10	5.75×10^5	8.22
5-FU/TMQ	10	5.87×10^5	8.16

5-FU (5-Fluorouracil); MTX (Methotrexate); MTX/5-FU (MTX given 2h prior to 5-FU); 5-FU/MTX (5-FU given 2h before MTX); TMQ (Trimetrexate); TMQ/5-FU (TMQ given 2h prior to 5-FU); 5-FU/TMQ (5-FU given 2h prior to TMQ)