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TITLE: Selectivity of Very High Dose Methotrexate in Mcf-7 and Normal Cells Using a Priming and Non-Toxic 5-Fluorouracil Dose

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### Abstract (Maximum 200 Words)

The growth inhibitory effects of high-dose methotrexate (MTX) and trimetrexate (TMQ) are maintained in Mcf-7 breast cancer but is decreased in Hs824.T human bone marrow by a priming- and nontoxic 5-fluorouracil (5-FU) dose. Incubation of MCF-7 breast cells with 10 μM MTX, 10 μM TMQ alone or in combination with 10 μM 5-FU (MTX or TMQ 2h prior to 5-FU [MTX/5-FU or TMQ/5-FU] or 5-FU 2h prior to MTX or TMQ [5-FU/MTX or 5-FU/TMQ]) resulted in similar inhibitory patterns but dissimilar effects occurred in bone marrow cells. These studies suggest that a) MTX or TMQ and 5-FU combinations on the growth of breast cancer cells are independent of sequence of administration and are best related to MTX and TMQ rather than 5-FU (since 5-FU had no effect which differed from control and sequential MTX or TMQ plus 5-FU had no effect which differed from MTX or TMQ alone), b) a priming- and nontoxic dose of 5-FU will protect bone marrow against MTX and TMQ cytotoxicity while not affecting the maximum inhibitory effects of MTX or TMQ in breast cancer cells, and c) the results from the use of the nonclassical and nonpolyglutamyl antifolate TMQ suggest that polyglutamation is not a critical determinant of MTX cytotoxicity in bone marrow.
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Dennell Bowen 1/12/2000
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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 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 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 μg/ml of insulin. Stock cultures were maintained in 75-cm² flasks and incubated at 37°C in the presence and absence of CO₂, respectively, for bone marrow. 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 μM 5-FU and 1-10 μM 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_{final} - T_{initial} / 3.32 (log cell no. T_{final} - log cell no. T_{initial}).
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.

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 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. Figure 1 (Anticancer Res. 1999;19:985-988) illustrates the effects of 1) high-dose MTX and the independence of MTX and 5-FU sequence of administration on the growth of MCF-7 breast cancer cells 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 2) (Anticancer Res. 1999; 19:985-988). 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). The inset of Figure 1 (MCF-7 cells) (Anticancer Res. 1999; 19:985-988) 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.) In bone marrow (Figure 2), 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). The inset of Figure 2 (Anticancer Res. 1999; 19:985-988) shows that the growth rate of MTX and MTX (2h) + 5-FU are 29.58 \( \pm \) 2.99 % and 31.39 \( \pm \) 1.77 % of control rates, respectively; while 5-FU (2h) + MTX rate is 79.66 \( \pm \) 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 aminomimidazolecarboxamide 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) 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 the polyglutamated antifolate MTX was made on the growth of MCF-7 human breast cancer cells and Hs 824.T bone marrow cells. Figure 1 (Anticancer Res. 1999; 19:3837-3840) illustrates the differential inhibitory effects of TMQ in the absence and presence of 5-FU on MCF-7 cells. 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 (Anticancer Res. 1999; 19:985-988) with MTX, 5-FU (2h) + MTX, and MTX (2h) + 5-FU was similar to TMQ and TMQ and 5-FU combinations. In bone marrow (Figure 2) [Anticancer Res. 1999; 19:3837-3840], the inhibitory effects of TMQ and TMQ plus 5-FU were very similar, but not 5-FU plus TMQ. The percentage differences among TMQ and TMQ (2h) + 5-FU, TMQ and 5-FU (2h) + TMQ, and TMQ (2h) + 5-FU and 5-FU (2h) + TMQ on the growth rates of MCF-7 breast cancer cells, respectively, are 3.56 %, 2.35 %, and 1.68 %. In bone marrow cells (Figure 2; inset; Anticancer Research 1999; 19:3837-3840), the differences among TMQ and TMQ (2h) + 5-FU, TMQ and 5-FU (2h) + TMQ, TMQ (2h) + 5-FU and 5-FU (2h) + TMQ on growth rates, respectively are 5.76 %, 30.03 % (significant protection, i.e. 5-FU (2h) + TMQ is less inhibitory than TMQ), and 35.78 % (sequence dependent).

Similar 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.

CONCLUSIONS

High-dose MTX cytotoxicity is maintained in MCF-7 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 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.

**Key Research Accomplishments**

1. Methotrexate (MTX) and 5-fluorouracil (5-FU) combination on the growth of MCF-7 human breast cancer line is independent of sequence.

2. A priming-dose of 5-FU will protect bone marrow from MTX and TMQ cytotoxicity but not breast cancer cells.

3. A priming and nontoxic dose of 5-FU and high-dose MTX may have maximum antineoplastic activity while at the same time provide protection to the hematopoietic system.

4. Polyglutamation may be a critical determinant of MTX activity in breast cancer but not in bone marrow.

**Reportable Outcomes**

Publications:


REFERENCES


5-Fluorouracil Simultaneously Maintains Methotrexate Antineoplastic Activity in Human Breast Cancer and Protects against Methotrexate Cytotoxicity in Human Bone Marrow*

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Abstract. High-dose methotrexate (MTX) cytotoxicity is maintained in MCF-7 breast cancer cells but reduced in HS824.T human bone marrow by a priming and nontoxic 5-fluorouracil (5-FU) dose. When MCF-7 breast or HS824.T bone marrow cells are incubated with 10 μM 5-FU and 10μM MTX for 48 h, the growth rates of breast cancer cells were 97.59 ± 0.97 % and 21.81 ± 3.33 % of the control rate, respectively, and the growth rates of bone marrow cells were 90.61 ± 3.71 % and 29.58 ± 2.99 % of the control rate. The combinations of 5-FU 2 h prior to MTX or MTX 2 h prior to 5-FU followed by a 48-h incubation, respectively, gave growth rates of 20.96 ± 2.44 % and 19.86 ± 2.56 % of the control rate for MCF-7 cells. In bone marrow cells, the combinations of 5-FU 2 h prior to MTX or MTX 2 h prior to 5-FU followed by a 48-h incubation, respectively, gave growth rates of 79.66 ± 7.41 % (protection) and 31.39 ± 1.77 % of the control rate. Similar patterns to bone marrow emerge in platelets. These studies suggest that: a) MTX and 5-FU combination on the growth of human MCF-7 breast cancer cells is independent of sequence; and b) a priming-dose of 5-FU will protect bone marrow from MTX cytotoxicity but not breast cancer cells. Therefore, a priming and non-toxic dose of 5-FU and MTX may have maximum antineoplastic activity while at the same time provide protection to the hematopoietic system.

Recently, the National Institutes of Health (NIH) convened Consensus Development Conferences on Adjuvant Therapy of Breast Cancer reached several conclusions regarding the use of adjuvant therapy which included the administration of methotrexate (MTX) and 5-fluorouracil (5-FU). One conclusion is that maximum tolerated doses should be used to the degree possible since dose reduction can compromise efficacy. However, an increased dose often increases toxicity. Dose reductions of adjuvant chemotherapy containing MTX and 5-FU are modified for thrombocytopenia and leukopenia. Major problems in the use of MTX and 5-FU are a) the lack of selectivity between diseased and normal cells and b) equitoxicity of sequential MTX and 5-FU in tumor and hematopoietic stem cells.

The combination of MTX and 5-FU has been the subject of detailed investigations (1,2), but key differences in MTX and 5-FU pharmacokinetics in tumor and hematopoietic cells (3-6) suggested that the parameters for optimal effectiveness (5-FU given prior to MTX) would not necessarily be identical in cancer and normal cells. Previous studies from this laboratory have illustrated that fluoropyrimidine antagonism to MTX was reversed in a dose-dependent manner by MTX (7). In vivo studies from this laboratory demonstrated that high-dose MTX produced no lethality or gastrointestinal toxicity (8) in animals given a priming bolus dose of 5-FU. The in vitro and in vivo studies suggest that high-dose MTX in combination with 5-FU is independent of sequence in cancer cells, but sequence-dependent in hematopoietic cells. We now report preliminary results that a priming-and nontoxic dose of 5-FU provides a means whereby high-dose MTX may be administered with selectivity to human breast cancer, i.e., 5-FU protects human bone marrow from MTX toxicity, but has no protective effect on MTX cytotoxicity in human breast cancer cells.

Materials and Methods

MTX, 5-FU, Dulbecco’s modified Eagles medium (DMEM) containing 100 units/ml penicillin, 100 μg streptomycin and 10 μg/ml insulin, 10 % fetal calf serum, and 1.0 μM sodium pyruvate were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. An early-passage human MCF-7 breast cancer cell line and human bone marrow (HS 824.T) from American Type Culture Collection, Manassas, VA, U.S.A. were used for these studies. The cells were grown as a continuous monolayer in 75 cm² plastic tissue culture flasks in DMEM. For each of the experimental points, 1 × 10⁶ MCF-7 and 1 × 10⁷ HS 824.T cells, respectively, were plated onto 25 cm² plastic tissue culture flasks containing: MTX, 5-FU, 5-FU 2 hours (2h) prior to MTX exposure [5-
Figure 1. Sequence independence of methotrexate (MTX) and 5-fluorouracil (5-FU) administration on the proliferation of human MCF-7 breast cancer cells. MCF-7 cells were exposed to 10 μM 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. Cells were then incubated for 48h, harvested, and counted. The symbols represent the mean ± the standard error of three different experiments and the inset represents the percentage of control growth rate for each drug treatment.

Figure 2. The effect of methotrexate (MTX) and 5-fluorouracil (5-FU) alone and in combination on the proliferation of human bone marrow. Hs292T human bone marrow cells were incubated with 10 μM MTX or 10 μM 5-FU alone or in combination (5-FU 2h prior to MTX and MTX prior to 5-FU) for 48h. 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). The symbols represent the mean ± the standard error of three different experiments and the inset represents the percentage of the control growth rate for each drug treatment.
FU (2h + MTX), MTX (2h) + 5-FU, and no drugs (control). The doses of 5-FU and MTX, respectively, were 10μM. After 48h incubation in a humidified atmosphere of 5% CO₂, the monolayers were washed with phosphate buffered saline, and cells were separated from the monolayers with 2 ml of 0.25% trypsin-EDTA. The density of cells were determined by microscopic counting of trypan blue treated cells in a hemacytometer.

Male CF-1 mice weighing 18-26 g (age 4-6 weeks) were obtained from Charles River Breeding Laboratories, Wilmington, MA, U.S.A. Upon arrival, mice were randomized and quarantined for at least one week. Solutions of MTX (245 mg/kg) and 5-FU (25 mg/kg) were prepared immediately before use in 0.9% NaCl and given as a single i.p. injection either alone or in combination. 0.9% NaCl was administered as the control. Animals surviving 3-14 days after MTX and/or 5-FU treatment were anesthetized and blood was collected by cardiac puncture in tubes containing EDTA for platelet determination. Platelet determinations were done on a Model ZB 1 Coulter Counter.

Results and Discussion

Selective effects of a priming-and nontoxic dose of 5-FU on high-dose MTX cytotoxicity. Logarithmically growing MCF-7 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 48 h. Figures 1 and 2, respectively, illustrate the effects of a) high-dose MTX and the independence of MTX and 5-FU sequence of administration on the growth of MCF-7 breast cancer cells (Figure 1) and b) 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 2). 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. In bone marrow, 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). The inset of Figure 1 shows that MTX as a single agent gave a growth rate of 21.81 ± 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 ± 2.44 %, 19.86 ± 2.56 %, and 19.86 ± 2.56 % of the control rates. (A priming-and nontoxic dose of 5-FU has no effect on growth; its rate is 97.59 ± 0.97 % of the control.) In bone marrow, the inset of Figure 2 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). A similar pattern to bone marrow emerges in peripheral blood cells in vivo (Figure 3). Thrombocytopenia occurs with MTX and MTX (2h) + 5-FU, but 5-FU protection occurs in the 5-FU (2h) + MTX regimen.

These results suggest that the incidence and the severity 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). However, 5-FU administered prior to MTX modulated MTX toxicity in bone marrow and platelets. The selective cytotoxic effect of MTX in breast cancer may result from the formation of MTX-polyglutamates (MTXPGs) (4).
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 μM MTX (4) — a concentration 1/5th 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 directly by MTX (such as aminopterin, leucovorin, and pteroylglutamic acid).

Whereas, bone marrow and/or peripheral blood cells form little or no MTXPGs when exposed to MTX (5,10); 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.

By preventing the oxidation of 5,10-methylenetetrahydrofolate (meTHF), 5-FU can conserve reduced-folates by altering the meTHF/DHF (dihydrofolate) ratio. Studies by Matthews and Baugh (11) indicate that regulation of the meTHF/DHF ratio might be of physiological importance in regulating the partitioning of meTHF into the competing pathways of dTMP biosynthesis and the regeneration of methionine from homocysteine. An increase in the meTHF/DHF ratio by 5-FU will spare, a) meTHF for reduction to 5-methyl tetrahydrofolate (m-THF) and b) m-THF for methionine and purine biosynthesis. Further, a diminution in DHF levels by a priming-and nontoxic 5-FU dose will decrease DHF inhibition of m-THF reductase (11) and allows for the continuance of THF production and purine and methionine biosynthesis.

Modulation of MTX cytotoxicity by 5-FU will only be of clinical use if it 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 therapeutic index since toxicity to normal cells may occur in a similar synergistic manner. Based on similar inhibitory effects of 5-FU + MTX, MTX + 5-FU, and MTX in MCF-7 breast cancer cells, sequential 5-FU + MTX appears to provide a cytotoxic advantage against breast cancer cells since hematopoietic cells are protected by 5-FU + MTX.

References

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Selectivity in Human Breast Cancer and Human Bone Marrow Using Trimetrexate in Combination with 5-Fluorouracil*

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Abstract. The growth inhibitory effect of trimetrexate (TMQ) is maintained in MCF-7 breast cancer but is decreased in Hs 824.T human bone marrow cells by a priming- and non-toxic 5-fluorouracil (5-FU) dose. Incubation of MCF-7 breast cells with 10 μM TMQ alone or in combination with 10μM 5-FU (TMQ 2h prior to 5-FU [TMQ/5-FU] or 5-FU 2h prior to TMQ[5-FU/TMQ]) resulted in similar inhibitory effects but dissimilar effects occurred in Hs 824.T bone marrow. In breast cancer, the percentage differences among TMQ and TMQ/5-FU, TMQ and 5-FU/TMQ, and TMQ/5-FU and 5-FU/TMQ on growth rates, respectively, were 3.56 %, 2.35 %, and 1.68 %. The percentage differences on growth rates of TMQ and TMQ/5-FU, TMQ and 5-FU/TMQ, and TMQ/5-FU and 5-FU/TMQ in bone marrow, respectively, were 5.76 %, 30.03 % (significant protection by 5-FU, i.e. the inhibitory effect of 5-FU/TMQ ≤ TMQ), and 35.78 % (sequence dependent). The growth rates of breast cancer and bone marrow cells in the presence of 5-FU were 96.03 ± 1.17 % and 94.59 ± 1.15 %, respectively, of control rates. These studies suggest that (a)TMQ and 5-FU combinations on the growth of MCF-7 breast cancer cells are independent of sequence of administration and best related to TMQ and (b) a priming- and non-toxic 5-FU dose protects against TMQ toxicity in human bone marrow while not affecting the maximum inhibitory effect of TMQ in breast cancer.

Trimetrexate (TMQ) is a non-classical, lipophilic, non-polyglutamyl antifolate which enters cells via passive diffusion (1,2) and binds tightly to dihydrofolate reductase (DHFR) (3,4). As a result of these properties, TMQ is effective against methotrexate (MTX) resistant cells by virtue of impaired transport and an increase in DHFR (5). In the clinic, TMQ has produced encouraging results (6-8). TMQ in combination with 5-fluorouracil (5-FU) can result in synergistic, additive, or antagonistic effects on tumor growth inhibition and cytotoxicity based on sequence and timing of drug exposure (9, 10). While synergistic interactions lead to improved antineoplastic effects, these interactions also enhance drug toxicity. The myelosuppressive effect of TMQ and 5-FU limits their use (11, 12). Recent preclinical and clinical studies (13, 14) have demonstrated that a priming and non-toxic dose of 5-FU protected bone marrow from high-dose MTX. The preclinical studies (13) showed that while 5-FU protected human bone marrow, there was no protective effect on MTX cytotoxicity in human breast cancer cells. These studies (13) suggest a similar approach could be used for TMQ and 5-FU and provide a means for increasing the therapeutic utility of TMQ in the treatment of breast cancer. We now report on (a) the independence of TMQ and 5-FU combination on sequence of administration in a human breast cancer line and (b) the importance of sequential TMQ and 5-FU in protecting human bone marrow from TMQ cytotoxicity.

Materials and Methods

Trimetrexate glucuronate was obtained from U.S. Bioscience, Inc., West Conshohocken, PA, U.S.A. 5-FU and Dulbecco's modified Eagles medium (DMEM) containing 100 units/ml penicillin, 100 mg streptomycin and 10 μg/ml insulin, 10% fetal calf serum, and 1.0 μM sodium pyruvate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. An early passage of the MCF-7 breast cancer line and human bone marrow (Hs 824.T) from American Type Culture Collection, Manassas, VA, U.S.A. were used for these studies. The cells were grown as a continuous monolayer in 75 cm² plastic tissue culture flasks in DMEM. For each of the experimental points, 1 × 10⁶
MCF-7 and $1 \times 10^4$ Hs824.T cells, respectively, were plated onto 25 cm$^2$ plastic tissue culture flasks containing: TMQ, 5-FU, TMQ 2 hours prior to 5-FU exposure (TMQ/5-FU), 5-FU 2 hours prior to TMQ exposure (5-FU/TMQ), and no drugs (control). The doses of TMQ and 5-FU, respectively, were 10 μM. After a 48h incubation in a humidified atmosphere of 5% CO₂, the monolayers were washed with phosphate-buffered saline, and cells were separated from the monolayer with 2 ml of 0.25% trypsin-EDTA. The density of cells was determined by microscopic counting of trypan blue stained cells in a hemacytometer.

Results and Discussion

Selectivity of a priming-and non-toxic dose of 5-FU and TMQ. Figures 1 and 2, respectively, illustrate the effects of (a) TMQ alone and the independence of TMQ and 5-FU sequence of administration on the growth of MCF-7 breast cancer cells (Figure 1) and (b) TMQ alone, the dependence of TMQ and 5-FU sequence of administration on Hs 824.T bone marrow growth, and the protective effect of a priming-and nontoxic 5-FU dose on bone marrow (Figure 2). In breast cancer cells, similar inhibitory effects of TMQ, TMQ/5-FU, and 5-FU/ TMQ (at the arrow) exist on cell number. In bone marrow, similar inhibitory effects of TMQ, and TMQ/5-FU exist on cell number, but a dissimilar (protective) effect occurs with 5-FU/TMQ (at the arrow).

The inset of Figures 1 and 2 show the percentage of control growth rates for TMQ alone, TMQ/5-FU, 5-FU/TMQ, and 5-FU alone. A priming-and nontoxic dose of 5-FU has no effect on growth rates; its rate is 96.03 ± 1.17 % and 94.59 ± 1.15 % of control rates, respectively, in breast cells and bone marrow. The percentage differences among TMQ and TMQ/5-FU, TMQ and 5-FU/TMQ, and TMQ/5-FU and 5-FU/TMQ on the growth rates of MCF-7 breast cancer cells, respectively, are 3.56 %, 2.35 %, and 1.68 %. In bone marrow cells (Figure 2; inset), the differences among TMQ and TMQ/5-FU, TMQ and 5-FU/TMQ, and TMQ/5-FU and 5-FU/TMQ on growth rates, respectively, are 5.76 %, 30.03 % (significant protection, i.e. 5-FU/TMQ is less inhibitory than TMQ), and 35.78 % (sequence dependent).

These results suggest that the incidence and the severity of TMQ/5-FU and 5-FU/TMQ cytotoxicity in breast cancer cells are best related to TMQ rather than 5-FU (since 5-FU had no effect which differed from control and sequential TMQ and 5-FU had no effect which differed from TMQ alone). However, 5-FU given before TMQ modulated TMQ cytotoxicity in bone marrow. This study raises a new element in the potential for dihydrofolate (DHF) polyglutamates to influence the selective effects of a
priming-and nontoxic 5-FU dose and TMQ. The selective effect of TMQ in breast cancer may result from the formation of DHF polyglutamates and feedback inhibition of thymidylate synthase and aminomimidazolecarboxamide (AICAR) transformylase by DHF-polyglutamates (15,16). Whereas in bone marrow, little or no DHF-polyglutamates form when exposed to TMQ; and, therefore, feedback inhibition on thymidylate synthase and AICAR transformylase will be insignificant. Hence, sequence dependency in bone marrow may best be related to 5-FU conserving reduced-folates to protect against the direct effects of TMQ.

By preventing the oxidation of 5,10-methylenetetrahydrofolate (meTHF), 5-FU can conserve reduced-folates by changing the meTHF/DHF ratio. Studies by Matthews and Baugh (17) indicate that regulation of the meTHF/DHF ratio might be of physiological importance in regulating the partitioning of meTHF into the competing pathways of dTMP biosynthesis and the regeneration of methionine from homocysteine. An increase in the meTHF/DHF ratio by 5-FU will spare (a) meTHF for reduction to 5-methyltetrahydrofolate (m-THF) and (b) m-THF for methionine and purine biosynthesis. Further, a priming-and nontoxic 5-FU dose diminishes DHF levels and, therefore, decreases DHF inhibition of m-THF reductase (17) and allows for the production of THF.

In conclusion, a priming-and nontoxic 5-FU dose is effective in protecting bone marrow from TMQ toxicity but not breast cancer; and, therefore, 5-FU may provide a means for increasing the therapeutic utility of TMQ in breast cancer.

References


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