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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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### 4. TITLE AND SUBTITLE

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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### 13. ABSTRACT (Maximum 200 Words)

The purpose of this study is designed: 1) to improve the quality of life by exploiting differences in the biochemical pharmacology of methotrexate (MTX) in human MCF-7 and MDA-MB-436 breast cancer cells and human bone marrow (Hs 824.T) cells and 2) to provide a clear basis for intracellular protection of susceptible host cells from MTX toxicity when high-dose MTX is used in combination with a priming and nontoxic dose of 5-fluorouracil (5-FU). High-dose MTX cytotoxicity is maintained in MCF-7 and MDA-MB-436 breast cells but reduced in bone marrow cells by a priming and nontoxic 5-FU dose. The combinations of 5-FU 2h prior to MTX, MTX 2h prior to 5-FU, and MTX alone inhibited breast cancer cells to the same degree. In bone marrow cells, only MTX 2h prior to 5-FU and MTX alone affected growth similarly, but 5-FU 2h prior to MTX protected against MTX inhibitory effects. Similar patterns to bone marrow emerges in platelets. A comparison of the cell killing effects of MTX and the nonpolyglutamatable antifolate trimetrexate (TMX) alone and in combination with 5-FU was made in an attempt to indirectly explore the role of polyglutamylation in breast cancer and bone marrow cells. Significant protection occurred only in bone marrow when 5-FU was administered before MTX or TMX. It is unlikely that MTX-polyglutamylation plays a significant role in bone marrow. Hence, these studies suggest that a priming and nontoxic 5-FU dose in combination with high-dose MTX 1) sustains MTX cytotoxicity in breast cancer but 2) protects against MTX toxicity to bone marrow.
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Introduction

The objective of this proposal is designed: 1) to improve the quality of life by exploiting differences in the biochemical pharmacology of methotrexate (MTX) in human MCF-7 and MDA-MB-436 breast cancer cells and human bone marrow (HS 824.T) cells and 2) to provide one clear basis for intracellular protection of only susceptible host cells from MTX toxicity when high-dose MTX is used in combination with a priming-and non-toxic dose of 5-fluorouracil (5-FU). A significant aspect of MTX selectivity should be the preferential build up and retention of MTX-polyglutamyl forms in susceptible breast cancer cells as compared to host cells such as bone marrow. By conserving cellular reduced-folates with 5-FU, there should be sufficient intracellular levels of reduced-folates to protect normal cells against MTX, but insufficient reduced-folate levels to protect cancer cells against depletion of tetrahydrofolate/reduced-folates by both MTX and MTX-polyglutamates (MTXPGs). To further assess the role of a priming and non-toxic dose of 5-FU and polyglutamation in selectivity, the non-polyglutamyl antifolate trimetrexate (TMQ) was used in combination with 5-FU. A comparison of priming- nontoxic 5-FU plus MTX and priming-nontoxic 5-FU plus TMQ on the hematopoietic system, bone marrow, and MCF-7 and MDA-MB-436 breast cancer was used to evaluate selectivity and toxicity.

Body

The in vitro and in vivo studies suggest that high-dose MTX in combination with 5-FU is independent of sequence in MCF-7 breast cancer cells, but sequence-dependent in human bone marrow and mouse platelet cells. Hence, 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 (see appended publication in Anticancer Research 19: 985-988, 1999).

Trimetrexate (TMQ) is a non-classical, lipophilic, non-polyglutamyl antifolate which enters cells via passive diffusion and binds tightly to dihydrofolate reductase (DHFR). TMQ in combination with 5-FU can result in synergistic, additive, or antagonistic effects on tumor growth inhibition and cytotoxicity based on sequence and timing of drug exposure. The myelosuppressive effect of TMQ and 5-FU limits their use. A similar approach to MTX and 5-FU could be used for TMQ and 5-FU. Hence, in vitro studies with human MCF-7 breast cancer cells and human HS 824.T bone marrow cells 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 nontoxic 5-FU dose protects against TMQ toxicity in human bone marrow while not affecting the maximum inhibitory effect of TMQ in breast cancer (see appended publication in Anticancer Research 19: 3837-3840, 1999).

To further investigate the basis of differential effects of MTX in human breast cancer and bone marrow cells, (a) the effects of high concentrations of MTX in combination with a nontoxic
concentration of 5-FU were determined in the metastatic MDA-MB-436 human adenocarcinoma breast cancer cells and (b) a comparison of the nonclassical antifolate TMQ and MTX in combination with 5-FU was determined both in breast and bone marrow cells. Key differences between MTX and TMQ metabolism suggest that parameters for maximal inhibition by MTX and TMQ would be different in MDA-MB-436 breast cancer cells but similar in Hs 824.T bone marrow. TMQ is not polyglutamated, whereas MTX undergoes polyglutamation and interacts with enzymatic sites other than DHFR. The comparison of TMQ and MTX alone or in combination with 5-FU provide, indirectly, information on the role of MTX polyglutamates in selectivity and 5-FU protection in human breast cancer and bone marrow. The above studies also suggest that the maximal achievable MTX concentration appears to be 100 micro molar, where the threshold level for maximum inhibition in breast cancer is 10 micro molar. (See publication in Cancer Detection and Prevention 24 (5): 453-459, 2000; Galley attached.)

As a result of studying the importance of sequencing MTX with other agents to treat breast cancer and the emergence of tamoxifen (TAM) as a chemopreventive agent in breast cancer treatment, a new study is underway which shows the antagonistic and an synergistic interactions of MTX and TAM in human MCF-7 breast cancer cells (see appended publication Anticancer Research 20: 1415-1418, 2000).

Key Research Accomplishments

- MTX and 5-FU combination on the growth of human MCF-7 breast cancer cells is independent of sequence

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

- 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

- TMQ, the nonclassical antifolate, and 5-FU combinations on the growth of MCF-7 cells are independent of sequence of administration and best related to TMQ

- A priming and nontoxic 5-FU dose protects against TMQ toxicity in human bone marrow in culture while not affecting the maximum inhibitory effect of TMQ in breast cancer

- The maximal achievable MTX concentration in MDA-MB-436 breast cells is 100 micro molar

- A priming and nontoxic concentration of 5-FU provided protection in bone marrow cells where the MTX concentration is 10 times that required for leucovorin rescue
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- Similar effects of TMQ and MTX in bone marrow cells suggest that they interact with the same target site, and MTX polyglutamates play no significant role in bone marrow.

- A comparison of TMQ and MTX revealed that MTX cytotoxicity exceeds that of TMQ by more than 20% in MDA-MB-436 breast cancer cells.

Reportable Outcomes

Manuscripts:

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Conclusions

The assumption that there is a lack of efficacy of regimens in which MTX follows 5-FU may not be valid. The therapeutic effect and the quality of life may even enhanced when using regimens in which high-dose MTX follows a priming and nontoxic 5-FU dose. Biomodulation only occurs in bone marrow and not in breast cancer when 5-FU precedes a high-concentration of MTX. The difference in biomodulation in bone marrow and cancer cells may result from conservation of reduced folates and formation of MTX-polyglutamates. The lack of protection against MTX cytotoxicity in breast cancer cells may be the result of the levels of reduced-folates necessary to prevent the inhibitory actions of MTX and MTX-polyglutamates. Bone marrow forms little or no MTX-polyglutamates when exposed to MTX, and, therefore, certain folate-requiring enzymes are not inhibited due to the absence or very low levels of MTX-polyglutamates.

To address the problem that differential effects observed in this study may be attributed to MTX-polyglutamates, a comparison of the nonpolyglutamated antifolate TMQ and MTX revealed that a priming and nontoxic 5-FU dose protected significantly against significantly the cytotoxicity of
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TMQ and MTX. It is noteworthy that the effects of TMQ and MTX alone or in combination with 5-FU are similar in bone marrow. Computer analyses from this laboratory indicate that the TMQ complex with dihydrofolate reductase is equally stable to MTX but less stable than MTX-triglutamate.

Finally, these studies raise a new element in the potential of high-dose MTX in the treatment of breast cancer when preceded by nontoxic 5-FU. If it is true that MTX behaves as two different drugs in breast cancer and as a single agent in bone marrow, the following may be predicted from our data: 5-FU before MTX should be more efficacious than MTX before 5-FU.

References


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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 48h, 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 2h prior to MTX or MTX 2h prior to 5-FU followed by a 48h 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 2h prior to MTX or MTX 2h prior to 5-FU followed by a 48h 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) equitoxicitiy 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 vivo 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 x 10⁶ MCF-7 and 1 x 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-}

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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. 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. Ha824.T 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 10 μM. 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 C3H/101 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 % 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-polyglutamatates (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 1/5 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 directly by MTX (such as aminolevulinatecarboxamidase ribonucleotide and formylglycinamid ribonucleotide transformylases (9)). 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
8 Robbins TJ, Bowen D, Bui QQ and Tran MT: Modulation of high-dose methotrexate toxicity by a non-toxic level of 5-fluorouracil: Toxicology 41: 61-73, 1986.
11 Matthews RG and Baugh CM: Interactions of pig liver methylene-
etrahydrofolate reductase with methyleneetrahydrodoperoxy-

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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 10 μM 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 3.36 %, 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 myelo-suppressive 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 μg 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 $1 \times 10^4$ Hs 824.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% CO$_2$, 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 trypsin blue treated 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 $96.03 \pm 1.17\%$ and $94.59 \pm 1.15\%$ 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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Sequence-Dependent Antagonism between Tamoxifen and Methotrexate in Human Breast Cancer Cells*

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Abstract. High-dose methotrexate (MTX) cytotoxicity is decreased in MCF-7 breast cancer cells when the chemoendocrine agent tamoxifen (TAM) is given to cells 24 hours prior to MTX (early TAM). However, when breast cancer cells are exposed to TAM 24 hours after MTX (delayed TAM), MTX cytotoxicity is enhanced by TAM. The growth of cells exposed to 10 μM TAM and 10 μM MTX alone or in combination with early TAM plus MTX had the following order: TAM > TAM (early) + MTX > MTX. The percentages of control rates for TAM, MTX, and TAM (early) + MTX are 74.71 ± 1.36 %, 22.13 ± 2.76 %, and 38.17 ± 2.75 %, respectively. The inhibitory sequence from cells exposed to MTX + TAM (delayed TAM) and TAM alone is MTX + TAM (delayed TAM) > MTX > TAM; and the percentages of control rates were 16.87 87 % (MTX + TAM [delayed TAM]), 25.92 ± 2.14 % (MTX), and 54.08 ± 14.79 % (TAM). These studies suggest that: (a) the interactions between TAM and MTX are sequence-dependent; (b) TAM antagonizes the effect of MTX when TAM administration precedes MTX; and (c) TAM enhances the effect of MTX when TAM administration follows MTX.

Tamoxifen (TAM) an antiestrogen is widely used in the treatment of breast cancer and recently was evaluated in large clinical trials as a preventative agent for breast cancer (1-4). Approximately two-thirds of breast cancer patients have estrogen-receptor positive tumors, only half respond to TAM treatment. In those cases where preventative TAM treatment fails or there’s a recurrence of breast cancer after TAM therapy, the subsequent use of chemotherapy may be compromised.

It is important to examine whether TAM alone has ever been superior or equivalent to chemotherapy and whether TAM in addition to chemotherapy is of additive benefit. The Steering Committee on Clinical Practice Guidelines for the Care and Treatment of Breast Cancer (5) made the following recommendations: 1) Tamoxifen should not be recommended as the sole treatment for premenopausal women with node-positive tumors. 2) Acceptable treatment regimens are those using methotrexate, 5-fluorouracil and cyclophosphamide. 3) Routine use of TAM after chemotherapy in premenopausal women cannot yet be recommended. 4) Women with estrogen receptor-positive tumors may gain a small additional benefit from taking chemotherapy in addition to TAM. 5) No recommendations about high-dose chemotherapy can yet be made.

Methotrexate, a classical antifolate, is used in a variety of chemotherapeutic combinations in the treatment of solid tumors (6,7). The combination of methotrexate (MTX) and TAM represents a reasonable therapeutic strategy for the treatment of breast cancer, in which both drugs are active, and since their mechanisms of action and clinical toxicity are different. The effects of exposure to TAM and high-dose methotrexate (MTX) in various sequences were studied in human breast cancer cells with estrogen-positive receptors to determine an optimal sequence.

Materials and Methods

Tamoxifen citrate, methotrexate, and Dulbecco's modified Eagles medium (DMEM) containing 100 units/ml penicillin, 100 mg streptomycin and 10 μg/ml insulin, 1% 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 from American Type Culture Collection, Manassas, VA, U.S.A. was used for these studies. The cells were grown as a continuous monolayer in 75 cm² plastic tissue culture flasks in DMEM. For each experimental point, 1 x 10⁴ were plated into 25 cm² tissue plastic culture flasks containing: 1) TAM, MTX, and TAM 24 hours prior to MTX or 2) TAM, MTX, and MTX 24 hours prior to TAM. Controls consisted of no drugs. The doses

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Key Words: Tamoxifen, methotrexate, interaction, human breast cancer cells.
of TAM and MTX, respectively, were 10 μM. After a 24h incubation for treated and non-treated cells, in a humidified atmosphere of 5% CO₂, the monolayer was washed with phosphate-buffered saline, and cells were separated from the monolayer with 2 ml of 0.25% trypsin-EDTA. The density of the cells was determined by microscopic counting of trypan blue treated cells in a hemocytometer.

Results and Discussion

Interactions between TAM and MTX. Figure 1 illustrates the effects of TAM, MTX, and the dependence of TAM and MTX sequence of administration on the growth of MCF-7 breast cancer cells. The greatest inhibitory effect is that due to MTX. The inhibitory effect on the growth of cancer cells is MTX > early TAM plus MTX > TAM. The inset to Figure 1 shows the percentage of control rates for MTX, TAM, and TAM preceding MTX. TAM and MTX alone gave growth rates of 74.71 ± 1.36 % and 22.13 ± 2.76 % of the control rates, respectively. The combination of TAM and MTX gave a growth rate of 38.17 ± 2.75 % of the control rates (an antagonistic interaction). Figure 2 illustrates the effects of TAM, MTX, or MTX and delayed TAM on the growth of MCF-7 breast cancer cells. The greatest inhibitory effect is now due to MTX and delayed TAM. Hence, the inhibitory effect on the growth of cancer cells is MTX plus delayed TAM > MTX > TAM. The inset to Figure 2 indicates that the percentage of control rates is 16.87 ± 1.78 % (MTX and delayed TAM), 25.92 ± 2.14 % (MTX), and 54.08 ± 14.79 % (TAM). Thus, TAM enhances the effect of MTX when TAM is administered 24h after MTX, but antagonizes the MTX effect when it (TAM) is given 24h before MTX.

These results suggest that a strong sequence-dependent interaction exists between TAM and MTX in MCF-7 breast cancer cells. Sequential 24-hour exposure to TAM followed by MTX led to antagonism of the MTX effect since the inhibitory action of MTX alone was greater than TAM. The opposite sequence was associated with an enhanced cytotoxic effect (again the effect of MTX alone on growth rate was greater than TAM). A plausible explanation for the sequence-dependent effects of TAM and MTX stem from their actions on the cell-cycle. The timing of S phase agents such as MTX and an agent that affects cells in G1 such as TAM (8) is hypothesized to be important. When MCF-7 cells are arrested at G1 by TAM first, fewer cells will progress to the S phase which will result in a decrease in the effect of MTX (a S phase specific agent). An enhanced MTX effect may come from inhibition of the growth rate in S phase first by MTX and a subsequent inhibition in growth is an arrest of cells in G1 by TAM. Regulated changes in the activity of cell cycle components that act within G1 have been closely associated
with alterations in the proliferation rate of transformed mammary epithelial and normal cells (9). Cyclins are key components of the cell cycle progression machinery. They activate cyclin-dependent kinases (CDKs) and possibly target them to respective proteins within the cell. One of the key endogenous substrates of the G1 CDKs is the retinoblastoma protein (Rb). Its phosphorylation is an important step in the transition between the G1 and S phases of the cell cycle; when phosphorylated, Rb releases a transcription factor of the E2F family that drives cells into S phase (10). Hence, TAM may interfere in part with the transition between G1 and S phases and a release of an E2F transcription factor thereby decreasing the activity of MTX.

Finally, this study provides information for a rational alternative to empiric designs for combination chemotherapy involving potential antagonism or possible synergism.

References


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Implications for Improved High-Dose Methotrexate Therapeutic Effects in Cultured Human Breast Cancer and Bone Marrow Cells*

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ABSTRACT: The cytotoxicity of high-dose methotrexate (MTX), 10 and 100 μM, and 5-fluorouracil (5-FU) combinations are independent of sequence in human MDA-MB-436 breast carcinoma cells. The growth inhibitory effects of 10 and 100 μM MTX are 22.54 ± 1.56% and 16.20 ± 0.74%, respectively, of the control rate. When the MTX and 5-FU concentrations are 10 μM, antiproliferative effects of MTX hr before 5-FU (MTX/5-FU) and 5-FU 2 h before MTX (5-FU/MTX) are 25.17 ± 1.23% and 25.60 ± 1.28% of the control rate, respectively. The percentage of control rates for 5-FU alone is 94.89 ± 1.35%. The growth rates of MDA-MB-436 cells in 100 μM MTX and 10 μM 5-FU are 15.19 ± 0.62% (MTX/5-FU) and 16.53 ± 0.85% (5-FU/MTX) of the control rate. The growth of cancer cells in the presence of 5-FU alone is 93.82 ± 1.69% of the control rate.

KEY WORDS: high-dose methotrexate, 5-fluorouracil, human breast cancer, bone marrow.

INTRODUCTION

The combination of methotrexate (MTX) and 5-fluorouracil (5-FU) has been the subject of detailed investigations.1–3 Several conclusions regarding the use of chemotherapy, which included MTX and 5-FU, were reached by the National Institutes of Health (NIH) convened Consensus Development Conferences on Adjuvant Therapy of Breast Cancer. One conclusion is that maximum tolerated doses should be used to the degree possible, because dose reduction can compromise efficacy. Dose reductions in the use of MTX and 5-FU are modified for bone marrow suppression.

Phase I clinical studies3,4 based on preclinical studies from this laboratory5,6 showed that 5-FU protected against MTX toxicity when MTX concentrations were five to six times the reported lethal dose.7 Perhaps the efficacy of the MTX/5-FU combination could be improved if a priming and nontoxic dose of 5-FU is administered with high-dose MTX (5-FU/MTX) instead of MTX administration before 5-FU (MTX/5-FU). (Synergism results when MTX is given before 5-FU,8 but synergism occurs not only in cancer cells but also in normal cells.) Some recent studies9 from this laboratory suggested that 5-FU in combination with high-dose MTX is independent of sequence in human MCF-7 breast cancer cells, but sequence dependent and protective in bone marrow, that is, a priming-and nontoxic 5-FU dose significantly prevented MTX toxicity to bone marrow, while not altering the toxicity to breast cancer. To further investigate the basis for the differential effects of MTX in human breast cancer and bone marrow cells, (1) the effects of high concentrations of MTX in combination with a nontoxic concentration of 5-FU were...
determined in the metastatic MDA-MB-436 human adenocarcinoma breast cancer cells, and (2) a comparison of the nonclassical antifolate trimetrexate (TMQ) and MTX in combination with 5-FU was determined both in breast and bone marrow cells and reported in this paper.

Like MTX, the cellular target for TMQ is dihydrofolate reductase (DHFR) to which it binds tightly.\textsuperscript{10,11} However, key differences between MTX and TMQ metabolism suggest that parameters for maximal inhibition by MTX and TMQ would be different in breast cells but similar in bone marrow. TMQ is not polyglutamated, whereas MTX undergoes polyglutamation and interacts with enzymatic sites\textsuperscript{12,13} other than DHFR. The comparison of TMQ and MTX alone or in combination with 5-FU may provide information on the role of MTX polyglutamates in selectivity and 5-FU protection in human breast cancer and bone marrow.

MATERIALS AND METHODS

Chemicals, Cells, and Cell Culture Conditions

MTX, 5-FU, Leibovitz’s L-15 medium and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Sigma Chemical Company (St. Louis). Trimetrexate glucuronate was obtained from U.S. Bioscience, Inc. (West Conshohocken, PA). An early passage of the human MDA-MB-436 breast cancer line and human Hs 824.T bone marrow line from American Type Culture Collection (Manassas, VA) was used for these studies. Breast cancer cells were grown in Leibovitz’s L-15 medium containing 10 µg/ml insulin, 16 µg/ml glutathione, and 10% fetal bovine serum. Bone marrow cells were grown in DMEM containing 10% fetal bovine calf serum, 100 units/ml of penicillin, 100 mg of streptomycin, 10 µg/ml of insulin, and 1.0 µg/ml sodium pyruvate. The cells were grown as a continuous monolayer in 75 cm\textsuperscript{2} plastic tissue culture flasks in Leibovitz’s L-15 medium for MDA-MB-436 cells and DMEM for Hs 824.T bone marrow cells. For each experimental point, 1 x 10\textsuperscript{4} breast cancer cells and 1 x 10\textsuperscript{4} bone marrow cells, respectively, were plated onto 25 cm\textsuperscript{2} flasks containing 5-FU, MTX, TMQ, 5-FU 2 hr before MTX exposure (5-FU/MITX), MTX 2 hr before 5-FU exposure (MTX/5-FU), 5-FU 2 hr before TMQ exposure (5-FU/TMQ), TMQ 2 hr before 5-FU exposure (TMQ/5-FU), and no drugs (control). The concentrations of 5-FU and TMQ, respectively, were 10 µM, whereas the concentrations of MTX were 10 and 100 µM. After 48-hr incubations in a humidified atmosphere for breast cells or in the presence of 5% CO\textsubscript{2} for bone marrow, the monolayers were washed with phosphate buffered saline, and the cells were separated from the monolayers with 2 ml of 0.25% trypsin-EDTA. The density of the cells was determined by microscopic counting of trypan blue treated cells in a hemacytometer.

RESULTS

Effect of High Concentrations of MTX in Combination with a Priming and Nontoxic 5-FU Dose

MDA-MB-436 breast cancer and Hs 824.T bone marrow cells were exposed to MTX alone or in combination with 5-FU and incubated for 48 hr. The independence of sequence of administration of MTX and 5-FU for breast cancer cells is shown in Figure 1. When the MTX concentration was 10 µM, pretreatment of breast cancer cells with 5-FU for 2 hr followed by MTX or MTX for 2 hr followed by 5-FU inhibited cellular growth to a similar degree as MTX alone. The percentages of control rate for MTX, 5-FU/MITX, MTX/5-FU are, respectively, 22.54 ± 1.56%, 25.60 ± 1.28%, and 25.17 ± 1.23%. 5-FU alone is 94.89 ± 1.35% of the control rate. With 100 µM of MTX, the percentages of control rates for MTX is 16.20 ± 0.74%; 5-FU/MITX is 16.53 ± 0.85%; and MTX/5-FU is 15.19 ± 0.62%. 5-FU alone is 93.82 ± 1.69% of the control rate.

Because the inhibitory effects of 10 µM and 100 µM MTX alone or in combination with 5-FU were 10% and less, bone marrow cells were incubated with 10 µM MTX alone or in combination with 5-FU (Fig. 2). As in previous studies,\textsuperscript{9} in which bone marrow was evaluated, similar inhibitory effects exist between MTX alone and MTX/5-FU, but a dissimilar effect occurs with 5-FU/MITX. The combination of 5-FU and MTX is sequence dependent in bone marrow. 5-FU/MITX appears to have a protective effect against MTX inhibition.
Comparison of the Effects of High Concentrations of TMQ and MTX in Combination with a Priming and Nontoxic Concentration of 5-FU

To ascertain a better understanding of the interaction of MTX and 5-FU in breast cancer and bone marrow cells, the effects of the nonpolyglutamyl and lipid soluble antifolate TMQ were compared simultaneously to MTX. Figure 3 illustrates (1) the independence of sequence of administration of TMQ in combination with 5-FU and (2) a decrease in the inhibition of growth rate by TMQ, and TMQ plus 5-FU when compared to MTX treated breast cancer cells alone or in combination with 5-FU. The cell numbers for TMQ, TMQ/5-FU, and 5-FU/TMQ are similar; they were also similar for MTX, MTX/5-FU, and 5-FU/MTX. However, MTX affected cell growth more than TMQ. <AU# 4> In MDA-MB-436 cells exposed to TMQ, the percentages of control rates for TMQ, TMQ/5-FU, and 5-FU/TMQ are, respectively, 47.81 ± 2.62%, 50.58 ± 2.23%, and 51.88 ± 1.54%. For MTX, MTX/5-FU, and 5-FU/MTX, the percentages of some control rates are 22.69 ± 1.11%, 24.97 ± 0.89%, and 25.55 ± 0.91%, respectively. The mean cumulative effect of TMQ, TMQ/5-FU, and 5-FU/TMQ is 50.02 ± 1.24% of the control rate and 24.40 ± 0.63% for MTX, MTX/5-FU, and 5-FU/MTX.

Figure 4 shows that (1) TMQ and MTX in combination with 5-FU are sequence dependent in bone marrow and (2) the results with <AU #5> TMQ and MTX, TMQ/5-FU and MTX/5-FU, and 5-FU/TMQ and 5-FU/MTX are similar. The percentages of con-
Figure 2. Sequence-dependent effects of methotrexate (MTX) and 5-fluorouracil (5-FU) combinations on the proliferation of human bone marrow cells. HS824T human bone marrow cells were incubated with 10 μM MTX and 10 μM 5-FU alone or in combination (MTX 2h prior to 5-FU [MTX/5-FU] and 5-FU 2h prior to MTX [5-FU/MTX]) for 48 h. Similar inhibitory effects on cell proliferation exist for MTX and MTX/5-FU, but a dissimilar antiproliferative effect (significant protection) occurs from 5-FU/MTX. The bars represent the mean ± the standard error of three different experiments.

Figure 3. A comparison of methotrexate (MTX), 5-FU 2h prior to MTX (5-FU/MTX), MTX 2h prior to 5-FU (MTX/5-FU) to trimetrexate (TMQ), 5-FU 2h prior to TMQ (5-FU/TMQ), TMQ 2h prior to 5-FU (TMQ/5-FU) in MDA-MB-436 breast cancer cells. Cells were incubated with 10 μM MTX and 10 μM of the nonpolyglutamated-antifolate TMQ alone and in combination with 10 μM 5-FU for 48 h. The bars represent the mean ± the standard error of four different experiments.
control rates are (1) 30.12 ± 4.77% and 30.71 ± 2.39% for TMQ and MTX; (2) 26.86 ± 5.03% and 30.59 ± 1.49% for TMQ/5-FU and MTX/5-FU; and (3) 63.17 ± 1.23% and 77.93 ± 5.51% for 5-FU/TMQ and 5-FU/MTX. The inhibitory effects of TMQ, MTX, TMQ/5-FU, and MTX/5-FU are also similar; and protection of growth inhibition by TMQ and MTX occurs when 5-FU precedes administration of TMQ or MTX.

The similar effects of TMQ and MTX suggest that they interact with the same target site, and MTX polyglutamates (MTXPGs) play no significant role in bone marrow.

DISCUSSION

The assumption that there is a lack of efficacy of regimens in which MTX follows 5-FU may not be valid. The therapeutic effect and the quality of life may even be enhanced when using regimens in which high-dose MTX follows a priming and nontoxic 5-FU dose. A priming and nontoxic concentration of 5-FU provided protection in bone marrow cells where the MTX concentration (10 μM) was 10 times that required for leucovorin rescue. (However, no protection was provided in breast cancer cells.) In MDA-MB-436 breast cancer cells, the inhibitory effect when 5-FU preceded MTX was not significantly different from MTX alone or when MTX preceded 5-FU. Previous studies from this laboratory reported similar effects in MCF-7 breast cancer cells.9 Hence, a therapeutic gain should be realized by giving a priming and nontoxic 5-FU dose with high-dose MTX. Protection against MTX toxicity should occur in bone marrow, but MTX cytotoxicity should be enhanced in breast cancer.

This study suggests that the severity or cytotoxicity of MTX and 5-FU combinations in breast cancer cells is best related to a high-concentration of MTX. The maximal achievable MTX concentration appears to be 100 μM, where the threshold level for maximum inhibition in breast cancer is 10 μM. The difference in the inhibitory effects in 10 μM and 100 μM MTX treated cancer cells is less than 10%.

Biomodulation only occurs in bone marrow and not in breast cancer, when 5-FU precedes a high-concentration of MTX. The difference in biomodulation in bone marrow and cancer cells may result from conser-
vation of reduced folates and formation of MTXPGs.14
Previously, we reported that the basal rate of thymidy-
late synthesis affects the inhibitory action of MTX on
dNA, RNA, protein synthesis by controlling the avail-
ability of reduced-folates for purine, and amino acid
synthesis.15,16 The basis for these findings was
attributed to 5-FU conversion to 5-FdUMP, which
inhibited thymidylate synthase, thus preventing the
depletion of cellular THF <AU #6> cofactors upon
the subsequent addition of MTX. Consequently, 5-
methyltetrahydrofolate will be utilized for methionine
and 5-formyltetrahydrofolate for purine biosynthesis
and allow for the continuance of THF production.17
The lack of protection against MTX cytotoxicity in
breast cancer cells may be the result of the levels of
reduced-folates necessary to prevent the inhibitory
actions of MTX and MTXPGs. The formation of
MTXPGs from MTX in human breast cancer cells14
allows for the inhibition of dihydrofolate reductase,
thymidylate synthase, and formylglycinamide ribonu-
cleotide and aminomimidazolecarboxamide transform-
lases, which are not affected directly by MTX.15 Bone
marrow forms little or no MTXPGs when exposed to
MTX,18,19 and, therefore, certain folate-requiring
enzymes are not inhibited due to the absence or very
low levels of MTXPGs.

To address the problem that differential effects
observed in this study may be attributed to MTXPGs,
a comparison of the nonpolyglutamated antifolate
TMQ and MTX revealed that a priming and nontoxic
5-FU dose protected significantly against the cytotox-
icity of TMQ and MTX. It is noteworthy that the
effects of TMQ and MTX alone or in combination
with 5-FU are similar in bone marrow. Computer
analyses from this laboratory indicate that the TMQ
complex with DHFR is equally stable to MTX but
less stable than MTXiriglutamate (unpublished
results). Hence, the similar inhibitory effects of TMQ
and MTX alone or in combinations with 5-FU suggest
that MTXPGs are not critical determinants in cytotoxic-
ity to bone marrow. A comparison of TMQ and
MTX revealed that MTX cytotoxicity exceeds that of
TMQ by more than 20% in breast cancer cells.

Finally, these studies raise a new element in the
potential of high-dose MTX in the treatment of breast
cancer when preceded by nontoxic 5-FU. If it is true
that MTX behaves as two different drugs in breast
cancer and as a single agent in bone marrow, the fol-
lowing may be predicted from our in vitro data: 5-FU
before MTX should be more efficacious than MTX
before 5-FU.

REFERENCES

1. Advanced Colorectal Cancer Meta-Analysis Project. Meta-
    Analysis of randomized testing the biochemical modulation
    of fluorouracil by methotrexate in metastatic cancer. J Clin
2. Sobrero J. Does biomodulation of 5-fluorouracil improve
3. White RM. 5-Fluorouracil modulates the toxicity of high dose
4. White RM. A phase I study of methotrexate administration
5. Bowen D, Bailey B, Guerney L. Rate-limiting steps in the
    interactions of fluoropyrimidines and methotrexate. Eur J
6. Robbins T J. Bowen D, Bui QQ, Tran M. Modulation of
    high-dose methotrexate toxicity by a nontoxic level of 5-fluo-
7. Brownman GP, Archibald SD, Young JEM, et al. Prospective
    randomized trial of one-hour sequential versus simultaneous
    methotrexate plus 5-fluorouracil in advanced recurrent squa-
    pretreatment on 5-fluorouracil metabolism in L1210 cells. J
9. Bowen D, Johnson DH, Southard WM, et al. 5-Fluo-
    rouracil maintains methotrexate antineoplastic activity in
    human breast cancer and protects against methotrexate cyto-
    toxicity in human bone marrow. AR <AU #7> 1999;
    19:985-988.
    cology of the lipophilic antifolate trimetrexate. Adv Enzyme
11. Bertino JR, Sawicki WL, Morison BA, et al. 2,4-Diamino-5-
    methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazolidone
    (TMQ), a potent non-classical folate antagonist inhibitor. I.
    Effect on dihydrofolate reductase and growth of rodent
tumors in vitro and in vivo. Biochem Pharmacol 1979;
    70:907-912.
13. Allegra CJ, Chabner BA, Drake JC, et al. Enhanced inhibi-
    tion of thymidylate synthase by methotrexate polyglutamates.
    Synthesis, retention, and biological activity of methotrexate
    polyglutamates in cultured human breast cancer cells. J Clin
    Invest 1982; 70:351-360.
15. Bowen D, Fosselch L, Guerney LA. Fluoropyrimidine-
    induced antagonism to free and tightly bound methotrexate:


SELECTIVITY OF HIGH-DOSE METHOTREXATE IN HUMAN BREAST CANCER AND BONE MARROW CELLS

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ABSTRACT: High-doses of methotrexate (MTX), 10 and 100 μM, and 5-fluorouracil (5-FU) combinations are independent of sequence in human MDA-MB-436 breast carcinoma cells. The inhibitory effects of 10 and 100 μM MTX are 22.54 ± 1.56 % and 16.20 ± 0.74 %, respectively, of the control rate. When the MTX and 5-FU doses are 10 μM, MTX 2h prior to 5-FU (MTX/5-FU) and 5-FU 2h prior to MTX (5-FU/MTX) antiproliferative effects are 25.17 ± 1.23 % and 25.60 ± 1.28 % of the control rate, respectively. The percentage of control rates is for 5-FU alone is 94.89 ± 1.35 %. The growth rates of MDA-MB-436 cells in 100 μM MTX and 10 μM 5-FU are 15.19 ± 0.62 % (MTX/5-FU) and 16.53 ± 0.85 % (5-FU/MTX) of control rate. The growth of cancer cells in the presence of 5-FU alone is 93.82 ± 1.69 % of the control rate. In Hs824.T human bone marrow cells, 10 μM MTX alone or in combinations with 10 μM 5-FU gave growth rates of a) 32.68 ± 1.94 % for MTX, b) 29.19 ± 0.69 % for MTX/5-FU, and c) 77.24 ± 7.34 % for 5-FU/MTX (a protective effect) of the control rate. 5-FU alone is 90.42 ± 3.57 % of the control rate for bone marrow. Similar patterns to bone marrow emerges in platelets. A comparison of the cell killing effects of MTX and the nonpolyglutamatable antifolate trimetrexate (TMQ) alone and in combination with 5-FU was made in an attempt to indirectly explore the role of polyglutamylation in breast cancer and bone marrow cells. The comparisons were made in equitoxic concentrations of (10 μM) of MTX and TMQ and the time of exposure was the same. The degree of interaction of TMQ, TMQ/5-FU, and 5-FU/TMQ in breast cancer cells was identical, but significantly less than MTX, MTX/5-FU, and 5-FU/MTX. The interaction between TMQ and MTX, TMQ/5-FU and MTX/5-FU, 5-FU/TMQ and 5-FU/MTX was quantitatively similar in bone marrow. As a result of the same interactions of 5-FU/MTX and 5-FU/TMQ in bone marrow it is unlikely that polyglutamylation plays a significant role. However, the greater inhibitory effect of MTX of MTX or MTX and 5-FU combinations when compared to TMQ or TMQ and 5-FU suggests that polyglutamylation may be important for MTX cytotoxicity in breast cancer. Hence, these studies suggest that a priming-and nontoxic 5-FU dose in combination with high-dose MTX a) enhances MTX cytotoxicity in breast cancer and b) simultaneously protects against MTX toxicity to bone marrow and platelets.

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POSITIONS

Howard University: Washington, D.C., Professor of Pharmacology, August, 1990 - Present.


Howard University: Washington, D.C., Assistant Professor of Pharmacology and Oncology, August, 1979 - July, 1983.

The Genesee Hospital (Affiliate of the University of Rochester): Rochester, N.Y., Chief, Cancer Research

POSITIONS (cont'd)


RESEARCH EXPERIENCE

Membrane Transport: I studied (a) the mechanism of the membrane transport and intracellular binding of actinomycin D in Ehrlich ascites tumor cells and the relationship between binding and inhibition of RNA synthesis, (b) the regulation of 5-fluorodeoxyuridine transport and metabolism in Ehrlich cells, and (c) interactions between fluoropyrimidines and methotrexate in the inhibition of DNA, RNA, and protein synthesis.

Pharmacodynamics: Further, I have investigated the interaction of methotrexate and 5-fluorouracil as well as the nonclassical antifolate trimetrexate and 5-fluorouracil in human breast cancer and human bone marrow.

Pharmacokinetics: I studied the pharmacokinetics of the immunomodulator swainsonine.

Phase I Study: Designed a toxicity study to evaluate the interaction of non-toxic 5-fluorouracil and high-dose methotrexate.

Peptide Synthesis: I worked on the synthesis of polyglutamates by solid phase peptide techniques from the triglutamic acid derivatives of pteroyl glutamates.

Classical Organic Synthesis: During my graduate studies, I worked on the development of techniques for the synthesis of phenanthrile and phenanthryne as a new approach to the synthesis of phenanthrocyclopropenes.

Environmental Toxicology: I worked with the Toxicology Branch, Office of Pesticide Programs (OPP), U.S. Environmental Protection Agency (EPA), in connection with OPP's registration of pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). In order for a pesticide to be registered for use, toxicological studies must be presented by the manufacturers in support of a petition to market their products. I reviewed toxicological studies submitted by industry to EPA as part of a validation effort being carried out by the Toxicology Branch.

MEMBERSHIP - SCIENTIFIC, HONORARY AND PROFESSIONAL SOCIETIES

American Society for Pharmacology and Experimental Therapeutics
American Association for Cancer Research
American Association for the Advancement of Science
Sigma Xi Research Society
Southeastern Cancer Research Association
National Institute of Science
New York Academy of Science
HONORS AND AWARDS

Recipient - Young Investigator's Award, National Cancer Institute, 1978-1980
NIH Predoctoral Fellow, 1970 - 1974
Moses Wharton Young Research Award, 1999
A Distinguished Faculty Author, Howard University, 2000

ACTIVITIES

Pharmacology

1. Director of Graduate Studies (1980-1995)
2. Member of:
   a. Executive Committee (1982-84; 1988-89; 1992-Present)
   b. Teaching Evaluation Committee (1986-Present)
   c. Admissions Committee (1981-Present)
   d. Graduate Dissertation Committee (1980-Present)
   e. Promotions Committee (1988-Present)
3. Mentor of Ph.D. graduate Students:
   a. Mofolorunso A. Enigbokan, Ph.D. (Post-doc. M.D. Anderson Cancer Center)
   b. Terry Robbins, Ph.D. (Post-doc. St. Jude Children's Research Hospital)
   c. Robin Willis
   d. Aida Guemie, M.D., Ph.D. (Post-doc NIH)

College of Medicine

1. Member of:
   a. Appointments, Promotion and Tenure Committee (1994-Present)
   b. Chairman, Search Committee for Cancer Center Director (1991-92)
   c. Admissions and Applicants Interview Committee (1981-90)
   d. Committee on Committees (1981-92)
   e. Committee on Graduate Education (1981-89)
   f. Task Force on Core Laboratory (1981-84)
   g. Sabbatical Leave Review Committee (1980-81)
   h. Team Evaluating Medical Student's Research (1980)
   i. Institutional Self-Study Task Force, Liaison Committee on Medical Education (Co-Chairman, Subcommittee on Graduate Basic Science Education 1983)
   j. Search Committee for the Chairman of Physiology (1984)
2. Chief Proctor, National Board Medical Examination (1981-90)
3. Sponsor, Summer Research Program for Medical Students (1985)

University

1. President, Sigma Xi, 1993
3. Member, Research Improvement at Minority Institutions Ad Hoc Committee, 1983
4. Howard University Research Development Study Section, 1984
5. Member, Committee to review Proposals for Graduate Program, 1983
6. Member, University-Wide Recruitment Team
7. Appointments and Promotions Committee (Graduate School), 1986-87
8. Advisory Committee, Patricia Robert Harris Fellowship Program, 1987
9. Evaluation Committee, Graduate School, Department of Communications Arts and Sciences, 1987
10. Evaluation Committee, Pharmacal Sciences, 1985
11. Member, Internal Quality Control Committee for Project One (Nutritional Status and the Outcome of Pregnancy), 1986-87
12. Evaluation Committee, Graduate School, Chemistry Department, 1990
14. Steering Committee of the Council of the University Senate, 1990-91
15. Council of the University Senate, 1991-96

National

1. Member, American Society for Pharmacology and Experimental Therapeutics Subcommittee on Affirmative Action, 1988-91.
2. Representative to American Society for Pharmacology and Experimental Therapeutics Ad Hoc Subcommittee on Affirmative Action
3. Member, Study Section and Special Review Committee (National Cancer Institute), 1983
4. Reviewer, Biochemical Pharmacology and Cancer Treatment Reports (1999)
5. Member, Steering Committee (National Meeting, Washington, D.C.) National Institute of Science, 1982
7. RFD/ADI Committee, EPA, Office of Pesticides and Toxic Substances, 1986-88
8. Reviewer (Grants), American Institute of Biological Sciences, 1986-1991
9. Reviewer Cancer Letters, 1999
10. DOD Breast Cancer Panel (Reviewer of Grants), 2000

PUBLICATIONS


Publications (cont’d)


PUBLICATIONS (cont’d)


ABSTRACTS


ABSTRACTS CONTINUED


REVIEWS


GRANT SUPPORT

"Polyglutamate and Antifolate Toxicity," from the National Cancer Institute, No. CA-24192, July 1, 1978 - June 30, 1979, $25,000.00, Principal Investigator.

"Methotrexate Polyglutamates and Antifol-Fluoropyrimidine Toxicity," from the National Cancer Institute, No. CA-28108, July 1, 1979 - August 31, 1980, $25,000.00, Principal Investigator.

"Cellular Pharmacokinetics of Methotrexate and the Fluoropyrimidines," from the National Cancer Institute, No. CA-28261, July 1, 1980 - June 30, 1983, $80,702.00, Principal Investigator.

"Pharmacokinetics of Methotrexate," from the National Cancer Institute, Division of Research Resources, June 1, 1983 - May 31, 1986, $70,296.00, Principal Investigator.

"Methotrexate Polyglutamates: A Determinant of Methotrexate Efficacy and Toxicity," from the Howard University Faculty Research Support Program HA-55, July 1, 1985 - June 30, 1986, $18,146.00, Principal Investigator.

"Evaluation of an Immunomodulator's Metabolism in a Microbial System," from the Howard University Faculty Research Support Program, I 88-14, May 19, 1988 -September 30, 1988, $30,387.00, Principal Investigator.

"Cellular Pharmacokinetics of the Antifols and Fluoropyrimidines," from the Procter and Gamble Company, July 1, 1984 - June 30, 1990, $32,500.00, Principal Investigator.

"Computational Research in the Biomedical Sciences Core," from Howard University Graduate School of Arts and Sciences (Collaborative Core Unit Program), Feb. 1, 1990 - June 30, 1992, $90,102.00, Co-Investigator.

"A Basis for Methotrexate Toxicity and Selectivity," from the National Institute of General Medical Sciences (MARC Predoctoral Fellowship), No. 1F31GM14103-01, March 22, 1991 - June 30, 1995, $93,934.00, Sponsor.


“Selectivity of Very High Dose Methotrexate in MCF-7 and Normal Cells Using a Priming and Non-Toxic 5-Fluorouracil Dose,” from the U.S. Army Research and Material Command, No. DAMD 17-96-1-6291, September 16, 1996 - September 15, 2000, $430,141.00 (Total Cost), Principal Investigator.

“A Primary and Nontoxic 5-Fluorouracil Dose Modulates High-Dose Methotrexate and Methotrexate-Polyglutamate Toxicity in MCF-7 and MDA-MB-436 Breast Cancer Cells, and Myeloid and Erythroid Hematopoietic Progenitors In Vitro,” from Latham Foundation, March 16, 1998 - March 16, 1999, $9,000.00, Principal Investigator.
SELECTIVITY OF HIGH-DOSE METHOTREXATE IN HUMAN BREAST CANCER AND BONE MARROW CELLS

D. Bowen, W.M. Southerland, M. Hawkins, Jr., D. E. Hughes, and D. H. Johnson

Departments of Pharmacology, Biochemistry and Molecular Biology, Microbiology, and Drug Discovery Unit, Howard University College of Medicine, Washington, D.C.20059
dbowen@facHoward.edu

ABSTRACT: High-doses of methotrexate (MTX), 10 and 100 μM, and 5-fluorouracil (5-FU) combinations are independent of sequence in human MDA-MB-436 breast carcinoma cells. The inhibitory effects of 10 and 100 μM MTX are 22.54 ± 1.56 % and 16.20 ± 0.74 %, respectively, of the control rate. When the MTX and 5-FU doses are 10 μM, MTX 2h prior to 5-FU (MTX/5-FU) and 5-FU 2h prior to MTX (5-FU/MTX) antiproliferative effects are 25.17 ± 1.23 % and 25.60 ± 1.28 % of the control rate, respectively. The percentage of control rates is for 5-FU alone is 94.89 ± 1.35 %. The growth rates of MDA-MB-436 cells in 100 μM MTX and 10 μM 5-FU are 15.19 ± 0.62 % (MTX/5-FU) and 16.53 ± 0.85 % (5-FU/MTX) of control rate. The growth of cancer cells in the presence of 5-FU alone is 93.82 ± 1.69 % of the control rate. In HS824.T human bone marrow cells, 10 μM MTX alone or in combinations with 10 μM 5-FU gave growth rates of a) 32.68 ± 1.94 % for MTX, b) 29.19 ± 0.69 % for MTX/5-FU, and c) 77.24 ± 7.34 % for 5-FU/MTX (a protective effect) of the control rate. 5-FU alone is 90.42 ± 3.57 % of the control rate for bone marrow. Similar patterns to bone marrow emerges in platelets. A comparison of the cell killing effects of MTX and the nonpolyglutamable antifolate trimetrexate (TMQ) alone and in combination with 5-FU was made in an attempt to indirectly explore the role of polyglutamylation in breast cancer and bone marrow cells. The comparisons were made in equitoxic concentrations of (10 μM) of MTX and TMQ and the time of exposure was the same. The degree of interaction of TMQ, TMQ/5-FU, and 5-FU/TMQ in breast cancer cells was identical, but significantly less than MTX, MTX/5-FU, and 5-FU/MTX. The interaction between TMQ and MTX, TMQ/5-FU and MTX/5-FU, 5-FU/TMQ and 5-FU/MTX was quantitatively similar in bone marrow. As a result of the same interactions of 5-FU/MTX and 5-FU/TMQ in bone marrow it is unlikely that polyglutamylation plays a significant role. However, the greater inhibitory effect of MTX of MTX or MTX and 5-FU combinations when compared to TMQ or TMQ and 5-FU suggests that polyglutamylation may be important for MTX cytotoxicity in breast cancer. Hence, these studies suggest that a priming-and nontoxic 5-FU dose in combination with high-dose MTX a) enhances MTX cytotoxicity in breast cancer and b) simultaneously protects against MTX toxicity to bone marrow and platelets.

The U.S. Army Medical Research and Material Command under DAMD17-96-1-6291 supported this work.