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SEQUENTIAL COMBINATION CHEMOTHERAPY IN HUMAN BREAST CANCER: A BASIS FOR INCREASED ANTINEOPLASTIC ACTIVITY AND BONE MARROW PROTECTION

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Abstract – These studies were designed to develop procedures that would capitalize on the growth inhibitory effects of tamoxifen (Tam) and methotrexate (MTX) in breast cancer, while protecting bone marrow with a priming dose of 5-fluorouracil (5-FU). High-dose MTX (10µM) cytotoxicity is maintained in MCF-7 breast cancer cells but reduced in human bone marrow by a priming and nontoxic dose of 5-FU (10µM). MTX cytotoxicity is decreased in MCF-7 breast cancer cells when the selective estrogen receptor modulator (SERM) Tam (10µM) is administered 24 hours prior to 5-FU (10µM) followed two hours later by MTX (early Tam) resulting in a growth rate of 57.42 ± 4.38% of the control rate. However, when breast cancer cells are exposed to Tam 24 hours after 5-FU + MTX (late Tam), the interaction between MTX and Tam is not antagonistic, the percentage of the control is 29.47 ± 4.54%. Bone marrow exposure to these drug combinations exhibits a protective effect to the MTX cytotoxicity, with the early Tam combination yielding 59.45 ± 16.38% of the control for MTX alone. These studies suggest that a) Tam in combination with a priming dose of 5-FU protects bone marrow from MTX cytotoxicity, b) the interactions between Tam and MTX are sequence-dependent, c) Tam decreases the effect of MTX when Tam administration precedes MTX.

Key words: tamoxifen, priming dose of 5-fluorouracil, methotrexate

This publication is dedicated to the late Dr. Donnell Bowen, a consummate teacher who dedicated his life to cancer research. Dr. Donnell Bowen was loved and respected by many scientists. His untimely passing will be a loss to all. His dedication to cancer research is well known. We therefore dedicate this paper to his work.

INTRODUCTION

The nonsteriodal antiestrogen tamoxifen (Tam) significantly reduces the incidence of breast cancer in those individuals with a family history of such a disease. Tam is also the front-line endocrine treatment for breast cancer, but disease recurrence is common. In those cases where prophylactic use of Tam fails or there is recurrence of breast cancer, the subsequent administration and effectiveness of standard doses of chemotherapy may be compromised. Preliminary studies have demonstrated that Tam administration before the chemotherapeutic agent MTX antagonizes the effect of MTX on the growth of MCF-7 breast cancer cells[3]. Conversely, these studies show that the effect of MTX followed by Tam is greater that MTX or Tam alone. The greatest inhibitory effect on the growth of MCF-7 cells occurred when high-dose MTX administration preceded Tam by 24 h. Therefore, the timing of S-phase agents such as MTX and an agent that affects cells in the G_1 phase such as Tam is important.

Many studies have been conducted comparing the effects of Tam and adjuvant chemotherapy utilizing cyclophosphamide, MTX, 5-FU (CMF). The National Surgical Adjuvant Breast and Bowel Project has conducted studies over the past twenty years that evaluated the benefits of adjuvant therapy involving Tam (T) alone, the B-14 study, as well as CMFT and MFT combinations, the B-20 study, in estrogen receptor positive tumors. The B-20 study which involved more that 2,300 women compared the effects of CMFT and MFT to Tam alone. This study revealed that chemotherapy plus Tam significantly improved

disease-free survival compared to Tam alone [5]. Although this study proved that chemotherapy combined with Tam was more advantageous than Tam treatment alone, it does not evaluate the importance of the sequence of administration of these agents.

Combination chemotherapy is universally employed in the treatment of breast cancer. One of the major concerns in utilizing combination chemotherapy is increased side effects. It is well known that the major dose limiting effects of adjuvant chemotherapy include toxicities to rapidly proliferating cells, which include hair, intestinal, and hematopoietic cells [14]. Toxicities to the hematopoietic system include anemia, leukopenia, and thromobocytopenia. New treatment regimens are being developed for patients with advanced breast cancer utilizing high doses of multiple drugs, followed by bone marrow transplantation [1, 2, 17, 18]. Methods for effectively treating breast cancer with combination chemotherapy while protecting bone marrow from toxicity are outlined in this study.

MATERIALS AND METHODS

To determine the sequential effects of Tam on MCF-7 and Hs-5 cells (ATCC, Manassas, Va.) in combination with MTX (Sigma, St. Louis, MO) and 5-FU (Sigma, St. Louis, MO), MCF-7 cells were grown in monolayer to 70-80% confluency in 75cm^2 flasks in RPMI media in the presence and absence of phenol red, supplemented with 10% fetal bovine serum, in a water-jacketed incubator at 37ºC in a 5% CO_2 atmosphere. The media was protected from microbial contamination with 0.1 U/ml of penicillin G sodium and 0.1 µg/ml of streptomycin. Cells were plated in six well plates and exposed to no drug in the first control well, 10.0µM of MTX in the second well, 10.0 µM of 5-FU in the third well, 10.0µM Tam in the fourth well, the fifth well contained 10.0µM of 5-FU followed two hours later by 10.0µM of MTX, well number six contained 10.0µM of 5-FU followed two hours later by 10.0µM of MTX followed twenty-four hours later by 10.0µM Tam, and well number seven was treated with 10.0µM of Tam followed twenty-four hours later by 5-FU followed two hours later by MTX the plate was incubated for 48 hours at 37ºC. Cell viability was determined utilizing trypan blue dye exclusion.

In order to determine the sequence dependent effects of MTX in combination with Tam and a priming dose of 5-fluorouracil on the phosphorylation of the retinoblastoma protein and the entrance of cells into the S-phase of the cell cycle, MCF-7 cells were exposed to the following dosing regimens: 1) 5-FU 2 hours before MTX followed 24 hours later by Tam and 2) Tam 24 hours prior to 5-FU followed 2hours later by MTX. These treated cells then underwent Western blot analysis to evaluate the phosphorylation of the retinoblastoma protein. Gel electrophoresis and blotting was carried out as previously described [12] utilizing purified mouse Anti-Human Rb antibody (BD Pharmingen, San Diego, California) at a concentration 2µg/ml for one hour as the primary antibody and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, California) at the appropriate concentration as the secondary antibody.

The treated cells were also stained with BrdU and analyzed utilizing flow cytometric techniques to determine the percentage of cells entering into the S phase of the cell cycle. One million cells were transferred to flow cytometry tubes, fixed and permeabilized by resuspension in 100 µl of BD Cytofix/Cytoperm Buffer per tube. The cells were incubated for 30 minutes on ice. The cells were then washed in 1 ml BD Perm/Wash Buffer and centrifuged at 4°C for five minutes and the supernatant was discarded. The cells were next resuspended in 100 µl of BD Cytoperm Plus Buffer per tube and incubated for 10 minutes on ice. The cells were washed by adding 1 ml of BD Perm/Wash Buffer and resuspended in 100 µl of BD Cytofix/Cytoperm Buffer per tube. They were allowed to incubate for 5 minutes on ice. Cells were again washed in 1 ml of BD Perm/Wash Buffer. Resuspension of cells was performed in 100 µl of diluted DNase per tube and incubated for 1 hour at 37°C. Cells were rewashed in 1 ml of BD Perm/Wash Buffer and resuspended in 50 µl of BD Perm/Wash Buffer containing diluted fluorescent anti-BrdU and incubated for 20 minutes at room temperature. The cells were washed once again in 1 ml of 1× BD Perm/Wash Buffer. For flow cytometric analysis, 1 ml of staining buffer (1 X phosphate buffered saline, 2 % fetal bovine serum, and 0.1 % sodium azide) was added to each tube to resuspend cells. The stained cells were analyzed with a FACScan (Becton Dickinson, Franklin Lakes, New Jersey) flow cytometer, Figures 4 and 5, run at a rate no greater than 400 events/seconds and acquired.

RESULTS

Combination chemotherapy utilizing Tam 24 hours prior to 5-FU followed 2 hours later by MTX yielded a percent inhibition of 42.58% of the control. Whereas, combination chemotherapy utilizing 5-FU two hours prior to MTX followed 24 hours later by Tam inhibited cell growth by 70.53%. This growth inhibition was greater than any single agent and also greater than the sequence of early Tam, given before 5-FU and MTX. These data suggest that the sequence of administration of Tam is important in chemotherapy. Early Tam, Tam before 5-FU and MTX, is a less cytotoxic dosing regimen in comparison to the reverse sequence, late Tam. The late Tam combination is more cytotoxic than MTX alone, whereas early Tam is less cytotoxic than late Tam and MTX alone. The most cytotoxic dosing regimen in Figures 1A and 1B is late Tam, 5-FU two hours prior to MTX followed 24 hours later by Tam. A Student's t-test showed that there was a significant difference between early Tam and late Tam ($p = 0.027$).

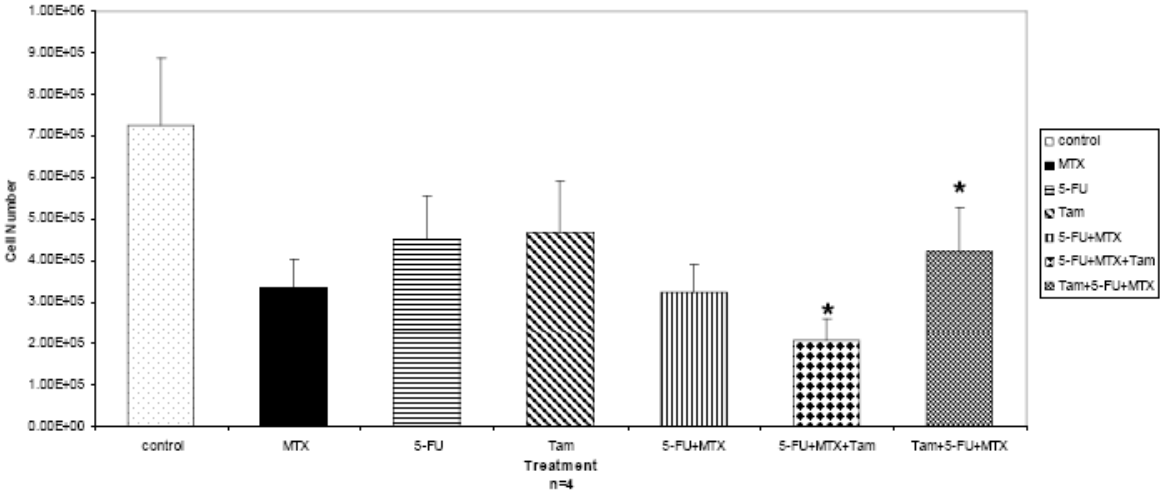

Figure 1A. The interaction between early and late tamoxifen (Tam, 10µM) in combination with methotrexate (MTX, 10µM) and 5-fluorouracil (5-FU, 10µM) in MCF-7 human breast cancer cells. (* represents significant difference, p=0.0027)

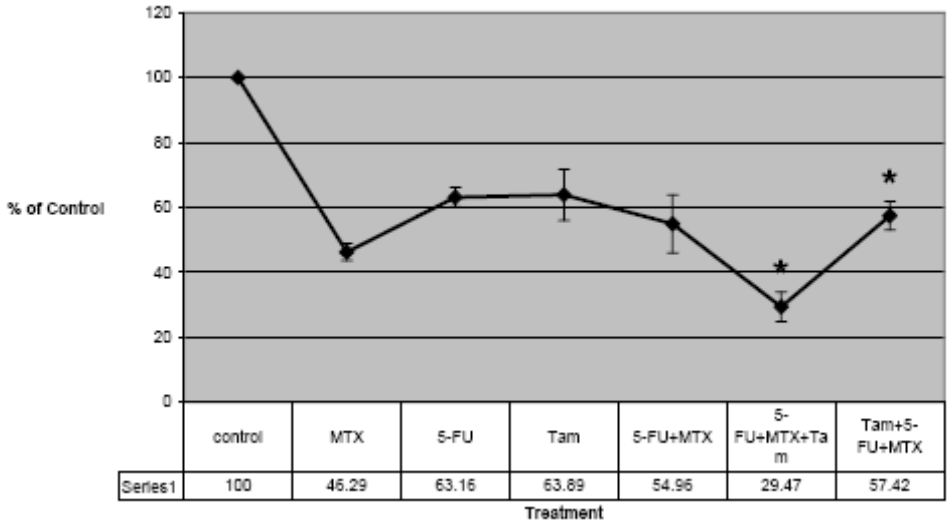

Figure 1B. The interaction between early and late tamoxifen (Tam, 10µM) in combination with methotrexate (MTX, 10µM) and 5-fluorouracil (5-FU, 10µM) in MCF-7 human breast cancer cells represented as the percent of the control.

Hs-5 human bone marrow cells were exposed to the above dosing regimens. Methotrexate was highly cytotoxic to these cells, resulting in a cell growth inhibition of 68.37% of the control (Figures 2A and 2B). Both early Tam and late Tam showed protection in bone marrow cells from the cytotoxicity of MTX, with a percent inhibition of 39.51.58% and 40.55% respectively. There was no significant difference between early Tam and late Tam in these cells, from a Student's t-test p = 0.50. These data suggests that unlike in human breast cancer cells, the sequence of administration of Tam is insignificant, the early Tam and late Tam combinations were less cytotoxic than MTX therefore, protection is observed in these human bone marrow cells by the addition of a priming dose of 5-FU. Combination chemotherapy utilizing 5-FU two hours prior to MTX, followed 24 hours later by Tam is the most efficacious dosing regimen, as it is the most cytotoxic combination in MCF-7 breast cancer cells while showing protection to the MTX toxicity in Hs-5 bone marrow cells.

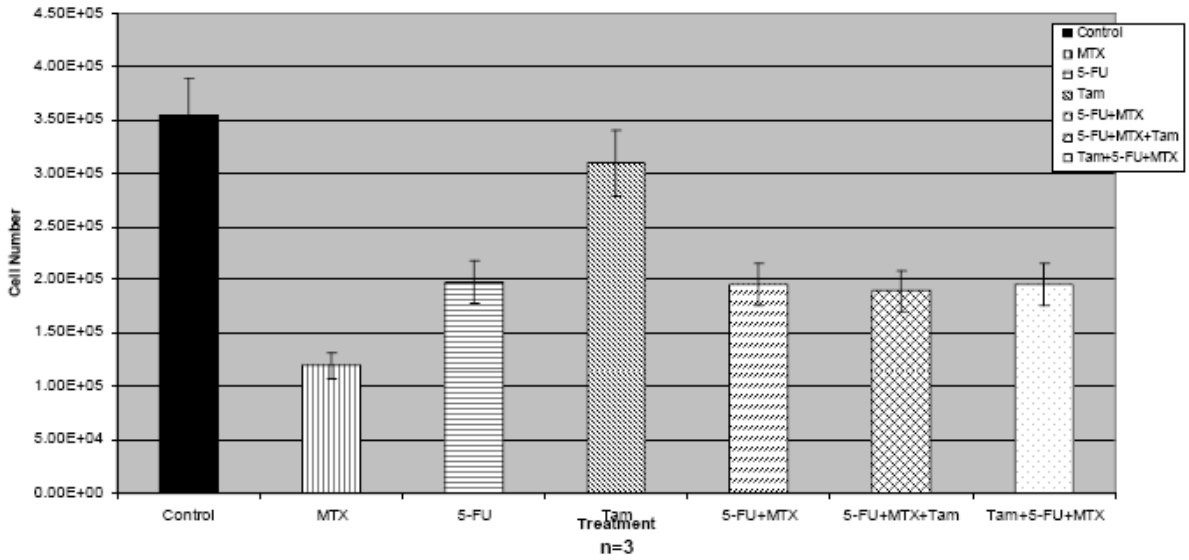

Figure 2A. The interaction between early and late tamoxifen (Tam, 10µM) in combination with methotrexate (MTX, 10µM) and 5-fluorouracil (5-FU, 10µM) in Hs-5 human bone marrow cells.

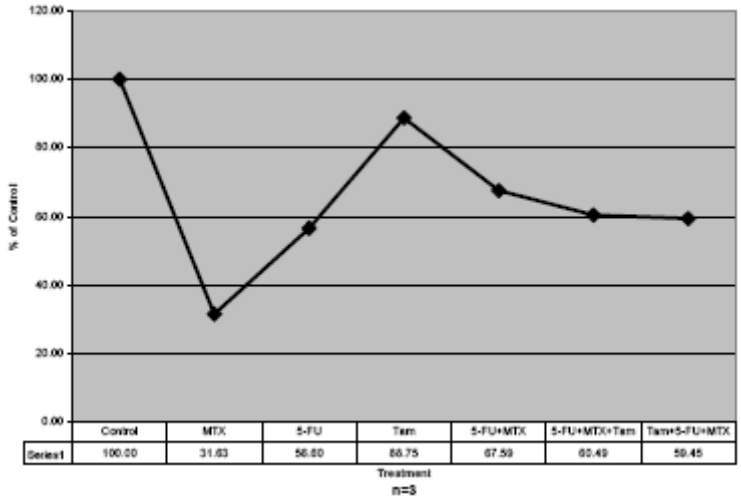

Figure 2B. The interaction between early and late tamoxifen (Tam, 10µM) in combination with methotrexate (MTX, 10µM) and 5-fluorouracil (5-FU, 10µM) in Hs-5 human bone marrow cells represented by the percent of the control.

Figure 3 illustrates the effects of late Tam, i.e. the administration of 5-FU two hours prior to MTX followed 24 hours later by Tam, in MCF-7 breast cancer cells and Hs-5 bone marrow cells. From this figure, a significant difference can be observed between the effects of late Tam in breast cancer cells and bone marrow cells. This drug combination inhibits the growth of MCF-7 breast cancer cells by 70.53% of the control; whereas in bone marrow the percent inhibition of cell growth in comparison to the control is 50.98%. There exists a significant difference between the administration of late Tam in MCF-7 breast cancer cells and Hs-5 bone marrow cells, p=0.0093. These data further illustrate the fact that the most cytotoxic dosing regimen in MCF-7 breast cancer cells, late Tam, which utilizes a dose of MTX ten times that required for leucovorin rescue, provides protection to Hs-5 human bone marrow cells.

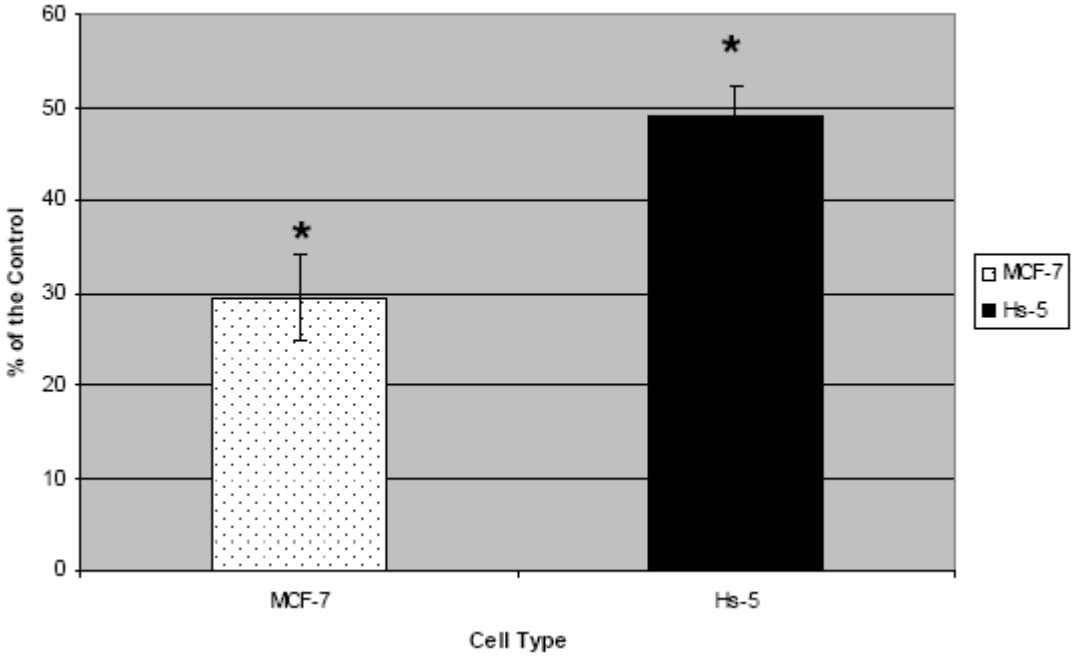

Figure 3. Comparison of the growth inhibitory effect of late tamoxifen in combination with 5-FU and MTX in MCF-7 breast cancer and Hs-5 bone marrow cells. (* represents a significant difference, p=0.0093).

Figure 4, illustrates data obtained from flow cytometric analyses of MCF-7 human breast cancer cells after exposure to various treatments. The first peak illustrated in bold on the control graph is the peak representative of the G_1 phase of the cell cycle. The second bold peak represents the cells that are in the S phase of the cell cycle. This area has been gated as gate M1, any cells in the peak following the M1 gate are considered to be in the S phase of the cell cycle. There are 39% of cells in the S phase of the cell cycle in the control panel. Panel two represents the cells treated with MTX alone at a concentration of 10μM. What is noticed in the second panel is that the second peak representative of G_1 has diminished and there is an increase in the number of cells in the S phase of the cell cycle, 48.55% in comparison to the control. This is consistent with the mechanism of action of MTX, in that it exerts its effect in the S phase of the cell cycle. This suggests that the cells enter into the S phase but are entrapped and do not progress out of this phase. The same trend is seen in panel three when 10μM of 5-FU is given alone; there is a decrease in the G_1 peak and an increase in the number of cells that entered into the S phase, 48.49%. In the fourth panel, a different phenomenon is seen when 10μM Tam was administered to MCF-7 cells. In the fourth panel there is an increase in the number of cells in the G_1 phase of the cell cycle shown by an increase in the first peak in the graph in comparison to the control, MTX, and 5-FU alone. However in panel four there is a decrease in the number of cells in the S phase of the cell cycle 25.89% compared to the control. This panel indicates that when cells are treated with Tam they enter into the G_1 phase of the cell cycle, however they do not progress out of this phase into the S phase of the cycle. The administration of 5-FU two hours before MTX is shown in the fifth panel; there are 40.06% of cells in the S phase of the cell cycle. The sixth panel represents late Tam and there are 35.22% of MCF-7 cells in the S phase of the cycle. This is a lesser number of cells in the S phase compared to the control, MTX and 5-FU alone. The same trend is seen in panel seven for early Tam where there are 36.78% of cells in the S phase of the cycle. Just as with late Tam in panel six there in an increase in the number of cells in the G_1 phase and a decrease of treated cells in the S phase of the cycle, suggesting that whenever Tam is introduced to the system, cells enter into G_1 but are unable to exit out of that phase and progress into the S phase. This further substantiates the hypothesis that Tam arrests cells in the G_1 phase of the cycle and because of this action interacts with the S phase agents.

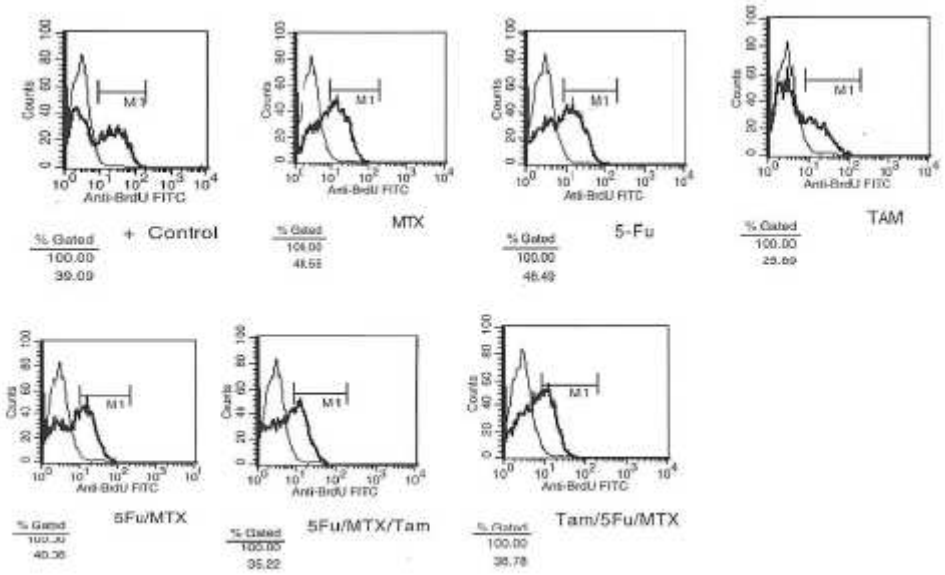

Figure 4. Flow cytometric analysis of Anti-BrdU-FITC stained cells. Cells were treated with MTX, 5-FU, Tam, 5-FU 2h before MTX, 5-FU 2h before MTX followed by Tam 24h, and Tam followed 24 h by 5-FU and MTX 2 h. Anti-BrdU- FITC staining was consistent with the tissue culture graphs in Fig. 1.

Western blot analysis of the phosphorylation of the retinoblastoma protein in MCF-7 breast cancer cells corroborated the data obtained by tissue culture analysis in this cell line. As demonstrated in Figure 6, the control visualized in lane one, depicts heavy smearing in the range of 181.5 to 115.5 kD, characteristic of the various levels of hyperphosphorylation of the retinoblastoma protein (pRb). This suggests that pRB is hyperphosphorylated and the cells are progressing through the cell cycle and proliferating. Lane two represents MTX given alone at 10μM. This lane is absent of the heavy smearing therefore suggesting that the cells in this lane do not contain the hyperphosphorylated form of pRb, and therefore are not proliferating, identical to that seen in the tissue culture data. A non-toxic dose of 5-FU given alone, is shown in lane three, similar to the control, there is heavy smearing in this lane suggesting that these cells contain the hyperphosphorylated form of the retinoblastoma protein and are progressing through the cell cycle. Lane four is Tam when given alone, there is noticeable smearing in this lane, however the smearing is more than that seen with MTX alone and on the other hand, less than that seen in the control. The data in this lane suggests that there are cells progressing through the cell cycle, however not as many as in the control. There are also more cells progressing through the cycle when Tam is given alone as compared to when MTX is given alone. This is consistent with the data obtained when Tam was given alone in the tissue culture studies. Lanes six and seven are the two most important lanes; they illustrate the sequence dependent effects of Tam administration. Lane six shows the effects of late Tam which is Tam's administration 24 hours following 5-FU and MTX. There is heavier smearing in lane seven in comparison to late Tam in lane six. This indicates that the cells in lane seven contain the hyperphosphorylated form of the retinoblastoma protein and are progressing through the cell cycle. However the cells in lane six exhibit no heavy smearing suggesting that these cells are not progressing through the cell cycle. This data is consistent with that seen in the tissue culture data in reference to the effects of early and late Tam administration on human breast cancer cells. Tamoxifen's administration 24 hours before 5-FU and MTX is less cytotoxic to these cells than its administration 24 hours following 5-FU and MTX. As shown in lanes six and seven, there are more cells progressing through the cell cycle in lane seven than in lane six, implying that there is less cell kill in lane seven (early Tam).

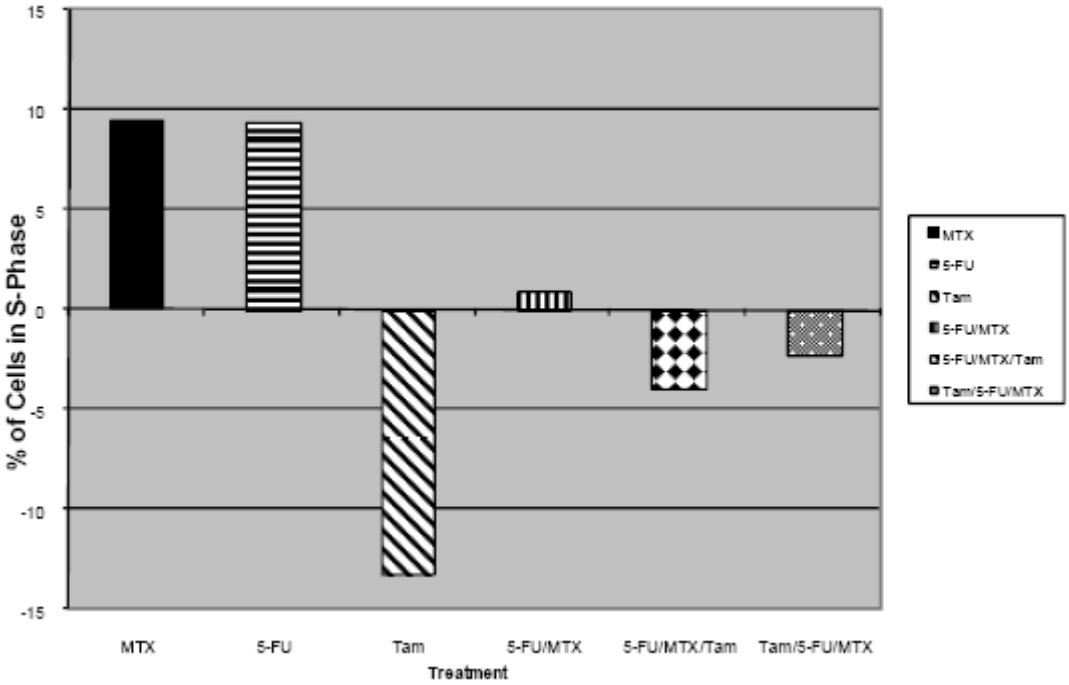

Figure 5. Bar graph representation for the flow cytometric analysis of the percent of cells in the S-phase of the cell cycle after MCF-7 exposure to early and late tamoxifen.

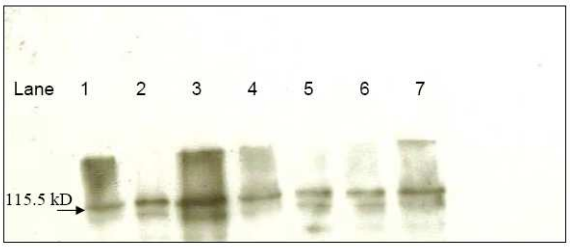

Figure 6. Western blot analysis of the effects of early and late tamoxifen on the phosphorylation of the retinoblastoma protein in MCF-7 breast cancer cells. Lane 1- Control, Lane 2- MTX, Lane 3- 5-FU Lane 4- Tam, Lane 5- 5-FU+MTX, Lane 6- 5-FU+MTX+Tam, Lane 7- Tam+5-FU+MTX

DISCUSSION

The aim of this study was to evaluate the sequence and time dependent effects of MTX in combination with Tam and a priming dose of 5-FU on the growth of MCF-7 breast cancer cells and Hs-5 bone marrow cells. It was demonstrated that the sequence of administration of these agents was important in achieving optimal cancer cytotoxicity while at the same time protecting bone marrow from MTX toxicity (Fig. 3).

Polychemotherapy has been shown to be superior to monotherapy. One of the paramount reasons polychemotherapy is employed is to avoid acquired resistance to the chemotherapeutic agent. By combining agents that exert their effects via different mechanisms, such as combining agents that inhibit cells in different phases of the cell cycle, drug resistance is delayed and minimized. [6, 9, 11, 19] Many tumors are able to salvage extracellular nucleosides and circumvent antifolate toxicity by bypassing the *de novo* nucleotide synthesis pathway [15]. Patel *et al.* have shown that the salvage of extracellular nucleosides protects bone marrow progenitor cells from many antifolate drugs inclusive of MTX.

Inhibition of pRb can cause the dissociation of the complex between pRb and the E2F transcription factors resulting in an increase in DHFR transcription and resistance to MTX. When the Rb protein is phosphorylated it releases the E2F transcriptional activators and activation of the S phase genes *dhfr* and *ts* which correlates to MTX resistance[10]. This may perhaps explain the decrease in the inhibitory effects of MTX that is observed when combined with early Tam. This is in comparison to the enhanced effect observed in the late Tam combination with 5-FU and MTX.

The data from this study suggests that the sequence of administration of chemotherapeutic agents is important for achieving maximal results. Not only does the sequence of administration of Tam and 5-FU assist in enhancing the antitumor effects of MTX, but it also protects normal cells that otherwise might be

negatively affected by MTX. This research also illustrates the benefits of a priming dose of 5-FU on bone marrow cells. By administering a priming dose of 5-FU the antitumor effects of MTX are maintained in breast cancer cells while at the same time protection is seen in the bone marrow. The explanation for this effect could lay in the understanding of salvage pathways. Bone marrow cells form little to no MTX polyglutamates in comparison to human breast cancer cells [4, 8]. Therefore, MTX is unable to inhibit as many enzymes in the folate biosynthesis pathway, including, AICAR and GAR transformylases in bone marrow cells. With this being the case, 5-FU can conserve reduced folates in bone marrow and protect against the direct effects of MTX. By administering a priming dose of 5-FU, the oxidation of 5,10-methylenetetrahydrofolate is inhibited. This leads to an increase in 5,10-methylenetetrahydrofolate (meTHF) and an increase in the meTHF/DHF ratio. This ratio increase will lead to an increase in the levels of 5-methyltetrahydrofolate (mTHF) therefore generating methionine from homocysteine and increasing the production of THF [13]. This production of THF from mTHF allows the bone marrow cells to bypass the effects of methotrexate's inhibition of DHFR and the continual production of purine and methionine biosynthesis by salvage pathways.

This study also focused on the molecular aspects of the importance of sequential administration of 5-FU, Tam, and MTX. The retinoblastoma gene (Rb) is known to be a tumor suppressor gene. In most human tumors, it is either absent or mutated. The 110-116kDa product acts as a cell cycle checkpoint between the G_1 and S phases of the cell cycle. The Rb protein is considered to be in its active state when it is hypophosphorylated which typically occurs when the cell is in the resting state or when the cell is fully differentiated. The protein becomes phosphorylated throughout the cell cycle until late mitosis. In this hyperphosphorylated state, Rb releases the E2F transcription factor and allows cells to progress into the S-phase of the cell cycle. Therefore the phosphorylated form predominates in proliferating cells [7]. Western blot analysis of the phosphorylation of pRb illustrated a distinct difference in the phosphorylation of the Rb protein between late Tam treatment and early Tam treatment. Late Tam treatment showed a decrease in the level of Rb phosphorylation in comparison to early Tam which showed an increased level of phosphorylation. Tamoxifen has been noted to have effects in the G_1 phase of the cell cycle. Therefore it is important to evaluate whether or not the cell cycle activity could influence the efficacy of chemotherapy treatment which involve S-phase agents and tamoxifen. It is rational to assume that if tamoxifen inhibits the progression of cells into the S phase of the cell cycle, and tamoxifen is administered prior to S phase specific agents, that the effects of those S phase agents, specifically MTX would be lessened. For this reason it is important to study the dynamics involved with sequence of administration of cell cycle specific agents. Flow cytometric analysis of the effects of 5-FU, Tam, and MTX alone and in combination revealed an accumulation of a discrete subpopulation of cells in the G_1 cell cycle region and a decrease in the S cell cycle region when Tam was added to any regimen. This data explains the significance in sequence dependent administration of chemotherapeutic agents, and notably MTX, 5-FU, and Tam. Tamoxifen's ability to decrease the number of cells in S phase and cause an accumulation in G_1 could be due in part to an inhibition of the release of the E2F transcription factor which in turn causes an inhibition of proteins such as dihydrofolate reductase and thymidylate synthase, the enzymes that are targeted by MTX and 5-FU [16].

Based on the following study, the administration of a priming and non-toxic dose of 5-FU prior to MTX protected human bone marrow cells from the cytotoxic effects of MTX. It has also been proven that the administration of Tam prior to 5-FU and MTX will lessen the antitumor effects of MTX. However when this sequence is reversed and Tam is given following 5-FU and MTX, the antitumor effects of MTX are enhanced. These studies defined a pharmacodynamic relationship between high-dose MTX, 5-FU, and Tam in ER-positive breast cancer and bone marrow cells. High-dose MTX, 5-FU, and Tam as part of a regimen in which the cellular rate is altered, killed more cancer cells because these rapidly cycling breast cancer cells (ER-positive) can be further growth inhibited by Tam. However, bone marrow cells were protected by a nontoxic dose of 5-FU. Alternatively, a priming and nontoxic dose of 5-FU may allow for antifolate dose escalation without bone marrow transplantation. This study

will add to the knowledge base of combination chemotherapy and elucidate the importance of sequence dependent chemotherapy.

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REFERENCES

1. Bergh, J., Where next with stem-cell-supported high-dose therapy for breast cancer? *Lancet*, 2000. **355**(9208): p. 944-5.
2. Bergh, J., Wiklund, T., Erikstein, B., Lidbrink, E., Lindman, H., Malmstrom, P., Kellokumpu-Lehtinen, P., Bengtsson, N.O., Soderlund, G., Anker, G., Wist, E., Ottosson, S., Salminen, E., Ljungman, P., Holte, H., Nilsson, J., Blomqvist, C., and Wilking, N., Tailored fluorouracil, epirubicin, and cyclophosphamide compared with marrow-supported high-dose chemotherapy as adjuvant treatment for high-risk breast cancer: a randomised trial. Scandinavian Breast Group 9401 study. *Lancet*, 2000. **356**(9239): p. 1384-91.
3. Bowen, D., Southerland, W.M., Hawkins, M., Jr., and Johnson, D.H., Sequence-dependent antagonism between tamoxifen and methotrexate in human breast cancer cells. *Anticancer Res*, 2000. **20**(3A): p. 1415-7.
4. Fabre, I., Fabre, G., and Goldman, I.D., Polyglutamylation, an important element in methotrexate cytotoxicity and selectivity in tumor versus murine granulocytic progenitor cells in vitro. *Cancer Res*, 1984. **44**(8): p. 3190-5.
5. Fisher, B., Highlights from recent National Surgical Adjuvant Breast and Bowel Project studies in the treatment and prevention of breast cancer. *CA Cancer J Clin*, 1999. **49**(3): p. 159-77.
6. Fox, M., Boyle, J.M., and Kinsella, A.R., Nucleoside salvage and resistance to antimetabolite anticancer agents. *Br J Cancer*, 1991. **64**(3): p. 428-36.
7. Harbour, J.W. and Dean, D.C., The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev*, 2000. **14**(19): p. 2393-409.
8. Jolivet, J., Schilsky, R.L., Bailey, B.D., Drake, J.C., and Chabner, B.A., Synthesis, retention, and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. *J Clin Invest*, 1982. **70**(2): p. 351-60.
9. Kinsella, A.R. and Haran, M.S., Decreasing sensitivity to cytotoxic agents parallels increasing tumorigenicity in human fibroblasts. *Cancer Res*, 1991. **51**(7): p. 1855-9.
10. Kinsella, A.R. and Smith, D., Tumor resistance to antimetabolites. *Gen Pharmacol*, 1998. **30**(5): p. 623-6.
11. Kinsella, A.R., Smith, D., and Pickard, M., Resistance to chemotherapeutic antimetabolites: a function of salvage pathway involvement and cellular response to DNA damage. *Br J Cancer*, 1997. **75**(7): p. 935-45.
12. Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970. **227**(5259): p. 680-5.
13. Matthews, R.G. and Baugh, C.M., Interactions of pig liver methylenetetrahydrofolate reductase with methylenetetrahydropteroylpolyglutamate substrates and with dihydropteroylpolyglutamate inhibitors. *Biochemistry*, 1980. **19**(10): p. 2040-5.
14. Partridge, A.H., Burstein, H.J., and Winer, E.P., Side effects of chemotherapy and combined chemohormonal therapy in women with early-stage breast cancer. *J Natl Cancer Inst Monogr*, 2001(30): p. 135-42.
15. Patel, D.H., Allay, J.A., Belt, J.A., and Sorrentino, B.P., Retroviral transfer of the hENT2 nucleoside transporter cDNA confers broad-spectrum antifolate resistance in murine bone marrow cells. *Blood*, 2000. **95**(7): p. 2356-63
16. Sandal, T., Molecular aspects of the mammalian cell cycle and cancer. *Oncologist*, 2002. **7**(1): p. 73-81.
17. Tallman, M.S. and Gradishar, W.J., High-dose chemotherapy and autologous stem cell transplantation as treatment for high-risk breast cancer. *Cancer Chemother Pharmacol*, 1998. **42 Suppl**: p. S60-7.
18. Tallman, M.S., Gray, R., Robert, N.J., LeMaistre, C.F., Osborne, C.K., Vaughan, W.P., Gradishar, W.J., Pisansky, T.M., Fetting, J., Paietta, E., and Lazarus, H.M., Conventional adjuvant chemotherapy with or without high-dose chemotherapy and autologous stem-cell transplantation in high-risk breast cancer. *N Engl J Med*, 2003. **349**(1): p. 17-26.
19 Weber, G. and Prajda, N., Targeted and non-targeted actions of anti-cancer drugs. *Adv Enzyme Regul*, 1994. **34**: p. 71-89.