Sequence-dependent Administration of 5-Fluorouracil Maintains Methotrexate Antineoplastic Activity in Human Estrogen-negative Breast Cancer and Protects against Methotrexate Cytotoxicity in Human Bone Marrow

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Abstract. *Background: Breast cancer is a leading cause of morbidity and mortality in women in developed countries and in increasingly developing countries. In general, estrogen receptor (ER)-positive breast cancers have a better prognosis and are often more responsive to anti-estrogen therapy. Unfortunately, ER-negative breast cancers are more aggressive and unresponsive to anti-estrogens. The aim of this investigation was to evaluate the 5-fluorouracil (5-FU) and methotrexate (MTX) combination to determine the most effective regimen considering the mechanism of action in treating ER-negative human breast cancer cells and at the same time mitigating methotrexate cytotoxicity in human bone marrow cells. Materials and Methods: In order to determine the sequence-dependent interaction between MTX and 5-FU on proliferation, cell viability was carried out using the Quick Cell Proliferation Assay by exposing the human estrogen negative (MDA-MB-436 and Hs-578T) and bone marrow (HS-5) cells to: (i) MTX and 5-FU alon; (ii) MTX 2 h prior to 5-FU (MTX/5-FU; (iii) 5-FU 2 h prior to MTX (5-FU/MTX). Results: The growth rate in MDA-MB-436 was 23.5±3.98%, in Hs-578T 30±5.9% and HS-5 32±3.1% of the control for MTX/5-FU. Whereas the growth rate in MDA-MB-436 was 28.5±4.1%, in Hs-578T 34.7±3.5% and HS-5 68.6±6.3% of the control for 5-FU/MTX combinations. The later combination exhibits significant protection against MTX cytotoxicity in bone marrow and at same time maintained maximum cytotoxicity in estrogen negative breast cancer cell lines. The findings were further supported by cell flow cytometry, apoptosis and Western blot analysis data. Conclusion: The combination of 5-FU/MTX effectively maintains the maximum inhibitory effect of MTX in ER-negative breast cancer and protects against MTX cytotoxicity in human bone marrow.*

Breast cancer is a leading cause of morbidity and mortality in women, in developed and also in increasingly developing countries (1). In the United States alone, it is estimated that 46,000 women will die of breast cancer and over 200,000 new cases will be diagnosed annually (2). In the new millennium, the expected number of annual new cases of breast cancer worldwide could exceed 1.5 million (3). These statistics emphasize the urgent need for improvements in detection, diagnosis, and treatment of breast cancer. Several studies have shown that the mortality rate of breast cancer is about three times higher in African-American women than in other populations (4, 5). In addition, the available data indicate that the tumors are very aggressive and poorly differentiated with a very low frequency of hormone receptors, a higher S-phase fraction and tumor necrosis (6).

Breast cancer is highly curable if diagnosed at early stage. It is now well-established that adjuvant systemic therapy improves survival in patients with early-stage breast cancer (7). Treatment options for early-stage breast cancer include chemotherapy (*e.g.* anthracyclines, taxanes) and hormone therapy (*e.g.* tamoxifen, aromatase inhibitor). Recent progress in diagnosis and therapy has increased the survival of women in estrogen-dependent (ER-positive) breast cancer. Unfortunately, ER-negative breast cancers are more aggressive and unresponsive to anti-estrogens. The treatment

Abbreviations: Methotrexate (MTX), 5-fluorouracil (5-FU), MTX polyglutamates (MTXPGs), 5,10-methylenetetrahydrofolate (meTHF), dihydrofolate (DHF), estrogen receptor (ER).

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Key Words: Methotrexate, 5-fluorouracil, estrogen-negative breast cancer cell lines.

options available for estrogen-independent tumors are far from satisfactory, and consequently lead to a poorer prognosis. The intricate details of breast cell growth control have not been completely elucidated. Estrogens are known to play the predominant role in breast cancer development and growth and much effort has been devoted to block estrogen formation and action (8). The most widely used therapy for breast cancer is the use of anti-estrogens, such as tamoxifen. However, the present breast cancer therapies achieve meaningful clinical results in only 30-40% of patients (9). Drug resistance is linked to the presence of estrogen-independent pathways for breast cancer cell growth (10, 11).

Adjuvant chemotherapy with methotrexate (MTX) and 5-fluorouracil (5-FU) are effective in inhibiting proliferation of breast cancer cells but frequently cause thrombocytopenia and leucopenia. Major problems with the use of MTX and 5-FU include: a) the lack of selectivity between diseased and normal cells, and b) equitoxicity of sequential MTX and 5-FU in both tumor and hematopoietic stem cells. Studies from this laboratory have shown that a high dose of MTX in combination with 5-FU was sequence-independent in human estrogen positive breast cancer cells in producing cytotoxicity but sequence-dependent in hematopoietic cells. These studies also suggested that a priming and non-toxic dose of 5-FU administered prior to MTX decreased toxicity to bone marrow and increased cytotoxic selectivity for estrogen positive breast cancer cells (MCF-7) (12-14). When 5-FU preceded MTX, 5-FU diminished the need for leucovorin rescue of MTX cytotoxicity. The goal of treatment for ER-negative breast cancer patients receiving chemotherapy and/or hormonal therapy is the same: to maximize cytotoxicity to breast cancer cells and to minimize cytotoxicity to bone marrow. Hence, the present study was designed to determine the role of sequence-dependent administration of MTX and 5-FU on the cytotoxicity to human ER-negative breast cancer and bone marrow cells.

Materials and Methods

Methotrexate, 5-fluorouracil, and Trypan blue dye were purchased from Sigma Chemical Company, St. Louis, MO, USA. An early passage of human estrogen negative breast cancer (MDA-MB-436 and Hs-578T) and bone marrow (HS-5) were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. The BCA Protein assay kit, PVDF membrane and Super Signal West Dura were purchased from Pierce, Rockford IL, USA. Quick Cell Proliferation Assay Kit was purchased from BioVision, CA, USA. Propidium iodide (PI), Annexin V and Primary (Rb) antibody were obtained from BD Biosciences, CA, USA.

Cell culture. Two human estrogen negative breast cancer cell lines (MDA-MB-436 and Hs-578T) were used in this study. Although these two cell lines belong to the same class, they possess different growth rate: the one is fast growing and the other is slow growing. The similar condition was also observed in tumor growth in humans. Stock cultures of human estrogen negative breast cancer and human bone marrow cells (HS-5) were grown in 75 cm^3 flasks and incubated in RPMI-1640 media (Cellgro, Mediatech Inc. VA, USA). For each experiment, $1x10^6$ cells were plated in 100 mm tissue culture Petri dishes. One group of cells was maintained without any drug and served as the control. The remaining groups were exposed to: (i) MTX and 5-FU alone; (ii) MTX 2 h prior to 5-FU (MTX/5-FU); (iii) 5-FU 2 h prior to MTX (5-FU/MTX). Drug concentrations were 10 μM MTX and 1 μM 5-FU. Cells were exposed for 48 h.

Assessment of cell viability using the quick cell proliferation assay. Cell viability studies were performed using the quick cell proliferation assay following the manufacturer's protocol. Briefly, the MDA-MB-436, Hs-578T and HS-5 ($1.5x10^4$) cells growing in 96-well plates in RPMI-1640 media in the presence or absence of drugs added in the same sequence as described above. The formazan dye produced by viable cells was quantified by measuring the absorbance of the dye solution at 440 nm using a microtiter plate reader. Furthermore, cells were stained with 0.2% Trypan blue dye and then counted in a heamocytometer.

Cell cycle analysis. Cell cycle perturbations induced by the drug were analyzed by propidium iodide (PI) DNA staining as described elsewhere (15). The cells were grown in 6-well plates ($1x10^5$ cells per well) in the presence or absence of the above-mentioned combinations. After 48 h of exposure cells were collected and evaluated after PI staining and cell cycle profiles were obtained using a BD FACS Calibur Cell flow Cytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed by ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

Western blot analysis. Western blot was performed as described elsewhere (15). The mouse anti-human retinoblastoma protein Rb monoclonal antibody was used in conjunction with horseradish peroxidase-conjugate. Antibody detection and densitometric analysis were performed as described elsewhere (15).

Determination of apoptotic cells. To drug-induced apoptosis was done using PI staining and followed by cell flow cytometry analysis. The cells with lower DNA content less than G1, as shown by PI staining, were defined to be apoptotic (sub-G_0/G_1 population) and were determined using a BD FACS Calibur Cell Flow Cytometer (Becton Dickinson). Data were analyzed by ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

Statistical analysis. Data were expressed as mean±standard error. Statistical differences within and between treatment groups were determined by one-way ANOVA followed by *post-hoc* Dunnett's comparison test. $P<0.05$ was considered statistically significant. Data were analyzed using Graphpad InStat (Graphpad Software Inc, San Diego, CA, USA).

Results

Effects of MTX and 5-FU on the growth of human ER-negative breast cancer and bone marrow cell lines. The growth rate in the estrogen negative breast cancer cell line (MDA-MB-436) for MTX and 5-FU alone was 22.5±2.67% and 94.9±8.56%

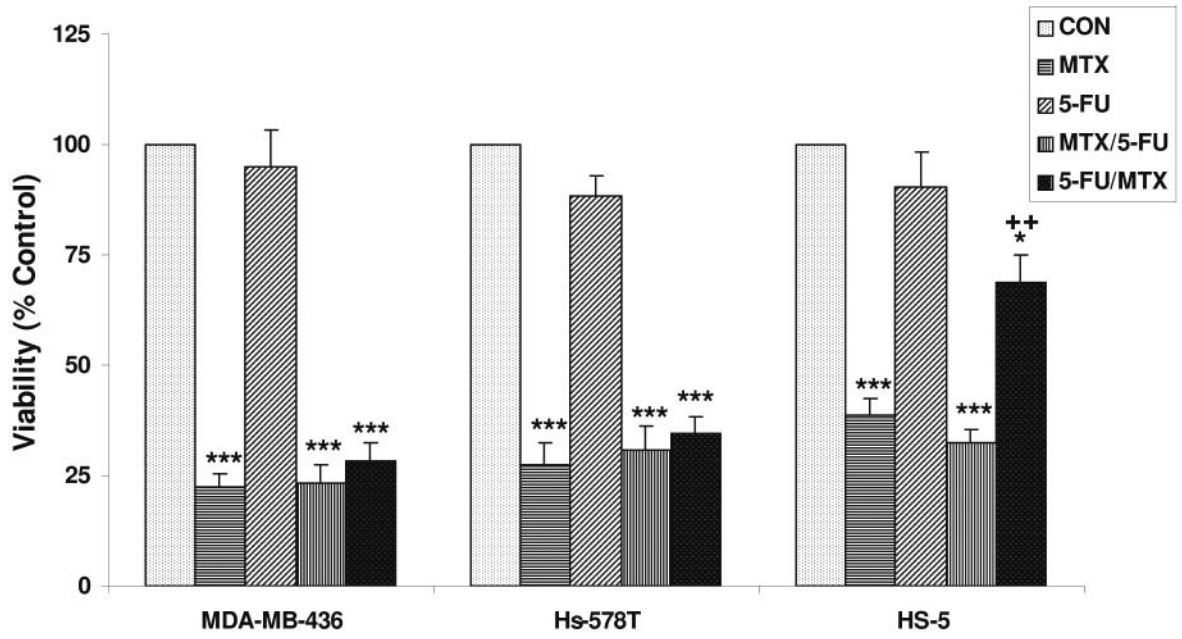

Figure 1. *The sequence-dependent interaction between MTX and 5-FU on the proliferation of human estrogen negative breast cancer (MDA-MB-436 and Hs-578T) and bone marrow HS-5 cell lines. Cells were exposed to: (i) 10 μM MTX and 1 μM 5-FU alone, (ii) MTX 2h prior to 5-FU, (iii) 5-FU 2 h prior to MTX. Total time of exposure was 48 h. Viability of cells was determined using the Quick Cell Proliferation assays. Results represent mean±SEM of five independent experiments. Analysis of variance indicated a significant reduction compared to the control (**p<0.01, ***p<0.001) and significant increase compared to MTX (+p<0.05, ++p<0.01, +++p<0.001).*

that of the control, respectively. Also in this cell line the growth rate for MTX/5-FU was 23.5±3.98% and for 5-FU/MTX 28.5±4.1% that of the control. The growth rate of Hs-578T cell line with MTX and 5-FU alone was 27.4±5.3% and 88.5±4.5% that of the control, respectively. The growth rate for MTX/5-FU was 30.9±5.2% and for 5-FU/MTX, 34.7±3.5% that of the control. With respect to the human bone marrow cell line (HS-5), MTX and 5-FU alone demonstrated a growth rate of 38.9±3.65% and 90.6±7.59% that of the control, respectively. The growth rate for HS-5 cells exposed to MTX/5-FU was 32.4±3.1% and for 5-FU/MTX was 68.6±6.3% that of the control. A significant reduction in cell growth compared to the control was observed with MTX in all cell lines (Figure 1), whereas 5-FU had no effect in cell growth in all the cell lines studied. The early MTX in combination 5-FU (MTX/5-FU) maintain the significant cytotoxicity in ER-negative breast cancer and bone marrow cell lines. In contrast late MTX (5-FU/MTX) exhibited a significant protective effect on the MTX cytotoxicity in bone marrow cells and at the same time maintained antineoplastic activity in human ER-negative breast cancer cells (Figure 2).

Effects of MTX and 5-FU on the cell cycle progression of human estrogen negative breast cancer and bone marrow cell line. Cell flow cytometry analysis was used to determine the effect of MTX and 5-FU on the progression of cells in

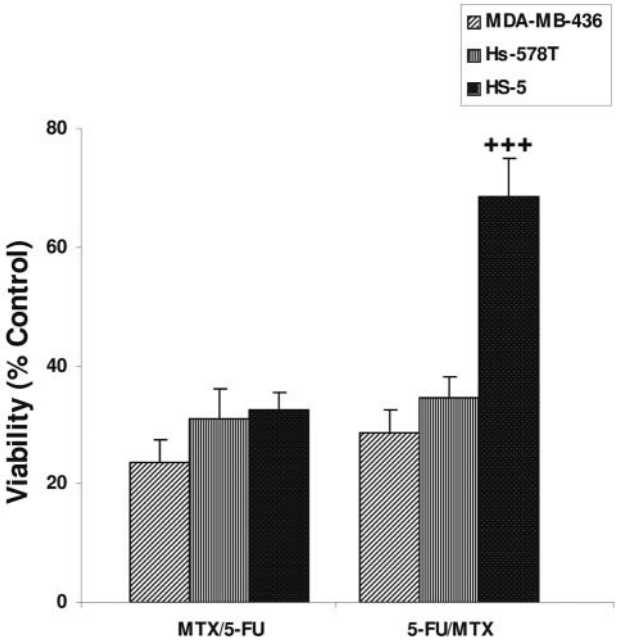

Figure 2. *Effects of MTX and 5-FU on the growth rate in human estrogen negative breast cancer (MDA-MB-436 and Hs-578T) and bone marrow HS-5 cells. Cells were exposed to: (i) MTX 2 h prior to 5-FU, (ii) 5-FU 2 h prior to MTX. Total time of exposure was 48 h. Analysis of variance indicated a significant increase when MTX/5-FU was compared to 5-FU/MTX (+++p<0.001).*

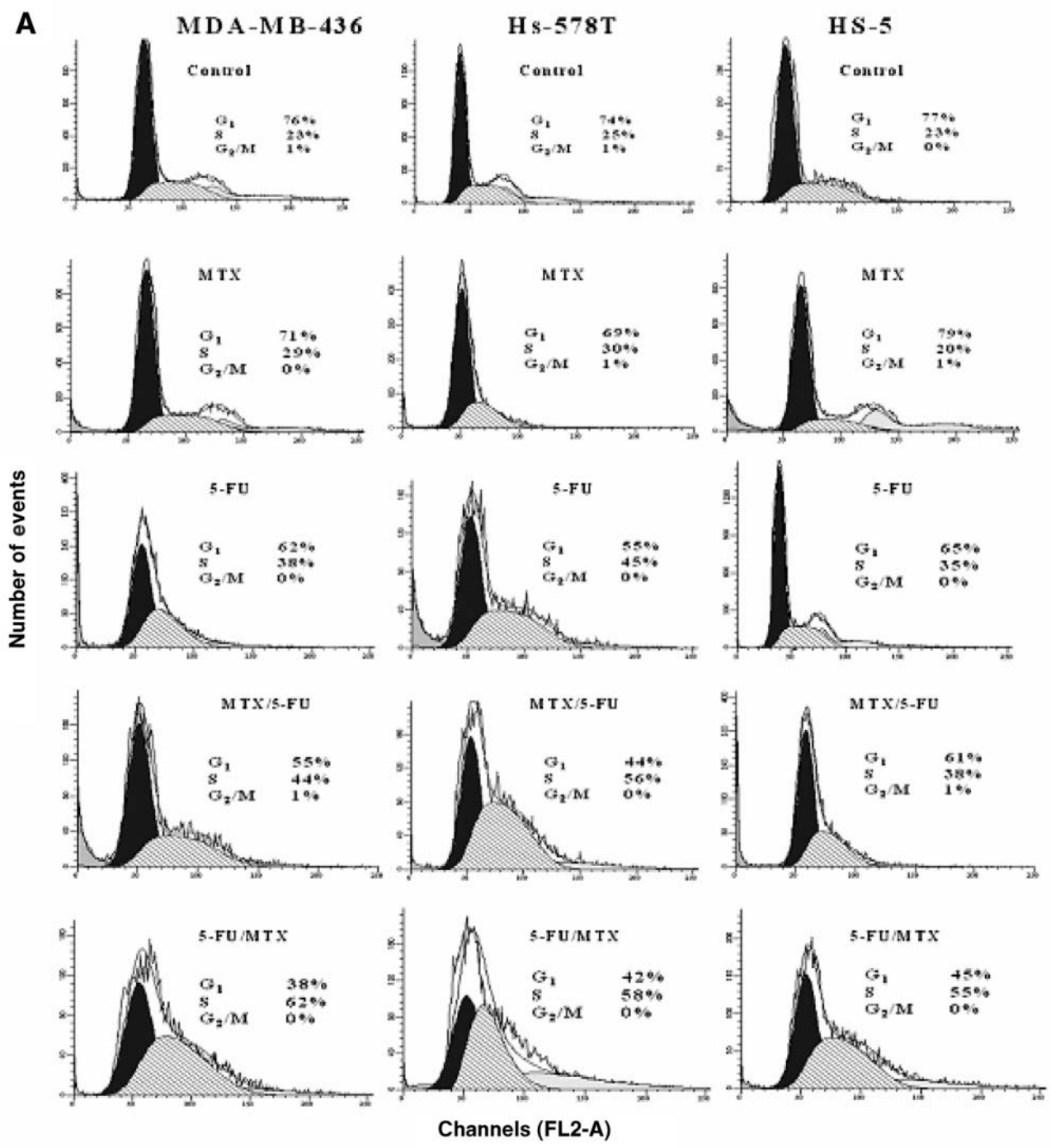

Figure 3. *continued*

human ER-negative breast cancer and bone marrow cell lines. The cell cycle profile (Figure 3A) is representative of three independent experiments using the four treatment groups in MDA-MB-436, Hs-578T and HS-5 cells. Figure 3B shows the percentage of cells in the S-phase. Analysis revealed that the highest number of cells progressing to the S-phase of the cell cycle for MDA-MB-436 was found in 5-FU/MTX (59±5.9%) followed by 5-FU alone (49±5.6%),

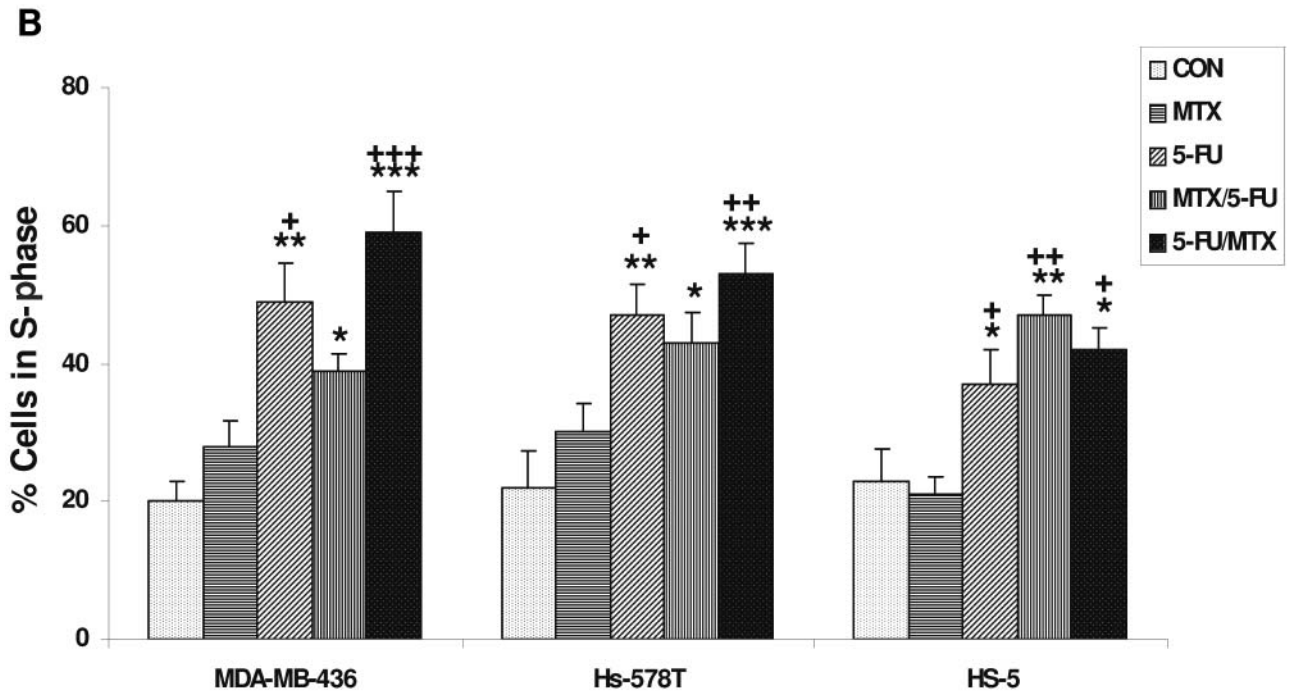

Figure 3. *The sequence-dependent interaction between MTX and 5-FU on the cell cycle progression of human estrogen negative breast cancer (MDA-MB-436 and Hs-578T) and bone marrow HS-5 cell lines. Cells were exposed to: (i) 10 μM MTX and 1 μM 5-FU alone, (ii) MTX 2 h prior to 5-FU, (iii) 5-FU 2 h prior to MTX. Total time of exposure was 48 h. A: The cell cycle profile is representative of three independent experiments. B: Percentage of cells in S-phase. Analysis of variance indicated a significant increase compared to control (*p<0.05, **p<0.01, ***p<0.001) and significant increase compared to MTX (+p<0.05, ++p<0.01, +++p<0.001).*

MTX/5-FU (39±2.9%) and then MTX (28±3.8%). The highest number of cells progressing to the S-phase of the cell cycle for Hs-578T was found in 5-FU/MTX (53±4.5%) followed by 5-FU alone (47±4.5%), MTX/5-FU (43±4.5%) and then MTX (30±4.1%). The highest number of cells progressing to the S-phase of the cell cycle for HS-5 was found in MTX/5-FU (47±2.9%) followed by 5-FU/MTX (42±3.1%), 5-FU alone (37±5.1%) and then MTX (21±2.5%). The 5-FU in combination with MTX showed significantly greater number of cells progressing to S-phase when compared to control.

Western blot analysis. The retinoblastoma protein (Rb) was used as a marker to determine the effects of MTX and 5-FU combination on cellular progression at the molecular level. The Western blot shown in (Figure 4A) is representative of five independent experiments using the four treatment groups. The relative optical density (ROD) is shown in Figure 4B. The highest density was observed in 5-FU, followed by late MTX (5-FU/MTX), indicating higher phosphorylation. The lowest density was found in MTX alone, indicating that fewer phosphorylated Rb proteins were present. Results revealed that the highest number of cells progressing to the S-phase of the cell cycle was found in 5-FU/MTX in estrogen negative cell lines. These differences were significant when compared to the control. Similar results were observed with the cell flow cytometry analysis. The observed differences may be due to the sequence of administration even though both drugs belong to S-phase target agents. In addition, the reason 5-FU was less cytotoxic when administered prior to MTX may be due to their targets on the folate synthesis pathway and 5-FU first allows cells to get around the MTX block of dihydrofolate reductase.

Determination of apoptotic cells. To drug-induced apoptosis was done using PI staining and followed by cell flow cytometry analysis. The cells with lower DNA content, as shown by PI staining less than G_1, were defined to be apoptotic (sub-G_0/G_1 population) and were determined using M1 gate as shown in Figure 5A. The highest number of apoptotic cells (sub-G_0/G_1 population) was observed in 5-FU/MTX for MDA-MB-436 was (40±4.1%) followed by Hs-578T (37±3.5%) and lowest in HS-5 (7±1.2%) as shown in

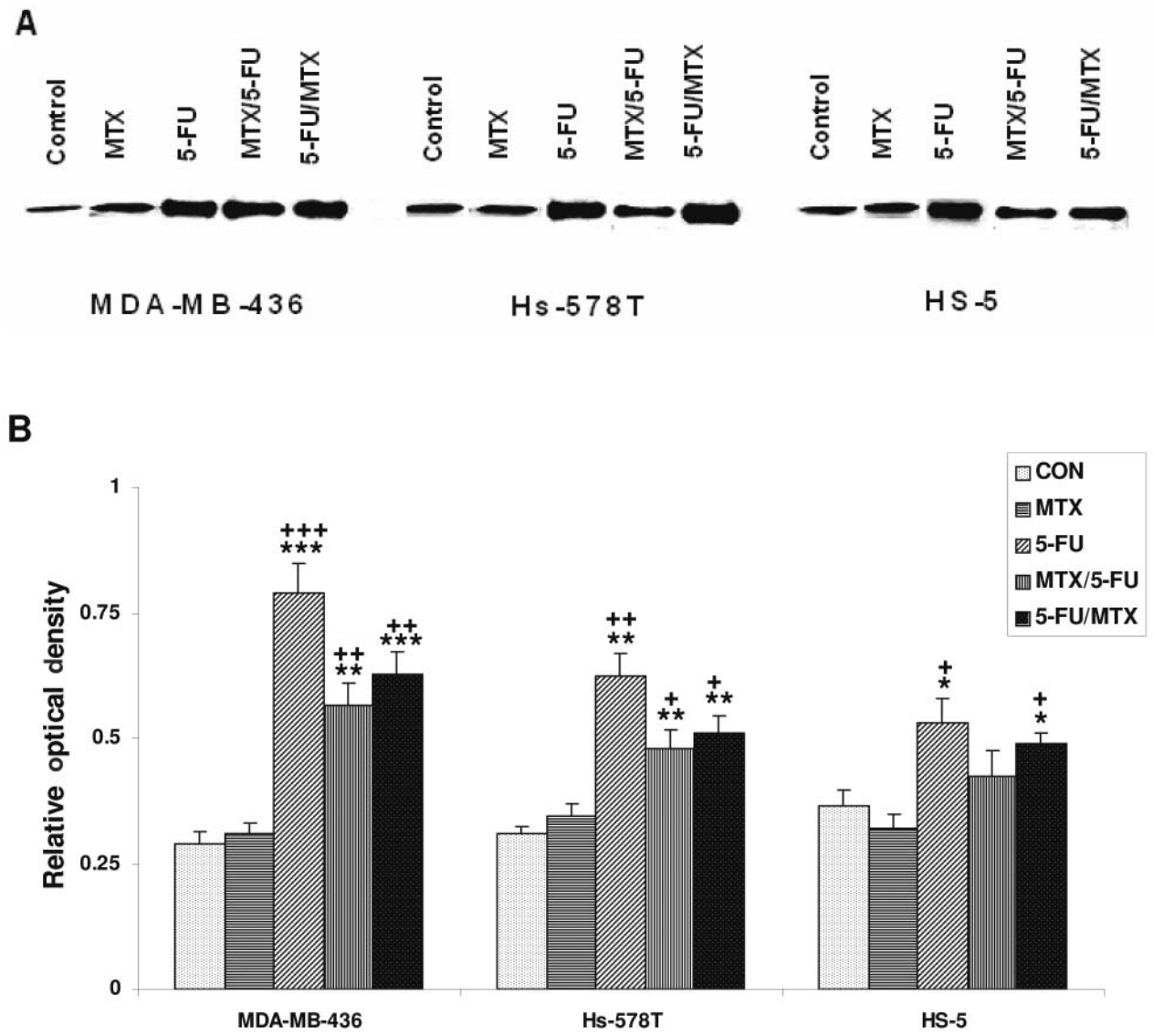

Figure 4. *Effects of MTX and 5-FU on the level of retinoblastoma protein (Rb) as phosphorylated protein marker in human estrogen negative breast cancer (MDA-MB-436 and Hs-578T) and bone marrow HS-5 cells. Cells were exposed to: (i) 10 μM MTX and 1 μM 5-FU alone, (ii) MTX 2 h prior to 5-FU, (iii) 5-FU 2 h prior to MTX. Total time of exposure was 48 h. A: The gel is representative of five independent experiments. B: Analysis of variance indicated a significant increase compared to control (*p<0.05, **p<0.01, ***p<0.001) and significant increase compared to MTX (+p<0.05, ++p<0.01, +++p<0.001).*

(Figure 5B). The significant protection of apoptotic cell death was observed in 5-FU/MTX combination in bone marrow compared to respective control. Whereas, MTX alone and MTX/5-FU did not show any protection of apoptotic cell death in bone marrow cells compared to control.

Discussion

Previous studies from this laboratory have shown that 5-FU was administered prior to MTX, not for additive cytotoxicity but to provide protection of bone marrow cells and eliminate the need for leucovorin rescue of MTX cytotoxicity in human estrogen positive breast cancer cells (12-14). The selective effect of MTX in breast cancer may be due to the formation of MTX-polyglutamates (MTXPGs) (16) in breast cancer cells and the inability of 5-FU to prevent the inhibitory effects of MTX and MTXPGs. The synthesis of MTXPGs increases with increasing drug concentration. The formation of MTXPGs allows for sustained inhibition of dihydrofolate reductase, thymidylate synthase, and other folate-requiring enzymes not affected directly by MTX (such as aminoimidazolecarboxamide

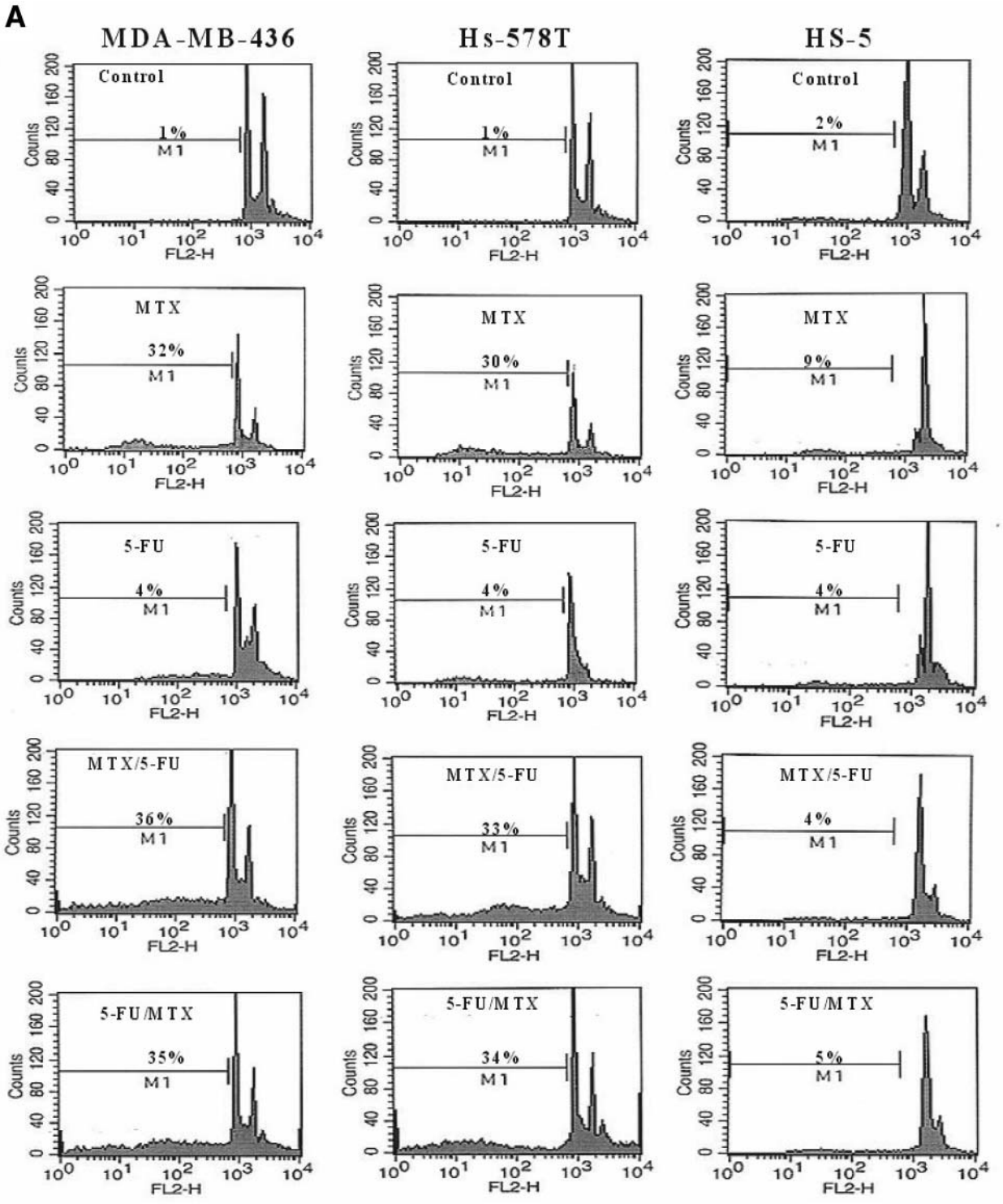

Figure 5. *continued*

ribonucleotide and formylglycinamide ribonucleotide transformylases) (17). The bone marrow forms little or no MTXPGs when exposed to MTX (18) and therefore, certain folate-requiring enzymes would not be inhibited due to the absence or very low levels of MTXPGs. Hence, sequence-dependency in bone marrow must be related to 5-FU

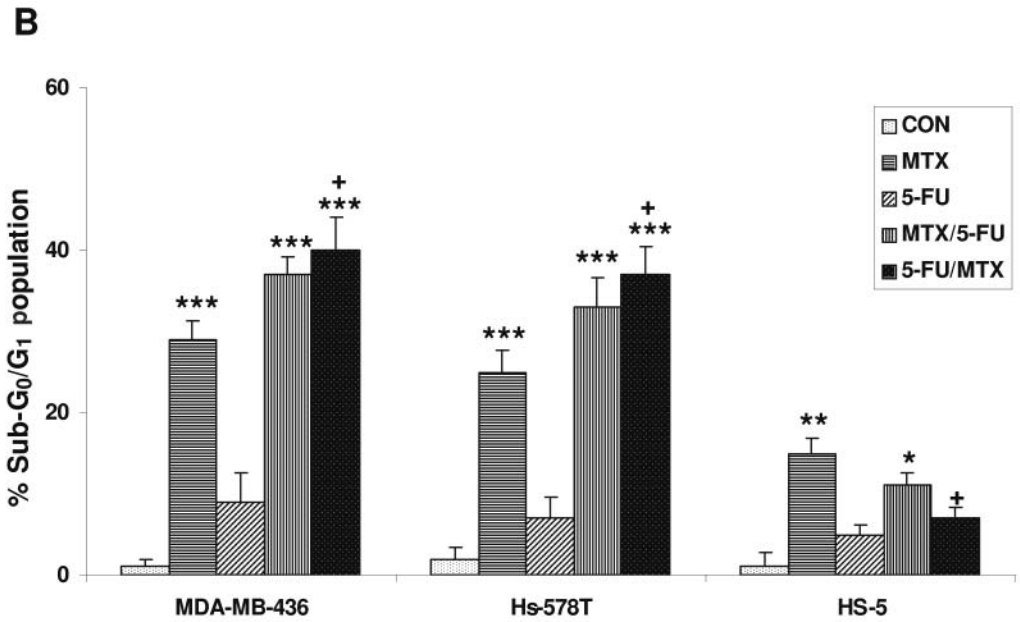

Figure 5. *Percentage of apoptosis calculated by measuring sub-G_0/G_1 population using cell flow cytometry in all the cell lines mentioned. The cells with lower DNA content showing less PI staining than G_1 were defined to be apoptotic. (A) The cell cycle profile with M1 gate is representative of three independent experiments. (B) Percent of apoptotic cells in the M1 gate (sub-G_0/G_1 population). Analysis of variance indicated a significant increase compared to control (*p<0.05, **p<0.01, ***p<0.001) and significant increase or decrease compared to MTX (+p<0.05, ++p<0.01, +++p<0.001).*

conserving reduced-folate to protect against the direct effect of MTX (12, 13). By preventing the oxidation of 5,10-methylenetetrahydrofolate (meTHF), 5-FU could conserve reduced-folates by altering the meTHF/DHF (dihydrofolate) ratio. In a previous report (19) it was indicated that regulation of the meTHF/DHF ratio might be of importance in regulating the partitioning of meTHF into the competing pathways of dTMP biosynthesis and regulation of methionine from homocysteine. An increase in the meTHF/DHF ratio by 5-FU would spare: a) meTHF for reduction to 5-methyl-tetrahydrofolate (m-THF), and b) m-THF for methionine and purine biosynthesis. Further, a diminution of DHF levels by a priming and non-toxic 5-FU dose would decrease DHF inhibition of m-THF reductase (19) and allow for the continuance of THF production and purine and methionine biosynthesis. The results from the present study suggested that the incidence and the severity of MTX/5-FU and 5-FU/MTX cytotoxicity in ER-negative breast cancer cells were 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 (5-FU/MTX) showed protection against MTX cytotoxicity in bone marrow cell line, which agrees with earlier observations (12).

5-FU and MTX exert their effects on the S-phase of the cell cycle. The percent of bone marrow cells, exposed to MTX/5-FU entering the S-phase of the cell cycle was statistically similar to estrogen negative cells. This suggested that the cytotoxicity observed in bone marrow when exposed to MTX/5-FU was due to MTX and 5-FU administration. The highest number of cells entering to S-phase was observed in estrogen negative breast cancer cells for 5-FU/MTX compared to MTX/5-FU. The later combination was equitoxic in ER-negative breast cancer cells and bone marrow cells no statistically significant. From our observation even though 5-FU and MTX exert their effects on the S-phase of the cell cycle but the highest number of cells entering to S-phase observed in 5-FU than MTX alone and 5-FU/MTX compared to MTX/5-FU there may be underlying mechanism(s) which need further investigation. Similar results were also observed in Western blot analysis. Therefore, the selectivity observed when administering 5-FU and MTX may primarily be due to toxicity not cell cycle specificity. As cells grow and migrate to next phase of cell cycle Rb has to be phosphorylated. So in a cytotoxic environment Rb may not be phosphorylated as much compared to less toxic environment and to control. The decreased cytotoxicity of 5-FU/MTX in bone marrow may be due to: i) conservation of reduced folates by 5-FU, ii)

formation of MTXPGs in estrogen negative breast cancer cells but not bone marrow, iii) or there may be other mechanism(s) that haven't been studied yet, which is supported by the data from the present study. It was also observed that the highest numbers of apoptotic cells were found in MDA-MB-436 followed by Hs-578T. There was no significant apoptotic cell death in bone marrow cells when treated with 5-FU/MTX compared to control. The results further confirmed that 5-FU/MTX combination maintained antineoplastic activity in human estrogen negative breast cancer cells and same time protect bone marrow cells from methotrexate cytotoxicity.

Our findings suggested that sequence-dependent administration of 5-FU/MTX provides a cytotoxic advantage against estrogen negative breast cancer cells since hematopoietic cells (bone marrow) were protected by 5-FU/MTX combination. The observations from this study have important implications regarding therapeutic dosing regime of MTX in combination with non-toxic dose of 5-FU in the treatment of estrogen negative breast cancer while protecting the bone marrow. In addition, African-American patients may be benefited from this drug combination regimen since a higher incidence of ER-negative breast cancers is reported for this population.

Acknowledgements

This work was supported by the RCMI grant G12RR003048-18. We are thankful to the Department of Pharmacology, College of Medicine, Howard University, Washington, D.C., U.S.A., for allowing us to use their core facilities to conduct the present research work.

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Received July 12, 2007
Accepted July 30, 2007