

## The Protection against Trimetrexate Cytotoxicity in Human Bone Marrow by Sequence-dependent Administration of Raloxifene, 5-Fluorouracil/Trimetrexate

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**Abstract.** *Background:* Currently, one of the most effective strategies for the treatment and prevention of breast cancer is the use of drugs that block estrogen action in the breast. The success of the first clinically relevant selective estrogen receptor modulator (SERM), tamoxifen, provided the foundation for further testing of this drug to reduce breast cancer incidence in high-risk women. However, the negative effects associated with the long-term use of tamoxifen have initiated the search for compounds that are more effective but less toxic. The discovery of raloxifene (RAL), which functions as a potent antiestrogen in the breast but an estrogen receptor (ER) agonist in the bone and cardiovascular system with very little uterotrophic activity, provided an alternative strategy to the targeted use of tamoxifen. The aim of this study was to evaluate RAL in combination with 5-fluorouracil (5-FU)/trimetrexate (TMX) to determine the most effective regimes and cellular mechanism of action to mitigate trimetrexate cytotoxicity in human bone marrow cells. *Materials and Methods:* The cell viability was performed using the Quick Cell Proliferation Assay by exposing the cells to TMX, 5-FU and RAL alone; RAL 24 h prior to 5-FU followed 2 h by TMX, and 5-FU 2 h prior to TMX followed 24 h by RAL determined the sequence-dependent

*interaction between TMX, 5-FU and RAL on the proliferation. Results:* The growth rate in MCF-7 in late RAL was  $34.75 \pm 4.79\%$  of the control, whereas in bone marrow the same drug combination exhibits a significant protection against TMX cytotoxicity with late RAL yielding  $51.25 \pm 4.43\%$  of the control. The findings were also supported by Cell flow cytometry and Western blot analysis. *Conclusion:* Sequence-dependent administration of RAL in combination with 5-FU/TMX can act against TMX toxicity in human bone marrow, while not affecting the maximum inhibitory effect of TMX in breast cancer.

*Abbreviations:* Raloxifene (RAL), trimetrexate (TMX), 5-fluorouracil (5-FU), TMX-polyglutamates (TMXPGs), dihydrofolate reductase (DHFR), aminoimidazolecarboxamide (AICAR), estrogen receptor (ER), selective estrogen receptor modulator (SERM).

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*Key Words:* Raloxifene, trimetrexate, 5-fluorouracil, estrogen receptor, selective estrogen receptor modulator, antifolate, dihydrofolate reductase.

Breast cancer remains a major health problem as incidence rates continue to increase despite recent improvements in the mortality rate. In the United States, breast cancer has the highest incidence (31%) and second highest mortality rate (15%) of all cancers among women (1). Clinical trials evaluating drugs for the treatment of breast cancer have been ongoing for many decades and have resulted in the development of several effective drugs that can reduce the risk of recurrence and death from this disease. There is convincing evidence that adjuvant systemic chemotherapy increases survival of patients with breast cancer (2). Selective estrogen receptor modulators (SERMs) play a key role in breast cancer chemoprevention (3). SERMs are non-steroidal compounds that exhibit a unique tissue-selective pharmacology: they are agonists in tissues (bone, liver and the cardiovascular system), antagonists in other tissues (brain and breast), and mixed agonists/antagonists in the uterus, through specific, high-affinity binding to estrogen receptor (ER). Tamoxifen, a triphenylethylene SERM, is the only agent approved for the treatment of breast cancer in women at high-risk for the disease (4). However, tamoxifen has been associated with increased risk of potentially life-threatening conditions, such as endometrial cancer uterine cancer and thromboembolic events (5). Raloxifene (RAL), a benzothiophene SERM that is

chemically distinct from tamoxifen and estradiol, is approved for the treatment and prevention of osteoporosis in post-menopausal women (6) and is currently being studied for breast cancer risk reduction effects in the Continuing Outcome Relevant to Evista (CORE) (7, 8). Additionally, Raloxifene Use for The Heart (RUTH) seems to have a positive effect on cardiac autonomic regulation in post-menopausal women with osteoporosis (9). The Study of Tamoxifen and Raloxifene (STAR) National Surgical Adjuvant Breast and Bowel Project (NSABP) trials are ongoing to reduce the incidence of breast cancer in post-menopausal women at high risk of breast cancer (10). In the Multiple Outcome of Raloxifene Evaluation (MORE) study, an osteoporosis treatment trial, RAL therapy was associated with a reduced incidence of invasive, ER-positive breast cancer compared with placebo 76%, without the increased risk of endometrial cancer (11). RAL also reduced spinal fracture risk in patients with low bone mineral density with (48%) or without (35%) prevalent vertebral fracture (12).

Trimetrexate (TMX) is a non-classical, lipophilic, non-polyglutamyl antifolate, which enters cells *via* passive diffusion (13, 14) and binds tightly to dihydrofolate reductase (DHFR) (15). As a result of these properties, TMX is effective against methotrexate (MTX) resistant cells by virtue of impaired transport and increase in DHFR (16). Clinical studies have shown that treatment with TMX had encouraging results. TMX in combination with 5-fluorouracil (5-FU) could result in synergistic, additive, or antagonistic effects on tumor growth inhibition and cytotoxicity based on sequence and timing of drug exposure (17). While synergistic interactions lead to improved antineoplastic effects, these interactions also enhance drug toxicity and the myelosuppressive effect of TMX and 5-FU limits their use (18). Previous studies from this laboratory (19) have demonstrated that a priming and non-toxic dose of 5-FU protect bone marrow from high-dose TMX cytotoxicity. Studies have shown that the sequence-dependent administration of RAL in combination with 5-FU/MTX has maximum antineoplastic activity in breast cancer, while at the same time provides protection to the human bone marrow (20). Recent studies from this laboratory also have shown that RAL attenuation of 5-FU/TMX cytotoxicity to breast cancer cells is sequence-dependent. In the present study a similar approach was used for TMX in combination with RAL and 5-FU to protect human bone marrow from TMX cytotoxicity and increase the therapeutic utility of TMX in the treatment of breast cancer.

## Materials and Methods

Trimetrexate glucuronate was obtained from US Bioscience, Inc. (West Conshohocken, PA, USA). 5-Fluorouracil, raloxifene hydrochloride and Trypan blue dye were purchased from Sigma Chemical Company (St. Louis, MO, USA). An early passage of

human bone marrow (HS-5) and breast cancer cell line (MCF-7) were obtained from 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) and Primary (Rb) antibody was from BD Biosciences, CA, USA.

*Cell culture.* Stock cultures of HS-5 bone marrow cells were grown in 150 cm<sup>2</sup> flasks and incubated in RPMI 1640 media (Cellgro, Mediatech Inc., VA, USA). For each experiment, 1x10<sup>6</sup> cells were plated in 100-mm-tissue culture Petri dishes. One group of cells maintained without any drugs served as the control and the remaining groups were exposed to TMX, 5-FU and RAL alone, RAL 24 h prior to 5-FU followed 2 h by TMX, and 5-FU 2 h prior to TMX followed 24 h by RAL. Drug concentrations were 10 μM TMX, 1 μM 5-FU and 10 μM RAL. Cells were exposed for 48 h.

*Assessment of cell viability by the Quick Cell Proliferation Assay and the Trypan blue dye exclusion assay.* Cell viability studies were performed using the Quick Cell Proliferation Assay, which is a spectrophotometric procedure that eliminates biasness. The Trypan blue dye exclusion assay is subject to the possible biasness of the observer. The HS-5 and MCF-7 (1.5x10<sup>4</sup> cells) growing in 96-well plates in RPMI 1640 media in presence or absence of drugs that were added in the same sequence as described above for viability assay. After 48 h of exposure, 10 μl of WST-1/ECS solution was added and incubated for a further 4 h at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. The assay was stopped by adding 10 μl of 1% SDS into each well and shaking thoroughly. The formazan dye produced by viable cells was quantified by measuring the absorbance of the dye solution at 440 nm using microtiter plate reader. The percent viability was calculated comparing the absorbance of treated cells to the control (corresponding to 100% viable cells). Furthermore, for the Trypan blue dye exclusion assay cells growing in 100 mm Petri dishes were exposed to different drug concentrations as mentioned above. After 48 h exposure cells were released by trypsinization, stained with 0.2% Trypan blue dye and counted in a hemocytometer.

*Cell cycle analysis.* Cell cycle perturbations induced by the inhibitor were analyzed by propidium iodide (PI) DNA staining as previously described (21). The PI intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that can be detected in the orange range of the spectrum using a 562-588 nm band pass filter. The cells were grown in six-well plates (1x10<sup>5</sup> cells per well) in the presence or absence of above-mentioned drugs. After 48 h of exposure cells were collected, plated and fixed in ice-cold 70% ethanol for 4 h at 4 °C. Ethanol-suspended cells were then centrifuged at 200 xg for 5 min and washed twice in PBS to remove residual ethanol. Pellets were suspended in 1 ml of propidium iodide/RNase A reagent and incubated at 37 °C for 30 min. Cell cycle profiles were obtained using a BD FacScan Cell flow Cytometer (Becton Dickinson Italia). Data were analyzed by ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

*Western blot analysis.* The cells were scraped from 100 mm Petri dishes following drug exposure and homogenized using lysis buffer containing protease inhibitor (cocktail tablet complete, from

Roche). The cell lysates were centrifuged at 5,000 xg for 10 min at 4°C. The supernatant was collected and protein concentration was measured using BCA Protein assay with bovine serum albumin as standard. Equal amounts of protein (20 µg) were loaded onto a 7.5% SDS-PAGE then transferred to a polyvinylidenedifluoride (PVDF) membrane. The membrane was probed with Primary antibody (mouse anti-human retinoblastoma protein Rb monoclonal antibody 1:1,000) and followed by horseradish peroxidase-conjugated Secondary antibody (Goat anti-mouse IgG, 1:10,000). Antibody detection was performed using enhanced Chemoluminescence reagents Super Signal West Dura. The membrane was exposed to Hyperfilm MP (Amersham Biosciences) and developed using Kodak GBX developer and fixer. Densitometric analysis was conducted using AIS densitometric computer based Imaging System program (Imaging Research Inc., Canada) to quantify the intensity of bands from five independent Western blots.

*Statistical analyses.* Data were expressed as mean±standard error. Statistical differences within and between treatment groups were determined in HS-5 and MCF-7 by One-way ANOVA followed by *post-hoc* Newman-Keuls Multiple comparison test. The  $p < 0.05$  was considered statistically significant. Data were analyzed for both control and treatment groups using Graphpad Prism 3 (Graphpad Software, Inc., San Diego, CA, USA).

## Results

*Effect of TMX, 5-FU and RAL on the growth of human bone marrow and breast cancer cell lines.* The growth rate in HS-5 for TMX, 5-FU and RAL alone was 38.5±3.11%, 67.3±4.56%, and 95.0±7.89% of the control, respectively. The growth rate for early RAL was 68.0±3.21% and for late RAL 51.25±4.43% of the control. A significant reduction in cells growth compared to the control, as well as RAL alone was observed with TMX and 5-FU. Late RAL showed increased cytotoxicity than early RAL and also showed significant reduction when compared with control. The consistent results were evidenced by the direct cell counts using the Trypan blue dye exclusion method and Quick Cell Proliferation assay (Figure 1A and B). RAL alone had no significant effect compared to control, whereas RAL with 5-FU and TMX combination showed significant decrease in growth rate compared to the control. The growth rate in MCF-7 with early RAL was 68.25±4.11%, and late RAL 34.75±4.79% of the control, whereas in bone marrow with early RAL combination yielding 68.0±3.21%, and late 51.25±4.43%, compared to the control. The late RAL combination exhibits a significant protective effect on the TMX cytotoxicity in bone marrow cells and at the same time maintains antineoplastic activity in breast cancer cells. The level of significance with late RAL was  $p < 0.001$  when MCF-7 cells were compared to bone marrow cells (Figure 2).

*Effect of TMX, 5-FU and RAL on the cell cycle progression of human bone marrow cell line.* Cell flow cytometry analysis:

Cell flow cytometry was used to determine the effect of RAL on the progression of cells when exposed to S-phase agents; the above-mentioned treatment groups were analyzed. The cell cycle profile in (Figure 3A) is representative of four independent experiments including the six treatment groups in HS-5. Figure 3B shows percent of cells in S-phase. Analysis revealed that the highest number of cells progressing to the S-phase of the cell cycle was found in 5-FU followed by early RAL, then late RAL. They were significant when compared to the control, as well as RAL alone. The lowest number of cells entering S-phase was observed in cells treated with RAL alone.

*Western blot analysis.* The retinoblastoma protein (Rb), a cell cycle regulator, which, when phosphorylated, allows the progression of cells from G<sub>1</sub>- to S-phase, was used as a marker to determine the effects of early RAL and late RAL on cellular progression at the molecular level. The Western blot (Figure 4A) is representative of five independent experiments including the six treatment groups. The relative optical density (ROD) is shown in Figure 4B. The highest density was observed in 5-FU and early RAL followed by late RAL and TMX indicated higher phosphorylation. The lowest density was found in RAL 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 and early RAL followed by, late RAL; they were significant when compared to the control, as well as RAL alone. The results coincide with the Cell flow cytometry results.

## Discussion

Results from this laboratory have previously shown that TMX and 5-FU combinations on the growth of MCF-7 breast cancer cells were independent of sequence administration, a priming- and non-toxic 5-FU dose protecting against TMX cytotoxicity in human bone marrow while not affecting the maximum inhibitory effect of TMX in breast cancer (19). This study raises a new element in the potential for dihydrofolate (DHF) polyglutamates to influence the selective effects of a priming- and non-toxic 5-FU dose and TMX. The selective effect of TMX in breast cancer might result from the formation of DHF polyglutamates and feedback inhibition of thymidylate synthase and aminoimidazolecarboxamide (AICAR) transformylase by DHF-polyglutamates (22). In bone marrow, little or no DHF-polyglutamates form when exposed to TMX, and, therefore, feedback inhibition on thymidylate synthase and AICAR transformylase would be insignificant. Hence, sequence dependency in bone marrow might best be related to 5-FU conserving reduced-folates to protect against the direct effects of TMX.

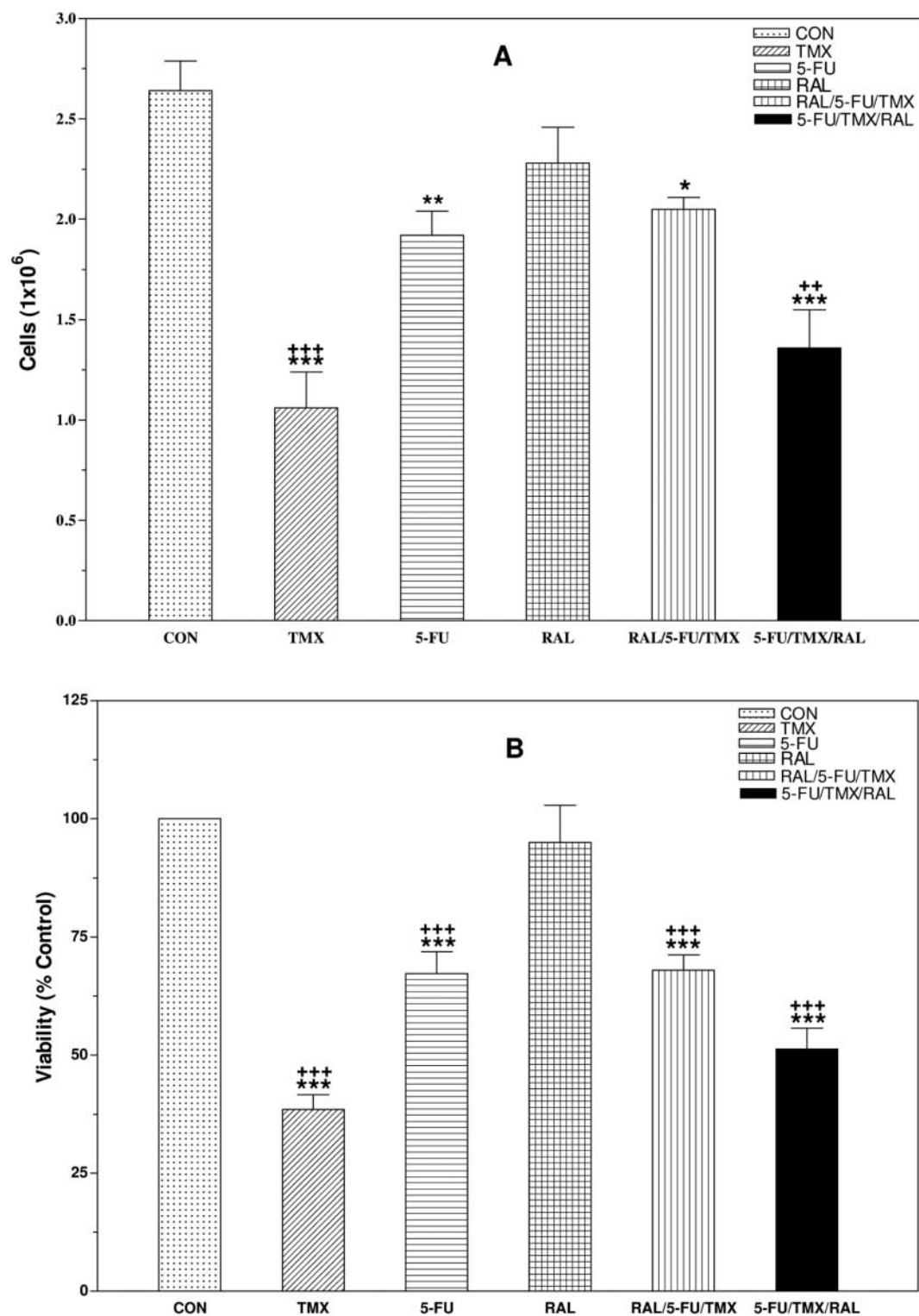


Figure 1. The sequence-dependent interaction between TMX, 5-FU and RAL on the proliferation of human bone marrow HS-5 cells. Cells were exposed to 10  $\mu$ M TMX, 1  $\mu$ M 5-FU and 10  $\mu$ M RAL alone, RAL 24 h prior to 5-FU followed 2 h by TMX, and 5-FU 2 h prior to TMX followed 24 h by RAL. Total time of exposure was 48 h, (A) counted using the Trypan blue dye. (B) Viability of cells was determined by the Quick Cell Proliferation assays. Results represent mean  $\pm$  SEM of five independent experiments. Analysis of variance indicated a significant reduction compare with control (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001) and compare with RAL (+ $p$ <0.05, ++ $p$ <0.01, +++ $p$ <0.001).



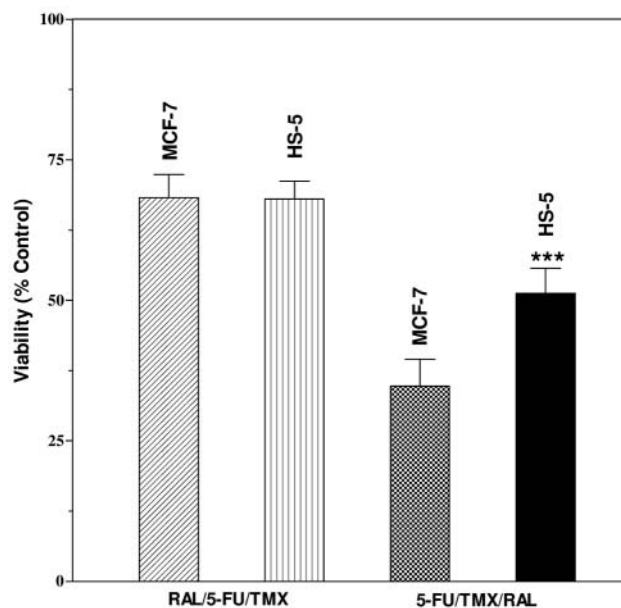


Figure 2. Effect of TMX, 5-FU and RAL on the growth of MCF-7 and HS-5 cells. Cells were exposed to 10  $\mu$ M TMX, 1  $\mu$ M 5-FU and 10  $\mu$ M RAL alone, RAL 24 h prior to 5-FU followed 2 h by TMX, and 5-FU 2 h prior to TMX followed 24 h by RAL. Total time of exposure was 48 h.

RAL alone did not exhibit significant toxicity, however, RAL combined with 5-FU and TMX showed significant toxicity. Late RAL was significantly more cytotoxic in MCF-7 breast cancer cells than HS-5 bone marrow cells. The selective cytotoxicity of late RAL may be due to the conservation of reduced folates in bone marrow by 5-FU as previously mentioned. Early RAL in the MCF-7 treated cells masked 5-FU/TMX cytotoxicity by binding to the estrogen receptor, inhibiting the progression of cells to the S-phase and inhibiting growth. RAL's effect on the estrogen receptor in bone marrow cells was not evident in these studies and may be due to a decreased amount of estrogen receptors in the bone marrow in comparison with the number of estrogen receptors found in breast cancer cells.

The mechanism of RAL's attenuation of 5-FU/TMX cytotoxicity became evident *via* Cell flow cytometry and Western blot analysis. RAL, like tamoxifen, (23) arrests cells in the G<sub>1</sub>-phase of the cell cycle, while 5-FU and TMX exert their effects on the S-phase. The percent of bone marrow cells exposed to early and late RAL entering the S-phase of the cell cycle showed similar pattern to MCF-7 cells exposed to early and late RAL. This suggests that the cytotoxicity observed in bone marrow when exposed to early and late RAL was not due to RAL interaction with the estrogen receptor but primarily to 5-FU and TMX administration. Late RAL was overall more cytotoxic than RAL alone or early RAL in both the MCF-7 breast cancer cells and HS-5 bone marrow cells. This may be

due to different mechanisms of cytotoxicity in the two cell types. Early RAL's inhibition of 5-FU/TMX cytotoxicity in MCF-7 breast cancer cells may be due to its inhibition of cellular progression to the S-phase of the cell cycle where 5-FU and TMX are most effective. Because RAL alone had no effect on the progression of bone marrow cells to the S-phase of the cell cycle and phosphorylation of the Rb protein, cytotoxicity of the late RAL regimen may be independent of RAL, but due to prolonged exposure of cells to 5-FU and TMX. In case of early RAL regimen cells were exposed for 24 h, whereas in late RAL combination cells were exposed for 48 h to 5-FU and TMX.

The retinoblastoma protein (Rb) is one of the key endogenous substrates of the G<sub>1</sub>-cyclin-dependent kinases (CDKs). The phosphorylation of Rb is an important step in the transition between G<sub>1</sub>- and S-phase of the cell cycle. Rb in its under-phosphorylated state prevents the progression of cells from G<sub>1</sub>- to S-phase. Following phosphorylation cells commit to progression through the remaining cycle (24). When phosphorylated, Rb releases a transcription factor of the E2F family that drives cells into S-phase. The results of Cell flow cytometry and Western blot, showed significantly fewer cells entering the S-phase of the cell cycle when cells treated with RAL alone. Early RAL in combination with 5-FU and TMX demonstrated a greater number of cells entering into S-phase compared with late RAL. Based on the sequence of RAL administration HS-5 cells were exposed for 48 h to the early RAL regimen and 24 h to the late RAL combination to RAL. Hence, RAL may, interfere with the transition between G<sub>1</sub>- and S-phase and a release of an E2F transcription factor, thereby, decreasing the activity of TMX, which arrest cells in S-phase. The growth rate of MCF-7 when compared to bone marrow the late RAL combination showed significant protection in bone marrow cell line. The findings suggest that sequence administration of late RAL provides a cytotoxic advantage against breast cancer cells since hematopoietic cells (bone marrow) are protected by a non toxic dose of 5-FU in combination with TMX.

In conclusion, the observations from this study have important implications regarding therapeutic dosing regime, which include TMX in combination with a priming non-toxic dose of 5-FU and RAL in the treatment of breast cancer while protecting the bone marrow. This may increase the therapeutic utility of TMX in breast cancer, given that TMX is most effective against methotrexate resistant cells by virtue of impaired transport and increase in binding to DHFR.

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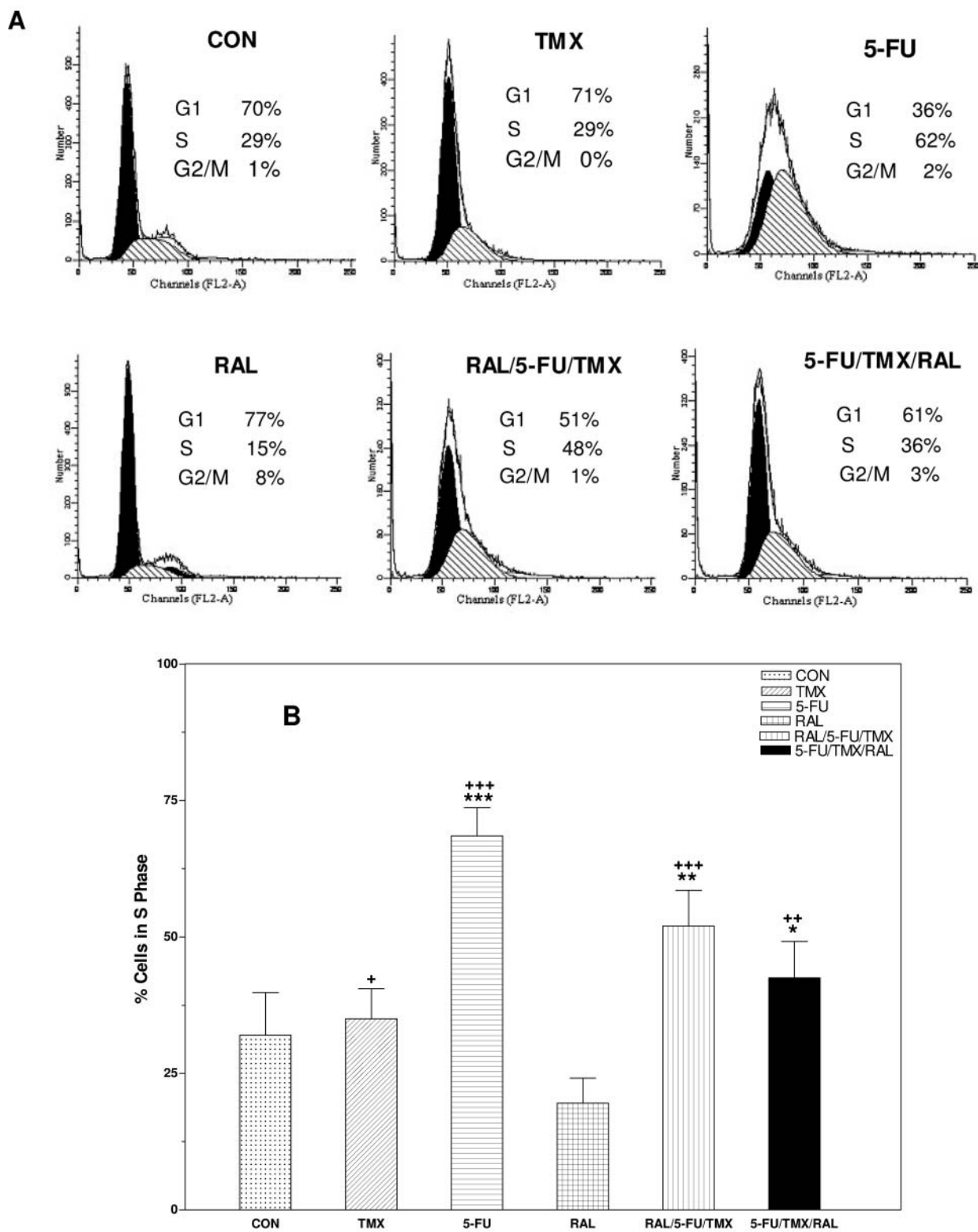


Figure 3. The sequence-dependent interaction between TMX, 5-FU and RAL on the cell cycle progression of human bone marrow HS-5 cells. Cells were exposed to 10  $\mu$ M TMX, 1  $\mu$ M 5-FU and 10  $\mu$ M RAL alone, RAL 24 h prior to 5-FU followed 2 h by TMX, and 5-FU 2 h prior to TMX followed 24 h by RAL. (A) The cell cycle profile is representative of four independent experiments. (B) Percent of cells in S-phase.

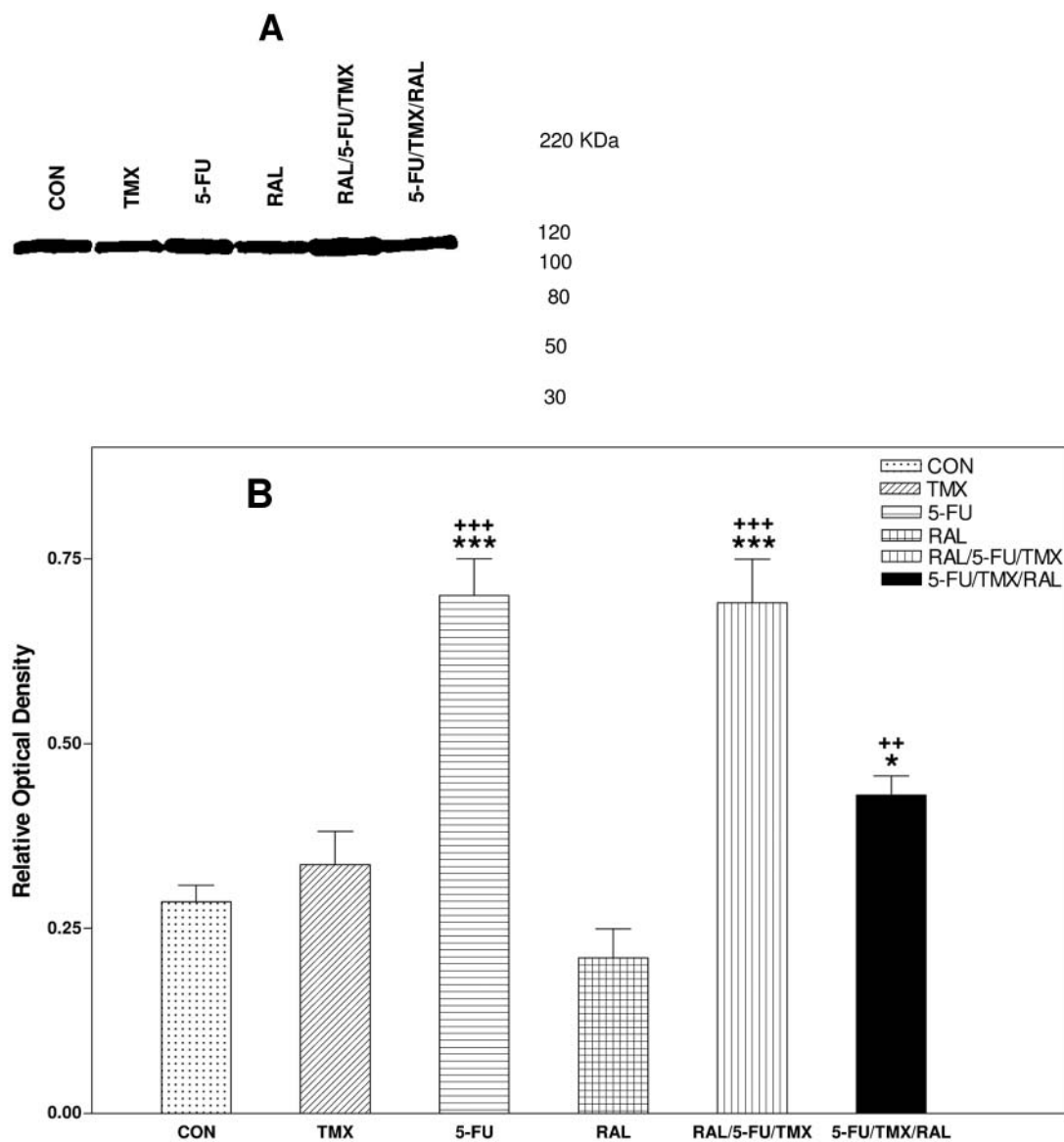


Figure 4. Effect of TMX, 5-FU and RAL on the level of retinoblastoma protein (Rb) as phosphorylated protein marker in human bone marrow HS-5 cells. Cells were exposed to 10  $\mu$ M TMX, 1  $\mu$ M 5-FU and 10  $\mu$ M RAL alone, RAL 24 h prior to 5-FU followed 2 h by TMX, and 5-FU 2 h prior to TMX followed 24 h by RAL. (A) The gel picture is the representative of five independent experiments. (B) Relative optical density.

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