

## Raloxifene and Selective Cell Cycle Specific Agents: A Case for the Inclusion of Raloxifene in Current Breast Cancer Treatment Therapies

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**Abstract.** *Background:* Breast cancer patients are at increased risk of osteoporosis. Contributing factors include age and/or chemotherapy. The selective estrogen modulator, raloxifene (RAL), effective in the prevention of breast cancer and approved for the treatment and prevention of osteoporosis, may prove beneficial in current breast cancer treatment modules. The purpose of this study was to evaluate RAL in combination with 5-fluorouracil (5-FU) and trimetrexate (TMX) to determine the most effective sequence in which to administer these cell cycle specific agents while taking into consideration the cellular mechanism of action. The goal was to maintain cytotoxicity to breast cancer cells and capitalize on the selective estrogen receptor modulatory effects of RAL. *Materials and Methods:* MCF-7 cells were exposed to (i) TMX, 5-FU or RAL alone, or (ii) RAL 24 h prior to 5-FU followed 2 h later by TMX, or (iii) 5-FU 2 h prior to TMX followed 24 h later by RAL. The cell viability was determined using the Quick Cell Proliferation Assay. *Results:* The growth rate of MCF-7 cells exposed to early RAL was  $68.25 \pm 4.11\%$  that of the control, however, late RAL exposure produced a growth of  $34.75 \pm 4.79\%$  that of the

control. Late RAL maintained the cytotoxicity of the regimen. The findings were further supported by cell flow cytometry and Western blot analysis data. *Conclusion:* RAL given prior to 5-FU/TMX significantly compromised cytotoxicity to breast cancer cells.

The selective estrogen receptor modulator (SERM) raloxifene (RAL), which binds to and selectively inhibits the estrogen receptor, is used for the prevention and treatment of osteoporosis (1-3). RAL is not only effective in the prevention of breast cancer (4, 5) but has also been shown to be as effective in breast cancer prevention as the prototype SERM, tamoxifen (6). The ability of RAL to treat osteoporosis and its selective antiestrogen properties could be especially beneficial to the postmenopausal breast cancer patient. Postmenopausal survivors of breast cancer are at increased risk of low bone mineral density (BMD), increased fractures and ultimately osteoporosis (7). Incorporating RAL into existing, effective antineoplastic combinations may improve the prognosis of the node-positive breast cancer patient; however, RAL in combination with other antineoplastic agents has not been extensively studied in breast cancer. A breast cancer treatment regimen of interest used in the clinic consists of the classic antifolate methotrexate (MTX) and the fluoropyrimidine, 5-fluorouracil (5-FU). 5-FU is administered prior to MTX and selective antifolates to diminish cytotoxicity to bone marrow while maintaining cytotoxicity to breast cancer cells (8-11). Previously, this laboratory has shown that the sequence of RAL administration, relative to MTX, can affect the overall cytotoxicity to proliferating breast cancer cells (12, 13). The lipid soluble antifolate trimetrexate (TMX) is used when breast cancer cells become MTX-resistant.

*Abbreviations:* RAL: raloxifene; TMX: trimetrexate; 5-FU: 5-fluorouracil; DHFR: dihydrofolate reductase; SERM: selective estrogen receptor modulator; Rb: retinoblastoma; BMD: bone mineral density.

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RAL arrests cells in the G<sub>1</sub>-phase of the cell cycle. 5-FU and TMX arrest cells in the S-phase of the cell cycle. Because RAL and the antimetabolites 5-FU and TMX target different phases of the cell cycle and exhibit different mechanisms of action and clinical toxicity, we investigated the *in vitro* effects of the sequence of their administration on the growth of MCF-7 human breast cancer cells.

## Materials and Methods

**Cell viability assays: assessment of cell viability by manual cell counting.** MCF-7 cells were obtained from the American Type Tissue Culture (Manassas, VA, USA). Trimetrexate glucuronate was obtained from US Bioscience Inc., West Conshohocken, PA, USA. 5-FU and RAL were purchased from Sigma (St. Louis, MO, USA). Stock cultures of MCF-7 cells were grown in 150 cm<sup>3</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. After 24 h incubation in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C, with the exception of an untreated control group, cells in each of five 100 mm Petri dishes were exposed to (i) 10 μM TMX, 1 μM 5-FU, 10 μM RAL alone, (ii) 10 μM RAL 24 h prior to 1 μM 5-FU followed 2 h later by 10 μM TMX (early RAL combination), or (iii) 1 μM 5-FU 2 h prior to 10 μM TMX followed 24 h later by 10 μM RAL (late RAL combination). Following 48 h of exposure, cells were harvested. The viability of cells was determined by microscopic counting of the Trypan blue exposed cells with a hemocytometer.

**Assessment of cell viability using the Quick Cell Proliferation Assay.** Additional cell viability studies were performed using the automated Quick Cell Proliferation Assay (BioVision Research Products, Mountain View, CA, USA) to complement the Trypan blue dye exclusion assay. MCF-7 cells (1.5x10<sup>4</sup> cells) were exposed to the previously mentioned drugs as described above. Following 48 h of exposure, 10 μL of WST-1/ECS solution was added and to the cells which were incubated for an additional 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 a microtiter plate reader. The percent viability was calculated comparing the absorbance of treated cells to the control (corresponding to 100% viable cells).

**Cell flow cytometry analysis.** Upon completion of cell viability assays, cells were prepared for cell flow cytometric analysis using a FITC (fluorescein isothiocyanate) BrdU Flow Kit from BD Biosciences Pharmingen (San Diego CA, USA). Cells were rinsed in 5 mL of 1x DPBS (Dulbecco's phosphate-buffered saline), trypsinized, stained with the Trypan blue and counted. After placing 8x10<sup>5</sup> cells in 5 mL centrifuge tubes, cells were centrifuged at 300 xg for 5 min in 1 mL of staining buffer. The cell pellets were resuspended in 100 μL of cytofix/cytoperm buffer containing 4% formaldehyde. Following a 30-min fixation and permeabilization at room temperature, cells were washed and centrifuged as before at 4°C with wash buffer containing FBS. The cell pellets were permeabilized a second time. Staining was enhanced by incubating the samples for 10 min on ice with 100 μL of a 10% DMSO solution. After washing and centrifugation, the cell pellet was refixed *via* resuspension in 100 μL of cytofix/cytoperm buffer and incubated for 5 min. Cells were washed, centrifuged as

before and treated with 30 μg of DNase for 1 h at 37°C to expose incorporated BrdU. Following DNase treatment cells were washed, centrifuged, resuspended in 1 mL of wash buffer, and stored at 4°C overnight. Twenty-four hours later, cell samples were centrifuged at 300 xg for 5 min as before. The cell pellets were resuspended in 50 μL of fluorochrome-conjugated anti-BrdU antibody and incubated at room temperature for 20 min. The cell pellets were washed, centrifuged, resuspended in 1 mL of staining buffer and analyzed with a BD FACScan cell flow cytometer (Becton Dickinson Italia).

**Western blot analysis.** Twenty μg of protein, as measured using BCA protein assay with bovine serum albumin as standard was isolated from MCF-7 cells and loaded onto a 7.5% SDS gel. Following SDS-PAGE separation, protein was transferred to a PVDF membrane, exposed to primary antibody (mouse anti-human retinoblastoma protein Rb monoclonal antibody 1:1000), followed by horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG, 1:10,000). Antibody detection was performed using enhanced chemiluminescent reagents Super Signal West Dura. The membrane was exposed to Hyperfilm MP (Amersham Biosciences UK Ltd., Buckinghamshire, Little Chalfont, UK) and developed using Kodak GBX developer and fixer. Densitometric analysis was conducted using an AIS densitometric computer based Imaging System (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 the MCF-7 cell line by one-way ANOVA followed by Newman-Keuls multiple comparison test. *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

**Effects of TMX, 5-FU and RAL on the proliferation of MCF-7 human breast cancer cells.** The growth rate of MCF-7 cells exposed to TMX, 5-FU or RAL alone was 39.75 ± 6.24%, 82.0 ± 12.09% and 63.0 ± 8.04% that of the control, respectively. The growth rate for early RAL was 68.25 ± 4.11% and for late RAL 34.75 ± 4.79% that of the control. Late RAL showed more cytotoxicity than early RAL and also showed a significant reduction when compared to the control. These results were supported by the direct cell counts using the Trypan blue dye exclusion method and Quick Cell Proliferation assay (Figure 1A and B).

**Effects of RAL, 5-FU, and TMX on cellular progression to the S-phase of the cell cycle.** 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 2A) is representative of five independent experiments using the six treatment groups. Early RAL administration corresponded with less cytotoxicity and fewer cells entering the S-phase of the cell cycle when compared to late RAL, which corresponded with greater cytotoxicity and an increased

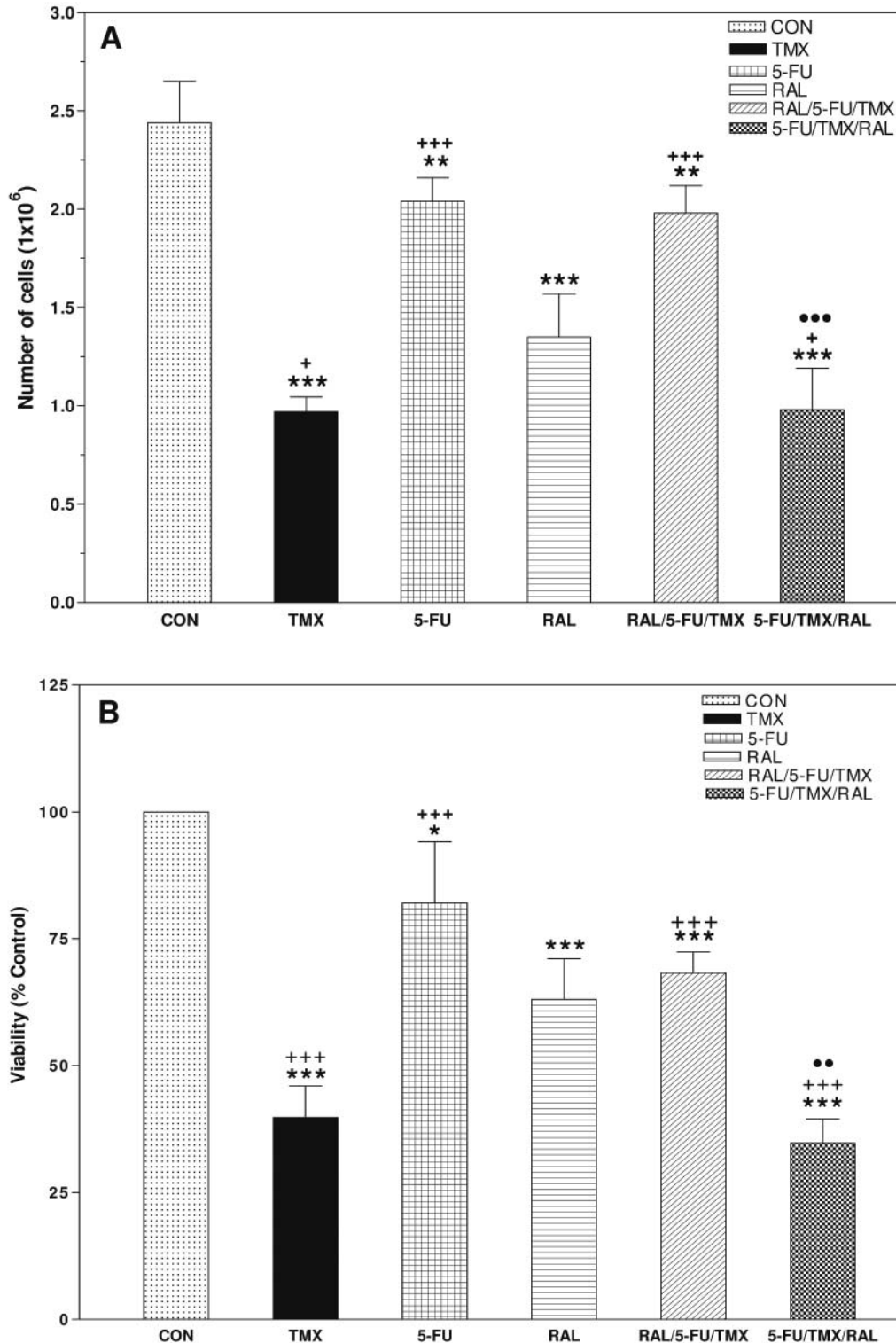


Figure 1. The sequence-dependent interaction between TMX, 5-FU and RAL on the proliferation of human breast cancer MCF-7 cells. Cells were exposed to (i) 10  $\mu$ M TMX, 1  $\mu$ M 5-FU or 10  $\mu$ M RAL alone, (ii) RAL 24 h prior to 5-FU followed 2 h later by TMX, or (iii) 5-FU 2 h prior to TMX followed 24 h later by RAL. Total time of exposure was 48 h. A: Cell number was counted using the Trypan blue dye. B: Viability of cells was determined by the Quick Cell Proliferation assay. Results represent mean  $\pm$  SEM of five independent experiments. Analysis of variance indicated a significant reduction compared with control (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001), compared with RAL (+ $p$ <0.05, ++ $p$ <0.01, +++ $p$ <0.001), and early RAL compared with late RAL (••• $p$ <0.01, •• $p$ <0.001).

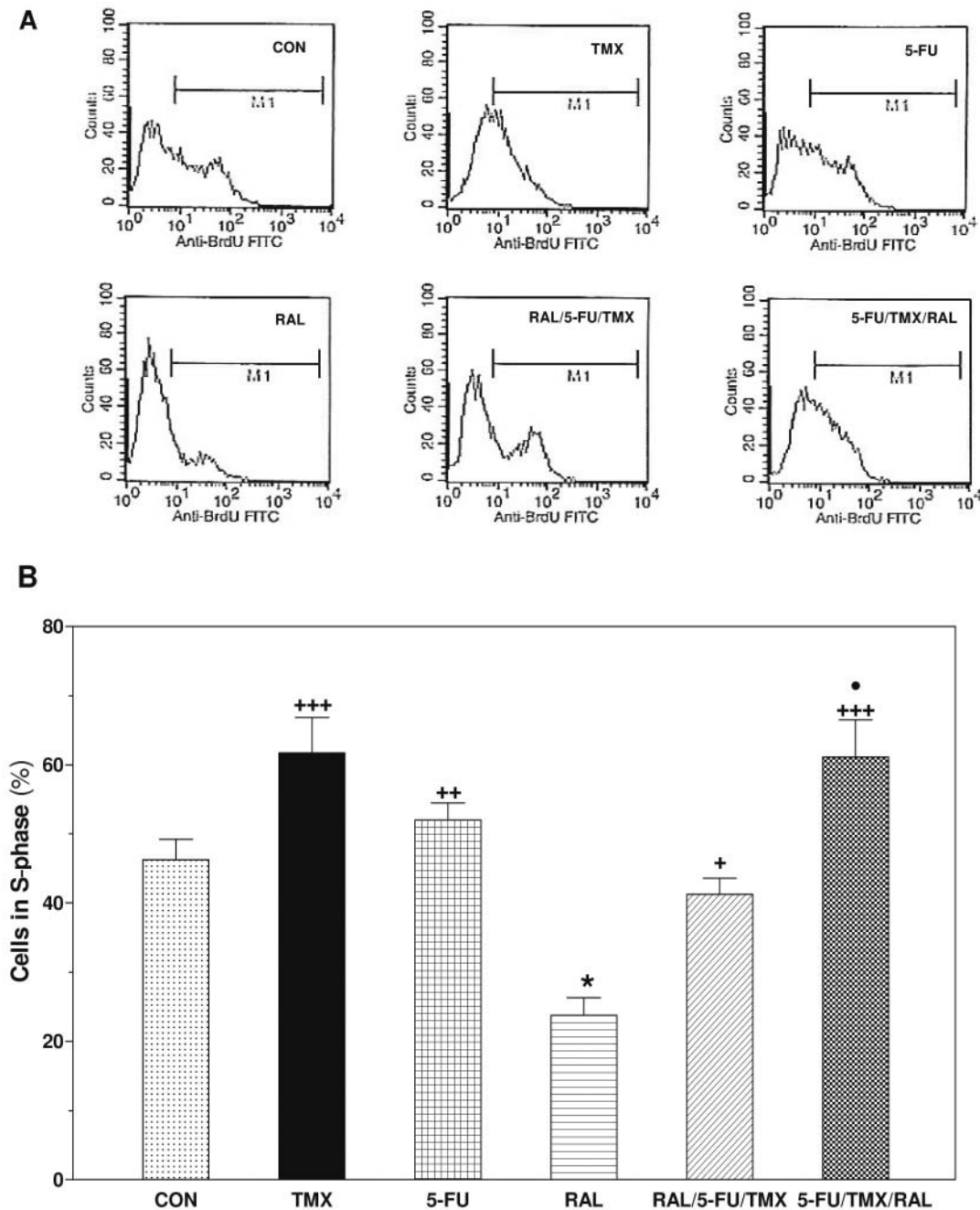


Figure 2. The sequence-dependent interaction between TMX, 5-FU and RAL on the cell cycle progression of human breast cancer MCF-7 cells. Cells were exposed to (i) 10 μM TMX, 1 μM 5-FU or 10 μM RAL alone, (ii) RAL 24 h prior to 5-FU followed 2 h later by TMX, or (iii) 5-FU 2 h prior to TMX followed 24 h later by RAL. A: The cell cycle profile is representative of four independent experiments. B: Percent of cells in S-phase. Analysis of variance indicated a significant increase compared with control (\* $p < 0.05$ ), compared with RAL (+ $p < 0.05$ , ++ $p < 0.01$ , +++ $p < 0.001$ ) and early RAL compared with late RAL (\* $p < 0.05$ ).

number of cells entering the S-phase of the cell cycle (Figure 2B). Late RAL showed significant increase of cells progressing to the S-phase compared to RAL alone and the early RAL combination.

*Western blot analysis.* The retinoblastoma protein (Rb), a cell cycle regulator which when phosphorylated allows the progression of cells from the G<sub>1</sub>- to the S-phase, was used as a marker to determine the effects of early RAL and late

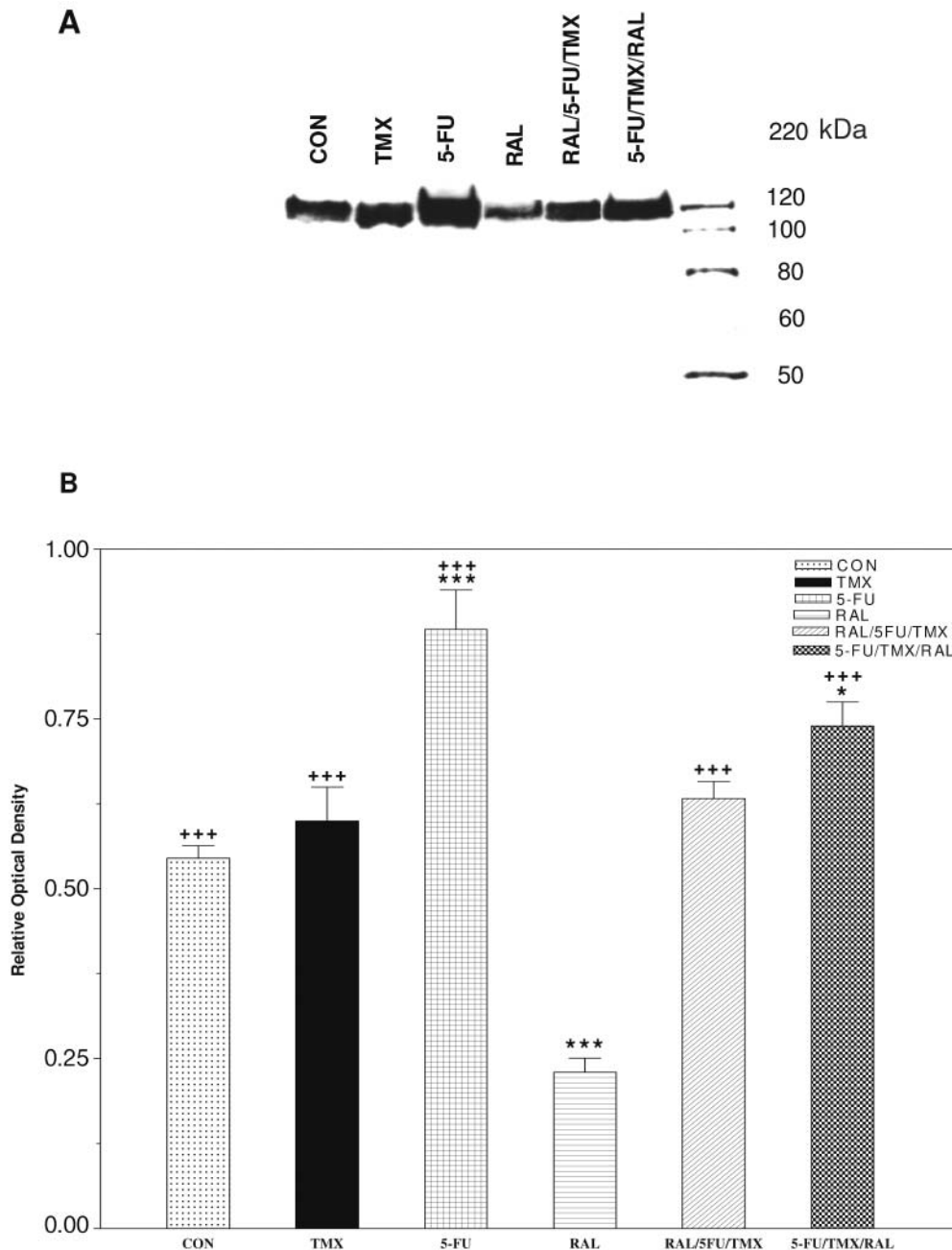


Figure 3. Effect of TMX, 5-FU and RAL on the level of retinoblastoma protein (Rb) as phosphorylated protein marker in human breast cancer MCF-7 cells. Cells were exposed to (i) 10  $\mu$ M TMX, 1  $\mu$ M 5-FU or 10  $\mu$ M RAL alone, (ii) RAL 24 h prior to 5-FU followed 2 h later by TMX, or (iii) 5-FU 2 h prior to TMX followed 24 h later by RAL. A: The gel image is the representative of five independent experiments. B: Relative optical density. Analysis of variance indicated a significant increase compared with control ( $*p < 0.05$ ,  $**p < 0.001$ ), compared with RAL ( $+++p < 0.001$ ).

RAL on cellular progression at the molecular level. The Western blot (Figure 3A) is representative of five independent experiments using the six treatment groups. The relative optical density (ROD) is shown in (Figure 3B). The optical density of the late RAL treatment group was

significantly greater than the early RAL treatment group, an indication of enhanced cellular progression to the S-phase of the cell cycle and greater phosphorylation. This observation corresponds with cell flow cytometry data which showed significantly fewer cells entering the S-phase of the

cell cycle for RAL alone and early RAL in combination with 5-FU and TMX than when RAL was administered last preceded by 5-FU and TMX.

## Discussion

The goal of this study was to determine if RAL could effectively be included in antifolate/fluoropyrimidine combination chemotherapy for the treatment of breast cancer, as well as to illustrate the relationship between the cytotoxicity of selective cell cycle specific agents and the sequence in which they are administered.

MCF-7 human breast cancer cells were assayed for viability to determine the cytotoxic effects of TMX, 5-FU, and RAL alone and in combination. Results showed early RAL was less cytotoxic to breast cancer cells than late RAL. Late RAL administration, preceded by 5-FU and TMX, exhibited enhanced cytotoxicity compared to RAL administration alone. The late RAL combination, due to greater cytotoxicity, is a more desirable combination for treating breast cancer than RAL alone or the early RAL regimen.

Cells exposed to TMX alone were one-half as viable as the control in this study. The early RAL combination did not demonstrate additive, enhanced, or synergistic cytotoxicity when compared to TMX alone. TMX alone and the late RAL combination were not significantly different. The early RAL combination appears to mask TMX cytotoxicity, which is manifested when RAL administration is reversed. In addition to the effect of late RAL on breast cancer cells, there may be several benefits of incorporating RAL into a 5-FU/TMX regimen such as the promotion of bone mineral density with RAL which will be discussed in further detail later.

The mechanism of early RAL attenuation of 5-FU/TMX cytotoxicity became evident *via* cell flow cytometric experiments. RAL, like tamoxifen, (14) arrests cells in the G<sub>1</sub>-phase of the cell cycle. 5-FU and TMX exert their effects on the S-phase of the cell cycle. RAL alone inhibited the progression of cells from the G<sub>1</sub>-phase of the cell cycle to the S-phase, in some cases by nearly 80%. Early RAL brought about a significant reduction in G<sub>1</sub>- to S-phase cellular progression when compared to the late RAL combination. When comparing early RAL and late RAL, the percent of cells entering the S-phase of the cell cycle was proportional to the cytotoxicity to breast cancer cells. Evaluating their effects on the phosphorylation of the Rb protein further elucidated the effects of the early RAL and late RAL combinations. Rb is a cell cycle regulating protein that exerts its effects during the early portion of the G<sub>1</sub>-phase. Rb in its underphosphorylated state prevents the progression of cells from the G<sub>1</sub>- to the S-phase. Following phosphorylation, cells commit to progression through the remaining cycle (15). Cells that lack the Rb protein contain inactivated Rb or alterations in the Rb activation pathway

and will enter the S-phase without regulation from Rb, as evident in numerous tumors including retinoblastoma, cervical carcinomas, esophageal and breast cancer (15). Analysis of Western blot studies illustrated the effect of early RAL and late RAL on the phosphorylation of Rb. Early RAL in combination with 5-FU and TMX caused less Rb phosphorylation than late RAL, providing further insight into the mechanism of action of early RAL attenuation of 5-FU/TMX at the molecular level.

The addition of RAL to a 5-FU and TMX regimen, based on the time of exposure used in this study, did not demonstrate an antineoplastic advantage over TMX alone. The utilization of RAL in a regimen inclusive of 5-FU and TMX to treat breast cancer will require further investigation however; we have shown in the present study that a regimen consisting of RAL followed by 5-FU and TMX is significantly cytotoxic to breast cancer cells. In addition to its toxico-therapeutic effects in the breast cancer patient when used in combination with 5-FU and TMX, RAL could be useful in a 5-FU/TMX regimen for patients undergoing treatment for breast cancer who are also at risk of developing osteoporosis. According to the National Institutes of Health Osteoporosis and Related Bone Diseases National Resource Center (NIH ORBD NRC), the majority of breast cancers occur in women in their fifties. The risk of breast cancer increases with age (16). This age group overlaps with a growing population at increased risk of developing osteoporosis. The National Osteoporosis Foundation estimates that osteoporosis is a major threat for 55% of people at 50 years of age and older. Postmenopausal breast cancer survivors are also at increased risk of osteoporosis (7). Breast cancer treatment can decrease circulating estrogen and induce early menopause for many premenopausal patients. These patients could benefit from the estrogenic bone density promoting properties of RAL.

Data from this study postulates that the sequence in which RAL is incorporated into existing effective antineoplastic therapies is very important. The pharmacokinetic properties of RAL must be taken into consideration before its inclusion in existing antineoplastic therapies *in vivo*. Raloxifene has been reported to have poor bioavailability (17). Similar studies will need to be conducted *in vivo* to fully ascertain the benefits of RAL incorporation into breast cancer treatment. However, if findings from this study are representative of the effect of RAL on 5-FU and TMX and their treatment of the breast cancer patient, the administration of RAL should follow a 5-FU/TMX regimen or the cytotoxicity of 5-FU/TMX may be compromised. This observation is not limited to 5-FU/TMX and may also be extrapolated to other antineoplastic agents specific for the S-phase. The observations from this study have important implications regarding therapeutic dosing schedules of antifolates in combination with 5-FU and RAL in the clinical setting.

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