Abstract. Background: Increasing evidence suggests that adjuvant systemic chemotherapy is necessary for the survival of breast cancer patients. Antitumor agents are more effective when used in combination with drugs exhibiting different mechanisms of action than when used alone. Previous studies from this laboratory have shown that raloxifene (RAL) attenuation of 5-fluorouracil/methotrexate (5-FU/MTX) cytotoxicity to breast cancer cells was sequence-dependent. The aim was to evaluate the same combination of RAL, 5-FU and MTX to determine the most effective regimes and cellular mechanisms of action to mitigate MTX cytotoxicity in human bone marrow cells.

Materials and Methods: The sequence-dependent interaction among MTX, 5-FU and RAL on the proliferation and viability of human bone marrow HS-5 cells was determined by the MTT assay and the Trypan blue dye exclusion assay by exposing the cells to MTX, 5-FU and RAL alone, RAL 24 h prior to 5-FU followed 2 h later by MTX, and 5-FU 2 h prior to MTX followed 24 h later by RAL. The control cells were untreated. Results: The growth rate in MCF-7 in early RAL was 68±3.07% and late RAL 37±2.05% of the control rate, whereas in bone marrow the same drug combinations exhibit a significant protection against MTX cytotoxicity, with the early RAL combination yielding 81±3.77% and late 54±2.74% of the control. The finding was further supported by cell flow cytometry and Western blot analysis.

Conclusion: Sequence-dependent administration of RAL in combination with 5-FU/MTX may have maximum antineoplastic activity in breast cancer while at the same time provide protection to human bone marrow.

Breast cancer is still a leading cause of cancer death in women in the United States. There is convincing evidence that adjuvant systemic chemotherapy increases the survival of patients with breast cancer (1, 2). In women with estrogen receptor-positive breast cancer, 5 years of adjuvant tamoxifene has been shown to reduce disease recurrence rates by almost half and decrease mortality by a third (3). However, tamoxifene has been associated with increased risk of potentially life-threatening conditions such as endometrial cancer, uterine cancer and thromboembolic events (for reviews see 4, 5 and 6). Raloxifene (RAL) is a nonsteroidal benzothiophene that, like tamoxifene, has been classified as a selective estrogen receptor modulator (SERM) on the basis of studies in which it produced both estrogen agonistic effects on bone and estrogen antagonistic effects on uterine endometrium and breast tissue (for reviews see 4, 5 and 6). Because of its ideal tissue selectivity, RAL may have fewer side effects than tamoxifene. RAL is also an agent for the prevention of osteoporosis in postmenopausal women.

Adjuvant chemotherapy with methotrexate (MTX) and 5-fluorouracil (5-FU) frequently causes thrombocytopenia and leucopenia. The major problems with the use of MTX and 5-FU include: i) the lack of selectivity between diseased and normal cells and ii) equitoxicity of sequential MTX and 5-FU in tumor and hematopoietic stem cells. Studies from this laboratory showed that a high dose of MTX in combination with 5-FU was independent of sequence in 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 the bone marrow and increased the cytotoxic selectivity for breast cancer cells (7-9). When 5-FU precedes MTX, 5-FU

Abbreviations: Methotrexate (MTX), 5-fluorouracil (5-FU), raloxifene (RAL), MTX-polyglutamates (MTXPGs).

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dimmishes the need for leucovorin rescue for MTX cytotoxicity. Desoto et al. (10) reported that RAL in combination with high-dose MTX increased cytotoxicity in a breast cancer cell line (MCF-7) in a sequence- and time-dependent manner. Recent studies from this laboratory (11) also showed that RAL attenuation of 5-FU/MTX cytotoxicity to breast cancer cells was sequence-dependent. Late RAL was more cytotoxic to the MCF-7 breast cancer cell line than early RAL. The goal of treatment for breast cancer patients receiving chemotherapy and/or hormonal therapy is to maximize cytotoxicity to the breast cancer cells and minimize cytotoxicity to the bone marrow. Hence, the present study was designed to determine the role of sequence-dependent administration of RAL, when used in combination with 5-FU/MTX on cytotoxicity to human breast cancer and protection of bone marrow cells.

Materials and Methods

Methotrexate hydrate, 5-fluouracil, raloxifene hydrochloride and Trypan blue dye were purchased from Sigma Chemical Company (St. Louis, MO, USA). Early passage human bone marrow (HS-5) and breast cancer cell lines (MCF-7) 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). The FITC BrdU Flow Kit and primary (Rb) antibody were from BD Biosciences.

Cell culture. Stock cultures of HS-5 bone marrow cells were grown in 150-cm² flasks and incubated in RPMI 1640 media (Cellgro, Mediatech Inc.). For each experimental point, 1x10⁶ 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 of cells were exposed to MTX, 5-FU and RAL alone, RAL 24 h prior to 5-FU followed 2 h later by MTX, and 5-FU 2 h prior to MTX followed 24 h later by RAL. The drug concentrations were 10 ìM MTX, 1 ìM 5-FU and 10 ìM RAL. The cells were exposed for 48 h.

Assessment of cell viability by the MTT assay and the Trypan blue dye exclusion assay. Cell viability studies were performed using a modified form of the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay (12). Briefly, 1.5x10⁴ cells of the HS-5 and MCF-7 cell lines, growing in 96-well plates in RPMI 1640 media in the presence or absence of drugs, were added in the same sequence as described above. After 48-h exposure, 30 ìl of MTT (5 mg/ml) was added and incubated for a further 4 h at 37°C in a humidified atmosphere with 5% CO₂. The media were removed and the formazan crystals were solubilized with 100 ìl of 0.04 M HCl in isopropanol for 15 min. Absorbance was measured at 550 nm. The percent viability was calculated by comparing the absorbance of the 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, the cells were released by trypsinization, stained with 0.2% Trypan blue and counted in a hemocytometer.

Cell cycle analysis. The cell cycle was analyzed by cell flow cytometry using the FITC BrdU Flow Kit. 8x10⁵ cells were fixed and permeabilized using cytofix/cytoperm reagent and treated with 30 µg of DNsperase for 1 h at 37°C to incorporate the BrdU. Following DNase treatment, the cells were incubated with 50 µl of fluorochrome-conjugated anti-BrdU antibody at room temperature for 20 min. The cell pellets were washed, centrifuged, re-suspended in 1 ml of staining buffer and analyzed with a BD FACScan cell flow cytometer. Data acquisition (10,000 events for each sample) was performed using CELLQuest Software (Becton Dickinson Italia).

Western blot analysis. The cells were scraped from the 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 5000 xg for 10 min at 4°C. The supernatant was collected and the protein concentration was measured using the 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 were probed with 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 the enhanced Chemoluminescence reagents Super Signal West Dura. The membrane was exposed to Hyperfilm MP (Amersham Biosciences) and developed using the Kodak GBX developer and fixer. Densitometric analysis was conducted using the AIS densitometric computer-based Imaging System (Imaging Research Inc., Canada) program to quantify the intensity of the bands from five independent Western blots.

Statistical analyses. The data were expressed as mean±standard error. Statistical differences within and between the treatment groups were determined in the HS-5 and MCF-7 cells by one-way ANOVA followed by post-hoc Newman-Keuls multiple comparison test. P<0.05 was considered statistically significant. The data were analyzed for both the control and treatment groups using Graphpad Prism 3 (Graphpad Software, Inc., San Diego, CA, USA). Cell flow cytometry data analysis was performed using CELLQuest Software.

Results

Effects of MTX, 5-FU and RAL on the growth of human bone marrow and breast cancer cell lines. The growth rate in HS-5 cells with MTX, 5-FU and RAL alone was 46±1.75%, 59±5.3% and 89±10.05% of the control rate, respectively. The growth rate for early RAL was 81±3.77% and for late RAL 54±2.74% of the control rate. A significant reduction in cell growth compared to the control was observed with MTX and 5-FU. Similar results were found when compared with RAL alone. Late RAL showed more cytotoxicity than early RAL. The consistent results were evidenced by the direct cell counts using the Trypan blue dye exclusion method and MTT assay (Figure 1 A, B). RAL alone had no significant effect compared with the control, whereas RAL with the 5-FU and MTX combination showed significantly
decreased growth rate compared with the control rate. The growth rate in MCF-7 with early RAL was 68±3.07%, and with late RAL 37±2.05% of the control rate, whereas in bone marrow the same drug combinations exhibited a significant protective effect on MTX cytotoxicity, with the early RAL combination yielding 81±3.77% and late RAL

Figure 1. The sequence-dependent interaction between MTX, 5-FU and RAL on the proliferation of human bone marrow HS-5 cells. The cells were exposed to 10 μM MTX, 1 μM 5-FU and 10 μM RAL alone, RAL 24 h prior to 5-FU followed 2 h later by MTX, and 5-FU 2 h prior to MTX followed 24 h later by RAL. The total exposure time was 48 h. The cells were harvested (A) and counted using Trypan blue. (B) The viability of the cells was determined by MTT assay. The results represent mean±SEM of four independent experiments. The analysis of variance indicated a significant increase or decrease compared with the control (*p<0.05, **p<0.01, ***p<0.001) and compared with RAL (*p<0.05, ++p<0.01, +++p<0.001).
Figure 2. The sequence-dependent interaction between MTX, 5-FU and RAL on the cell cycle progression of human bone marrow HS-5 cells. The cells were exposed to 10 μM MTX, 1 μM 5-FU and 10 μM RAL alone; RAL 24 h prior to 5-FU followed 2 h later by MTX, and 5-FU 2 h prior to MTX followed 24 h later by RAL. The total exposure time was 48 h. (A) Cell flow cytometry were performed using the BrdU Kit. The graph is representative of three independent experiments. (B) Percent of cells in S-phase.
54±2.74%, compared to the control. The level of significance with early RAL was $p<0.05$ and with late RAL was $p<0.01$ (Figure 3 A).

**Effect of MTX, 5-FU and RAL on the cell cycle progression of the 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 exposed to S-phase agents; the above-mentioned treatment groups were analyzed. The cell flow cytometry histogram in (Figure 2A) is representative of three independent experiments inclusive of the six treatment groups in HS-5. Analysis revealed that the highest number of cells progressing to the S-phase of the cell cycle was found with 5-FU followed by early RAL, then late RAL (Figure 2B). They were significant when compared to the control as well as RAL alone. The early and late RAL combinations involving HS-5 and MCF-7 cell flow cytometry data (11) showed a significant increase in early RAL and a non-significant increase in late RAL in the bone marrow.

**Western blot analysis.** The retinoblastoma protein (Rb), a cell cycle regulator, which when phosphorylated allows the progression of cells from $G_1$-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 the representative of five independent experiments inclusive of the six treatment groups. The relative optical density (ROD) is shown in Figure 4B. The highest ROD was observed in 5-FU and early RAL, followed by the control and late RAL, indicating higher phosphorylation. The lowest ROD was found for MTX followed by RAL, indicating fewer phosphorylated Rb proteins were present.

**Discussion**

In previous studies from this laboratory, 5-FU was administered prior to MTX, not for additive cytotoxicity but to protect the bone marrow cells and eliminate the need for leucovorin rescue of MTX cytotoxicity (7-9). The selective effect of MTX in breast cancer may be due to the formation of MTX-polyglutamates (MTXPGs) (13) and the
inability of 5-FU to prevent the inhibitory effects of MTX and MTXPGs. The formation of MTXPGs allows for the inhibition of dehydrofolate reductase, thymidylate synthase and other folate-requiring enzymes not directly affected by MTX (14). Bone marrow formed little or no MTXPG when exposed to MTX (15), meaning that certain folate-requiring enzymes would not have been inhibited. Hence, sequence-dependency in the bone marrow appears to be related to 5-FU conserving reduced-folate to protect against the direct effect of MTX (7, 8). RAL alone did not exhibit significant toxicity, however, RAL combined with 5-FU and MTX showed significant toxicity. Late RAL was significantly more cytotoxic in MCF-7 breast cancer cells than in HS-5 bone marrow cells. This selective cytotoxicity may be due to the conservation of reduced folates in bone marrow by 5-FU as described. The effect of RAL on bone marrow cells was similar to the control. Early RAL in the MCF-7-treated cells masked 5-FU/MTX cytotoxicity by binding to the estrogen receptor, inhibiting the progression of cells to the S-phase and inhibiting growth. Its 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 to the amount of estrogen receptors found in breast cancer cells.

The mechanism of early RAL’s attenuation of 5-FU/MTX cytotoxicity became evident via cell flow cytometric experiments (Figure 2 A, B). RAL, like tamoxifen (16), arrests cells in the G1-phase of the cell cycle. 5-FU and MTX exert their effects on the S-phase of the cell cycle. The percentage of bone marrow cells exposed to early and late RAL entering the S-phase of the cell cycle was statistically similar to the control. Early RAL in the MCF-7-treated cells masked 5-FU/MTX cytotoxicity by binding to the estrogen receptor, inhibiting the progression of cells to the S-phase and inhibiting growth. This suggests that the cytotoxicity observed in bone marrow when exposed to early and late RAL was not due to RAL’s interaction with the estrogen receptor, but primarily to 5-FU and MTX 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 differing mechanisms of cytotoxicity in the two cell types. Early RAL’s inhibition of 5-FU/MTX cytotoxicity in MCF-7 breast cancer cells may arise from its inhibition of cellular progression to the S-phase of the cell cycle where 5-FU and MTX are more effective. Because RAL alone or the sequence of its administration had no effect on the progression of bone marrow cells to the S-phase and the phosphorylation of the Rb protein, the cytotoxicity of the late RAL regimen may be independent of RAL. Cytotoxicity may occur because of prolonged exposure to 5-FU and MTX. Based on the sequence of RAL administration, the HS-5 cells were exposed for 24 h in the early RAL regimen and 48 h in the late RAL combination with 5-FU and MTX.

Rb is one of the key endogenous substrates of the G1 cyclin-dependent kinase (CDK). The phosphorylation of Rb is an important step in the transition between the G1- and S-phases of the cell cycle, under-phosphorylation of Rb preventing this progression. Following phosphorylation, cells commit to progression through the remaining cycle (17). Rb, when phosphorylated, released a transcription factor of the E2F family that drives cells into the S-phase. RAL may, therefore, interfere with the transition between the G1- and S-phases and release the E2F transcription factor, thereby decreasing the activity of MTX. This observation corresponds with the cell flow cytometry results, which showed significantly fewer cells entering the S-phase of the cell cycle when the cells were treated with RAL alone. Early RAL in combination with 5-FU and MTX demonstrated a greater number of cells entering into the S-phase compared with late RAL. Our findings suggest that sequence administration of late RAL provides a cytotoxic advantage against breast cancer cells since hematopoietic cells (bone marrow) are protected by 5-FU/MTX. These observations have important implications in terms of the therapeutic dosing regime of MTX in combination with a non-toxic dose of 5-FU and RAL in the treatment of breast cancer while protecting the bone marrow.

In addition to its toxico-therapeutic effects in the breast cancer patient when used in combination with 5-FU and MTX, RAL could be useful in a 5-FU/MTX regimen for patients undergoing treatment for breast cancer who are also at risk of developing osteoporosis.

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References