Sequence-dependent Administration of Raloxifene and 5-Fluorouracil/Pemetrexed Protects against Pemetrexed Cytotoxicity in Human Bone Marrow

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Abstract. *Background: Pemetrexed (Alimta®) is a new-generation multitargeted antifolate that inhibits several key enzymes in the de novo pathways of pyrimidine and purine biosynthesis, including thymidylate synthase (TS), dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT). Alimta® has demonstrated antitumor activity in a broad array of human malignancies, e.g. breast, non-small cell lung cancer, malignant pleural mesothelioma and pancreatic, colorectal, gastric, bladder, head and neck cancer, and is currently in phase III clinical trials. It has been reported that a dose of 600 mg/m^2 of pemetrexed showed toxicity to bone marrow and the gastrointestinal system. The aim of this investigation was to evaluate raloxifene (RAL) in combination with 5-fluorouracil (5-FU)/pemetrexed multitargeted antifolate (MTA) to determine the most effective regimens and cellular mechanism of action to mitigate pemetrexed cytotoxicity in human bone marrow cells. Materials and Methods: In order to determine the sequence-dependent interaction between MTA, 5-FU and RAL on proliferation, cell viability was carried out using the Quick Cell Proliferation Assay by exposing the HS-5 and MCF-7 cells to (i) MTA, 5-FU and RAL alone, or (ii) RAL 24 h prior to 5-FU followed 2 h later by MTA, or (iii) 5-FU 2 h prior to MTA followed 24 h later by RAL. Results: The growth rate in MCF-7 in early RAL was 69±8.65% and late RAL was 36±4.6% of the control, whereas in bone marrow early RAL was 78±8.65% and late RAL was 52±5.49% of the control. The late RAL exhibits significant protection against MTA cytotoxicity in bone marrow. The findings were further supported by cell flow cytometry, apoptosis and Western blot analysis data. Conclusion: This study suggests that sequence-dependent administration of RAL (5FU/MTA/RAL), in combination with 5-FU/MTA, protects against MTA toxicity in human bone marrow while maintaining the maximum inhibitory effect of pemetrexed in breast cancer.*

The antimetabolites were among the first antineoplastic agents discovered (1). Antimetabolites act by disrupting cellular replication and division either by direct incorporation into DNA, such as the purine and pyrimidine analogues, or by interfering with the metabolic processes necessary for DNA synthesis, as antifolates do. All cells, both eukaryotic and prokaryotic, require folates for cell growth. Folates transfer one-carbon units in the enzymatic reactions of purine, thymidine and amino acid (histidine, serine, methionine) biosynthesis (2). Folate-dependent pathways are key targets in the development of anticancer agents. Research into the development of antifolates as anticancer agents has led to the development of the multitargeted antifolate Alimta® (MTA) (3, 4).

Pemetrexed (Alimta®) is a new-generation multitargeted antifolate (4-6). Alimta® is a novel pyrrolo[2,3-d]pyrimidine based antifolate. Unlike the "classic" antimetabolic drug methotrexate, which selectively targets a single enzyme, pemetrexed exerts its action by disrupting several folate-dependent metabolic processes essential for cell replication through inhibiting multiple enzymes (7, 8). Both the reduced folate carrier and membrane folate binding protein transport systems transport pemetrexed into cells. Once in the cell, MTA is converted to polyglutamate forms by the enzyme folypolyglutamate synthesis. The polyglutamate forms are retained in cells. MTA and its tri-and pentaglutamate

Abbreviations: Raloxifene (RAL), pemetrexed (Alimta MTA), 5-fluorouracil (5-FU), thymidylate synthase (TS), dihydrofolate reductase (DHFR), glycinamide ribonucleotide formyltransferase (GARFT), 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT), MTA polyglutamates (MTAPGs).

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derivatives (MTAPGs) have significant inhibitory activity against thymidylate synthase (TS), glycinamide ribonucleotide formyltransferase (GARFT), and to a lesser extent 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT) and dihydrofolate reductase (DHFR) (7, 8). Polyglutamation is a time- and concentration-dependent process that occurs in tumor cells and, to a lesser extent, in normal tissues. Polyglutamated metabolites have an increased intracellular half-life resulting in prolonged drug action in malignant cells. Pemetrexed has demonstrated broad antitumor activity in several solid tumors, including breast (9, 10), non-small cell lung cancer (11), malignant pleural mesothelioma (12, 13), cervical cancer (14), colorectal (15), pancreatic (16), gastric (17), bladder (18), head and neck cancer (19).

Pemetrexed (Alimta®) is the first agent approved by the US Food and Drug Administration in February 2004 for the treatment of malignant pleural mesothelioma (MPM) in combination with cisplatin in patients with unresectable disease or for whom curative surgery is not an option. (4). It has a moderate toxicity profile at a dose of 500 mg/m^2 with myelosuppression being the chief symptom of dose limiting toxicity (20). A higher dose of 600 mg/m^2 showed toxicity to bone marrow and the gastrointestinal system (9, 21). Folic acid co-administered with MTA decreased its toxicity while maintaining antitumor activity (20). In August 2004, pemetrexed was approved as a second-line, single agent treatment of locally advanced or metastatic non-small cell lung cancer (NSCLC) (4). In addition, pemetrexed has been studied as a single agent and in combination regimens in patients with breast, pancreatic, colorectal, gastric, genitourinary, head and neck cancer and is currently in Phase III clinical trials (20, 5).

Inhibition of DHFR and TS has shown proven benefit in breast cancer, and methotrexate and 5-FU are used in combination regimens in the treatment of metastatic breast cancer and as adjuvant therapy (5). With a mechanism overlapping both methotrexate and 5-FU with additional enzyme targets, pemetrexed may prove active and offer additional benefit in the treatment of breast cancer. Clinical development has also focused on combining pemetrexed with other agents active against breast cancer to identify highly functioning, well-tolerated regimens for the adjuvant setting. Combining agents that target different oncogenic pathways or multiple steps of the same cascade is a promising strategy to explore in developing the best regimen that will increase the options available to a physician to treat breast cancer patients. Preclinical evidence suggested that pemetrexed had additive or synergistic activity when combined with many other clinically important anticancer agents, including gemcitabine (Gemzar®), fluorouracil, carboplatin (Paraplatin®), oxaliplatin (Eloxatin®), paclitaxel, and vinorelbine (Navelbine®) (5, 22). Studies have shown that the sequence-dependent administration of raloxifene (RAL) in combination with 5-FU/methotrexate has maximum antineoplastic activity in breast cancer while at the same time provides protection to the human bone marrow (HS-5) cell line (23). Recent studies from this laboratory also have shown that RAL attenuation of 5-FU/MTA cytotoxicity to breast cancer cells was sequence-dependent (24). In the present study a similar approach was used for pemetrexed (MTA) in combination with RAL and priming- and non-toxic 5-FU in protecting human bone marrow cells from MTA cytotoxicity through conservation of reduced folates and increasing the additional therapeutic utility of multitargeted pemetrexed in the treatment of breast cancer.

Materials and Methods

Pemetrexed (Alimta®) was obtained from Eli Lilly and Company Ltd., Indianapolis IN, USA. 5-FU, 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 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. Stock cultures of HS-5 bone marrow cells 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 60 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 (i) MTA, 5-FU and RAL alone, (ii) RAL 24 h prior to 5-FU followed 2 h later by MTA (early RAL), or (iii) 5-FU 2 h prior to MTA followed 24 h later by RAL (late RAL). Drug concentrations were 10 μM MTA, 1 μM 5-FU and 10 μM RAL. 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 HS-5 and MCF-7 (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, Trypan blue dye exclusion assay was performed by staining the cells with 0.2% Trypan blue dye and then counting cells in a heamocytometer.

Cell cycle analysis. Cell cycle perturbations induced by the inhibitor were analyzed by propidium iodide (PI) DNA staining as described elsewhere (23). The cells were grown in 6-well plates (1x10^5 cells per well) in the presence or absence of the above-mentioned drugs.

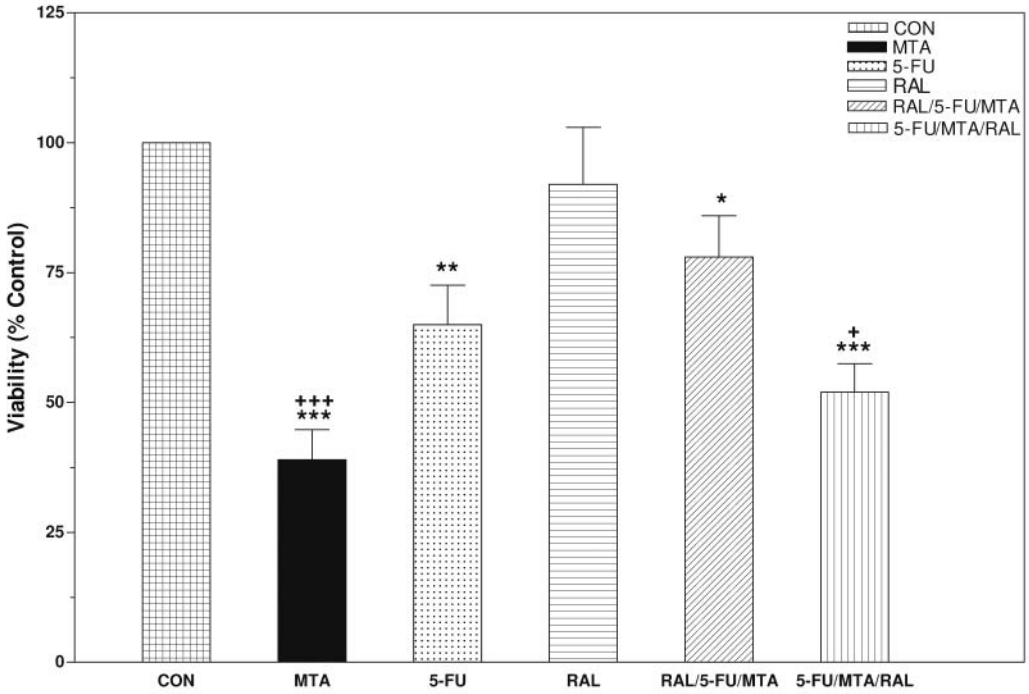

Figure 1. *The sequence-dependent interaction between MTA, 5-FU and RAL on the proliferation of human bone marrow HS-5 cells. Cells were exposed to (i) 10 μM MTA, 1 μM 5-FU and 10 μM RAL alone, (ii) RAL 24 h prior to 5-FU followed 2 h later by MTA, and (iii) 5-FU 2 h prior to MTA followed 24 h later by RAL. 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 with the control (*$p<0.05$, **$p<0.01$, ***$p<0.001$) and compared with RAL ($^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$).*

After 48 h of exposure cells were collected and evaluated after propidium iodide staining and 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).

Apoptosis assay. To quantify drug-induced apoptosis, annexin V/propidium iodide staining was carried out followed by flow cytometry. Briefly, after drug treatments, both floating and attached cells were combined and subjected to annexin V/propidium iodide staining using an annexin V-FITC apoptosis detection kit according to the protocol provided by the manufacturer. Untreated control cells were maintained in parallel to the drug treatment group. Double staining was used to distinguish between viable, early apoptotic and necrotic or late apoptotic cells. The resulting fluorescence was measured using flow cytometry using a FACScan flow cytometer (Becton Dickinson). According to this method, the lower left quadrant shows the viable cells, the upper left quadrant shows cell debris, the lower right quadrant shows the early apoptotic cells and the upper right quadrant shows the late apoptotic and necrotic cells.

Western blot analysis. Western blot was performed as described elsewhere (23). 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 (23).

Statistical analyses. Data were expressed as mean±standard error. Statistical differences within and between treatment groups were determined in HS-5 and MCF-7 cell lines 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

Effect of MTA, 5-FU and RAL on the growth of human bone marrow and breast cancer cell lines. The growth rate in HS-5 for MTA, 5-FU and RAL alone was 39±5.78%, 65±7.59%, and 92±10.98% that of the control, respectively. The growth rate for early RAL was 78±7.95% and for late RAL 52±5.49% that of the control. A significant reduction in cell growth compared to the control as well as RAL alone was observed with MTA and 5-FU. Late RAL showed greater cytotoxicity than early RAL and also showed significant growth reduction when compared with the control (Figure 1). RAL alone had no significant effect when compared with the control, whereas RAL with 5-FU and MTA combination significantly decreased the growth rate. The growth rate in

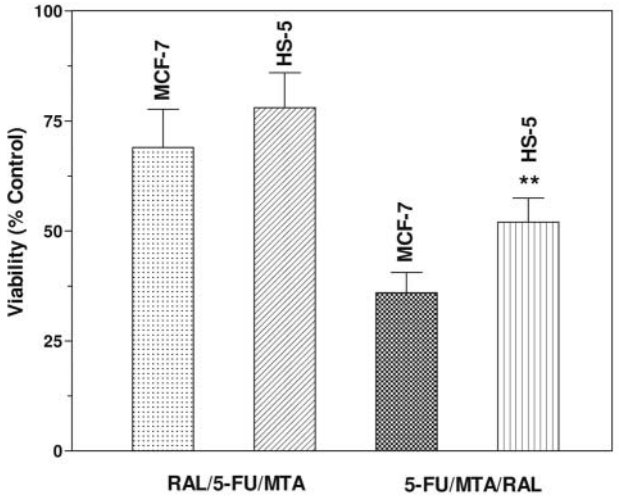

Figure 2. *Effects of MTA, 5-FU and RAL on the growth of MCF-7 and HS-5 cells. Cells were exposed to (i) 10 μM MTA, 1 μM 5-FU and 10 μM RAL alone, (ii) RAL 24 h prior to 5-FU followed 2 h later by MTA, and (iii) 5-FU 2 h prior to MTA followed 24 h later by RAL. Total time of exposure was 48 h. Analysis of variance indicated a significant increase in growth rate of HS-5 cells in late RAL compared with MCF-7 cells (**p<0.01).*

MCF-7 with early RAL was 69±8.65%, and late RAL 36±4.6% that of the control, whereas in bone marrow the early RAL combination yielded 78±7.95%, and the late 52±5.49%, compared to the control. The late RAL combination exhibited a significant protective effect on the MTA cytotoxity in bone marrow cells and same time maintained antineoplastic activity in breast cancer cells. The level of significance with late RAL was $p<0.01$ when MCF-7 cells were compared with bone marrow cells (Figure 2).

Effect of MTA, 5-FU and RAL on the cell cycle progression of human bone marrow cell line:

Cell flow cytometry analysis. Cell flow cytometry was used on the treatment groups to determine the effect of RAL on the progression of cells when exposed to S-phase agents. The cell cycle profile (Figure 3A) is representative of four independent experiments using the six treatment groups in 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 was found in 5-FU alone (52±5.21%) followed by MTA alone (38±3.5%), early RAL (32±4.15%) then late RAL (29±2.5%). These numbers were significantly greater when compared with RAL alone the lowest number of cells entering S-phase was observed in cells treated with RAL alone.

Apoptosis. Drug-induced apoptosis was measured using cell flow cytometry. The apoptosis profile (Figure 4A) is representative of four independent experiments using the six treatment groups in HS-5 cells. The percentage of early plus late apoptotic cells was highest with MTA alone (48±4.5%) followed by 5-FU alone (14±2.1%) then early RAL (10±1.5%) and late RAL (5±1%) as shown in Figure 4B. Significant reductions of apoptotic cell death were observed in early and late RAL combination compared with MTA and the late RAL combination showed no significant apoptosis when compared with control.

Western blot analysis. The retinoblastoma protein (Rb), a cell cycle regulator, which when phosphorylated allows the progression of cells from the G1-to the 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 shown in (Figure 5A) is representative of five independent experiments using the six treatment groups. The relative optical density (ROD) is shown in Figure 5B. The highest density was observed in 5-FU and early RAL, followed by late RAL and MTA, indicating higher phosphorylation. The lowest density was found in RAL alone, indicating 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. These differences were significant when compared to the control as well as to the results with RAL alone. Similar results were observed with the cell flow cytometry analysis.

Discussion

The pharmacological activity of pemetrexed is achieved after its conversion to a polyglutamated form; the pentaglutamated form is the most predominant (8). The formation of polyglutamated metabolites is essential for intracellular accumulation and retention of antimetabolites. Pemetrexed is one of the best known substrates for folypolyglutamate synthetase (FPGs) with an inhibition constant K_i of 1.6 μM, pemetrexed is polyglutamated 90-195 times more efficiently than methotrexate (25). In addition, the intracellular retention of the polyglutamated forms increases the affinity of pemetrexed to its cellular targets by several orders of magnitude.

Earlier studies from this laboratory showed that the effect RAL in combination with 5-FU/MTA on the growth of MCF-7 breast cancer cells was sequence-dependent (24). In the present study, the late RAL combination showed significant protection of the human bone marrow cells compared to the breast cancer cell line (Figure 2). This study raises a new element in the potential for dihydrofolate (DHF) polyglutamates to influence the selective effects of a priming non-toxic 5-FU dose with MTA. The selective cytotoxicity of MTA in breast cancer cells may be due to the formation of pentaglutamated

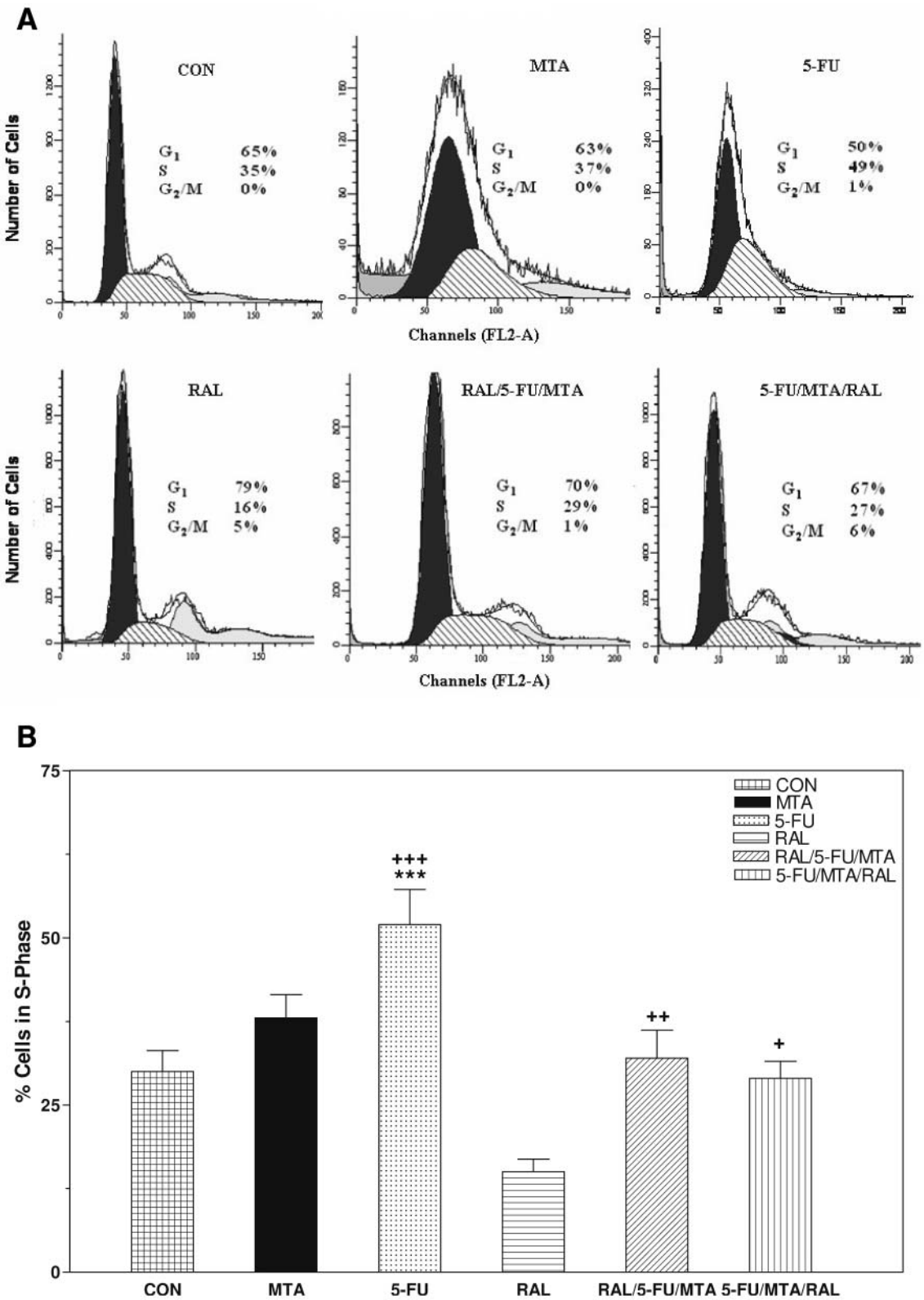

Figure 3. *The sequence-dependent interaction between MTA, 5-FU and RAL on the cell cycle progression of human bone marrow HS-5 cells. Cells were exposed to (i) 10 μM MTA, 1 μM 5-FU and 10 μM RAL alone, (ii) RAL 24 h prior to 5-FU followed 2 h later by MTA, and (iii) 5-FU 2 h prior to MTA followed 24 h later by RAL. A: The cell cycle profile is representative of four independent experiments. B: Percentage of cells in S-phase.*

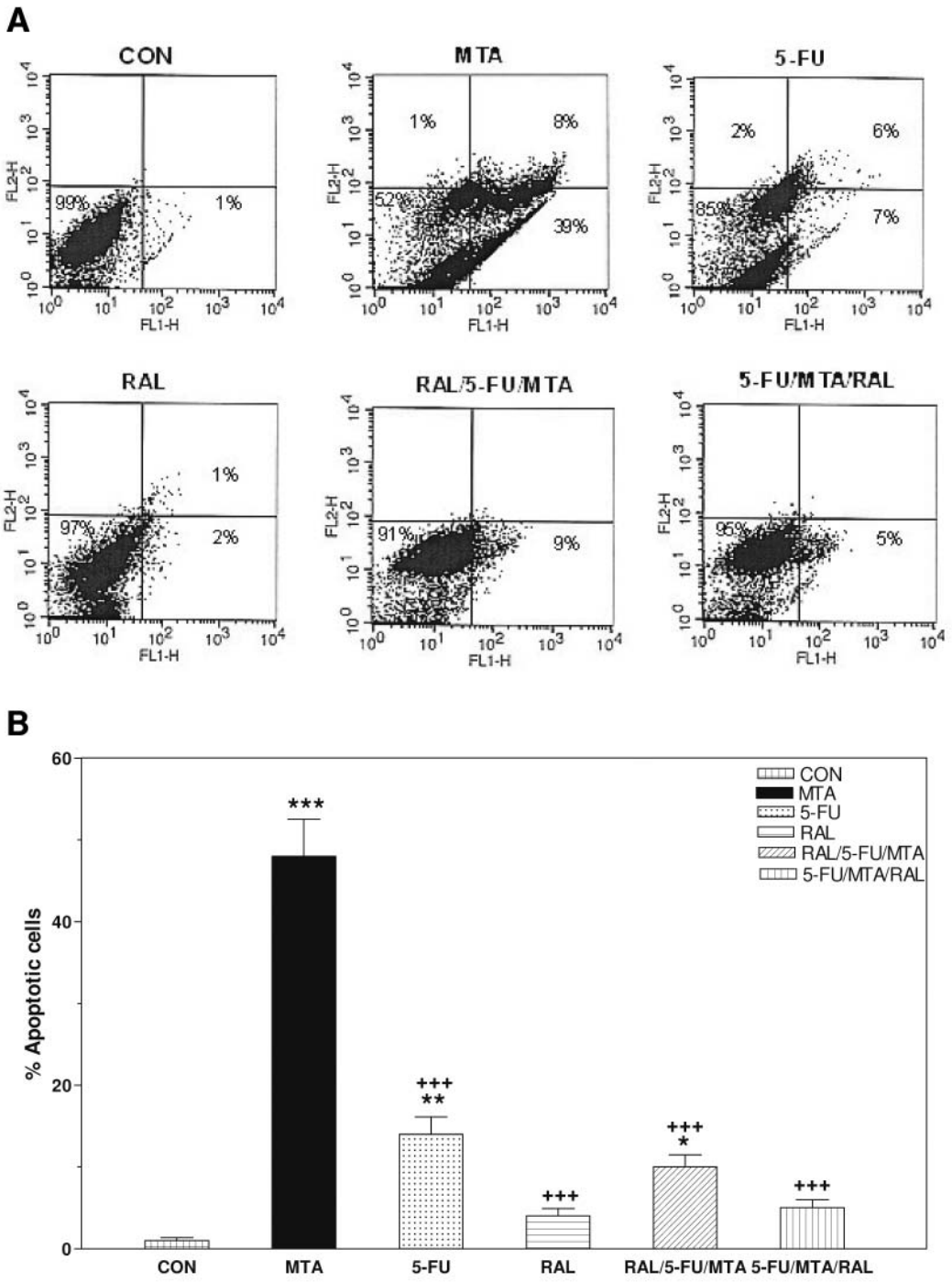

Figure 4. *Effects of MTA, 5-FU and RAL on the apoptosis of human bone marrow HS-5 cells. Cells were exposed to (i) 10 μM MTA, 1 μM 5-FU and 10 μM RAL alone, (ii) RAL 24 h prior to 5-FU followed 2 h later by MTA, and (iii) 5-FU 2 h prior to MTA followed 24 h later by RAL. A: Apoptosis analysis profile is representative of four independent experiments. B: Percent of early plus late apoptotic cells. Analysis of variance indicated a significant increase compared with control (*p<0.05, **p<0.01, ***p<0.001) or significant decrease compared with MTA ($^{+++}$p<0.001).*

pemetrexed and feedback inhibition of TS, GARFT, and to a lesser extent of AICARFT and DHFR (7, 8). Whereas in bone marrow, little or no DHF-polyglutamates form when exposed to MTA, and therefore, feedback inhibition on TS, GARFT and/or AICARFT would not be significant. Hence, sequence-dependency effect on bone marrow cells might best be related to 5-FU conserving reduced-folates to protect against the direct effects of

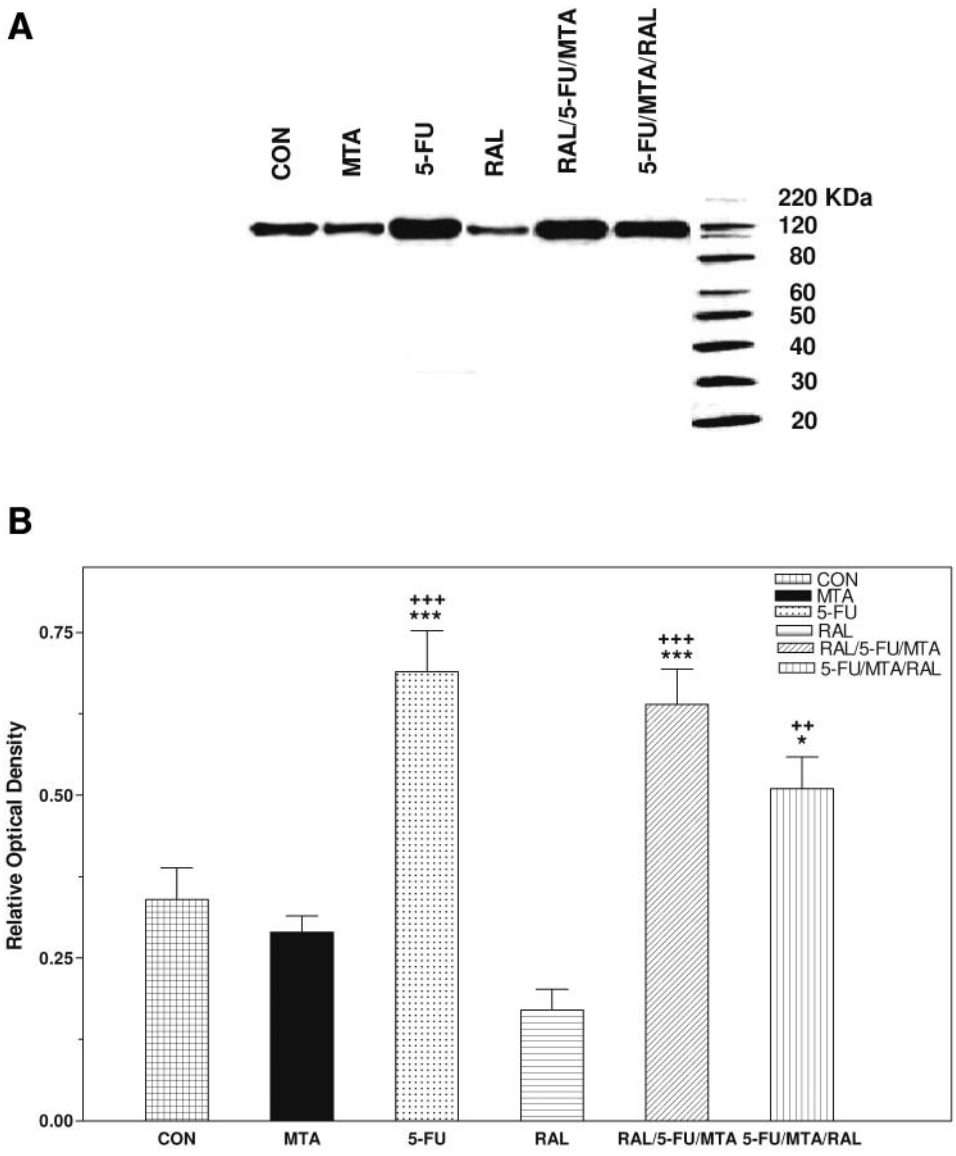

Figure 5. *Effect of MTA, 5-FU and RAL on the level of retinoblastoma protein (Rb) as phosphorylated protein marker in human bone marrow HS-5 cells. Cells were exposed to (i) 10 μM MTA, 1 μM 5-FU and 10 μM RAL alone, (ii) RAL 24 h prior to 5-FU followed 2 h later by MTA, and (iii) 5-FU 2 h prior to MTA followed 24 h later by RAL. A: The gel is representative of five independent experiments. B: Relative optical density.*

MTA. RAL alone did not exhibit significant toxicity, however, RAL combined with 5-FU and MTA 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.

The results of cell flow cytometry and Western blot showed significantly fewer cells entering the S-phase of the cell cycle when cells were treated with RAL alone. Early RAL in combination with 5-FU and MTA demonstrated a greater number of cells entering the S-phase compared with late RAL. Based on the sequence of RAL administration, HS-5 cells were exposed to RAL for 48 h with the early RAL regimen and 24 h with the late RAL regiman. Hence, RAL may interfere with the transition between the G1- and the S-phase and the release of an E2F transcription factor thereby decreasing the activity of MTA, which arrests cells in the S-phase. The growth rate of MCF-7 cells when compared with bone marrow cells using the late RAL combination showed significant protection in the bone marrow cell line. The findings suggest that the

administration of RAL late in the sequence provides a cytotoxic advantage for breast cancer cells since hematopoietic cells (bone marrow) are protected by a non-toxic dose of 5-FU in combination with MTA.

MTA forms a stable complex with TS and dUMP that inhibits binding of 5,10-methylene tetrahydrofolate to the enzyme (7, 8). TS inhibition causes nucleotide pool imbalances that result in S-phase cell cycle arrest and apoptosis. It is known that apoptosis-signaling pathways and cellular events controlling them have a profound effect both on cancer progression and in response to chemotherapy (26). Based on annexin V/propidium iodide staining it is clear that the high apoptotic cell death observed in MTA treated cells may have been due to MTA triggering or an enhanced apoptotic response in these cells. Pemetrexed was found to be a potent inducer of apoptosis in lymphocytic leukemia cells (27). The early and late RAL combination showed a significant reduction of apoptotic cell death compared to pemetrexed.

In conclusion, the observations from this study have important implications regarding therapeutic dosing regime, which include MTA 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 pemetrexed in breast cancer: it has a mechanism overlapping that of both methotrexate and 5-FU along with additional enzyme targets offering an additional benefit in the treatment of breast cancer while protecting the human bone marrow.

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