In Vitro Schedule-dependent Interactions between the Multitargeted Antifolate LY231514 and Gemcitabine in Human Colon Adenocarcinoma Cell Lines

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ABSTRACT

Purpose: Multitargeted antifolate (MTA) and gemcitabine (GEM) have shown preclinical and clinical activity in tumor histotypes such as colon, renal, small and non-small cell lung cancers, hepatomas and carcinoid tumors. In our study, we investigated the cytotoxic activity of MTA alone or in combination with GEM using different exposure schedules in three different colon cancer cell lines (LoVo, WiDr, and LRWZ).

Experimental Design: Cytotoxic activity was evaluated by sulforhodamine B assay, cell cycle perturbations and apoptosis were evaluated by flow cytometry, and thymidylate synthase expression was evaluated by immunohistochemical method.

Results: A 48-h exposure to MTA caused a minimal and no-dose-response effect on the three cell lines used. Flow cytometric analysis showed a cell accumulation in S phase that completely resolved in LoVo and LRWZ cell lines and persisted in WiDr cells after a 48-h washout. Moreover, a significant increase in thymidylate synthase expression was observed in all of the cell lines after MTA exposure.

Among the different combinations tested, the highest synergistic interaction, assessed using Kern’s method and expressed as the synergistic ratio index, was produced by pretreatment with GEM followed by MTA (ratio index: 1.3-6.7). It is possible that the depletion of nucleotide pools induced by MTA and required for DNA synthesis prevented cells from repairing DNA damage caused by GEM. The type and degree of drug interactions were not paralleled by apoptosis, which was almost always negligible, or by the type and persistency of the cell cycle perturbations.

Conclusions: Our results indicate that the sequential administration of GEM → MTA provides the greatest benefit in the clinical treatment of colon cancer.

INTRODUCTION

Rapidly proliferating cells are heavily dependent on an abundant supply of fully reduced folates, which are fundamental for the formation of nucleotides necessary for DNA synthesis and repair. This vulnerability has long been exploited in the area of anticancer research, and antifolates represent one of the most widely investigated classes of antineoplastic agents.

MTA2 (1) LY231514 (ALIMTA; Eli Lilly and Co., Indianapolis, IN) is representative of a new class of folate antimetabolites. Its antitumor effect works by inhibiting various enzymes of the folate pathways, including TS, dihydrofolate reductase, and glycaminide ribonucleotide formyltransferase (1). The involvement of these enzymatic targets in MTA cytotoxicity is supported by the fact that both thymidine and hypoxanthine are required to circumvent cellular death caused by MTA (1). Furthermore, MTA is an optimal substrate for foly polyglutamate synthetase, which causes extensive intracellular polyglutamation. This process converts the drug from a form that is rapidly extruded from the cell to one that is retained intracellularly for a prolonged period, producing a more sustained drug effect (2).

MTA has proven to be highly cytotoxic on a human leukemia cell line (1) and has also shown an anticolonogenic effect on tumor cells from different human histotypes, such as colon, renal, small and non-small cell lung cancers, hepatomas, and carcinoid tumors (3). Its ability to inhibit more than one enzymatic target may contribute to increase the spectrum of biochemical profiles of tumors potentially sensitive to the drug, thus avoiding the development of drug resistance.

GEM is a pyrimidine antimetabolite (difluorodeoxycytidine) that is anabolized sequentially to the monophosphate, diphosphate, and triphosphate nucleoside, intracellularly. The difluorodeoxycytidine triphosphate is incorporated into DNA, leading to chain termination. In addition, difluorodeoxycytidine diphosphate inhibits ribonucleotide reductase, an enzyme that catalyzes the formation of deoxynucleosides required for DNA synthesis, including dCTP. The reduction in the intracellular concentration of dCTP enhances the incorporation of GEM triphosphate into DNA (self-potentiation; Ref. 4). GEM has a wide-ranging activity in a variety of solid tumors (5) and is used for the treatment of pancreatic (6) and non-small cell lung (7) cancers. Furthermore, in experimental preclinical models, GEM

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2 The abbreviations used are: MTA, multitargeted antifolate; TS, thymidylate synthase; GEM, gemcitabine; RI, R index.
has proven effective against colon cancer cell lines, particularly in combination with other drugs (8–11).

The present study aimed to investigate the activity of the combination of two antimetabolites, MTA and GEM, which are capable of interfering with DNA through different mechanisms.

MATERIALS AND METHODS

Established Cell Lines. The study was performed using two established commercial colon adenocarcinoma cell lines (LoVo and WiDr) obtained from the American Type Culture Collection (Rockville, MD) and in a cell line (LRWZ) isolated in our laboratory and derived from a patient with a histologically proven diagnosis of colon adenocarcinoma.

Cells were maintained as a monolayer at 37°C and subcultured weekly. Culture medium was composed of DMEM:Ham’s F-12 (1:1) supplemented with FCS (10%), glutamine (2 mM), nonessential amino acids (1%; Mascia Brunelli s.p.a., Milan, Italy), and insulin (10 μg/ml; Sigma, Milan, Italy). Cells were used in the exponential growth phase for all of the experiments.

Drugs. MTA and GEM were supplied from Lilly (Sesto Fiorentino, Florence, Italy). GEM was diluted in sterile physiological solution at a concentration of 1 mg × ml⁻¹, and MTA was supplied at a concentration of 10 mg × ml⁻¹. The drugs were then divided into aliquots and stored at −70°C. Drug stocks were freshly diluted in culture medium before each experiment.

In Vitro Chemosensitivity Assay. Sulforhodamine B assay according to Skehan et al.’s method (12) was used. Briefly, cells were collected by trypsinization, counted, and plated at a density of 10,000 cells/well in 96-well, flat-bottomed microtitre plates (100 μl of cell suspension/well). Experiments were run in octuplet, and each experiment was repeated three times. After plating (18–24 h; a sufficient time for exponential growth recovery), 100 μl of culture medium containing or not the specific drugs were added to the wells. At the end of drug exposure, cells were fixed with 50% trichloroacetic acid at 4°C (50 μl/well, final concentration 10%) for 1 h. After five washes with tap water, cells were stained with 0.4% sulforhodamine B, dissolved in 1% acetic acid (100 μl/well) for 30 min, and subsequently washed four times with 1% acetic acid to remove unbound stain. Plates were air dried, and protein-bound stain was solubilized with 100 μl of 10 mM unbuffered Tris base. The absorbance of treated cells was determined at a wavelength of 540 nm by means of a fluorescence plate reader.

Single Drug Exposure. In the chemosensitivity assay, MTA and GEM were tested singly at scalar concentrations of 0.01, 0.1, 1, and 10 μg/ml. An exposure time of 48 h for each agent was chosen from the dose survival curves of single drugs as the time that produced the maximum effect. The cytotoxic effect was evaluated immediately after the end of drug exposure.

Drug Combinations. In simultaneous experiments, treatment exposure time was 48 h. In the sequential schemes, exposure time to each drug was 48 h, and the first drug was washed out before adding the second. At the end of the 48-h exposure to the first drug, the medium was removed from all of the wells using a multichannel pipette. Fresh culture medium (50 μl) was then added to all of the wells containing treated and untreated cells and was once again removed and discarded.

Finally, fresh culture medium containing or not the second drug was added to the treated samples and control cells, respectively, for a final volume of 200 μl/well.

The cytotoxic effect was evaluated immediately after the end of drug exposure in both simultaneous and sequential treatment schemes.

In all combination experiments, both drugs were tested at the four different concentrations used for single drug exposure (i.e., 0.01, 0.1, 1, and 10 μg/ml), at a ratio of 1:1.

Statistical Analysis. Differences between treatments (dose-response survival curves) were assessed by variance analysis (ANOVA). A P < 0.05 was considered significant.

Drug Interaction Analysis. Several methods have been proposed to evaluate the interaction between drugs, as critically analyzed by Zoli et al. (13), but most of them, such as the classic isobologram (14, 15), Steel and Peckham’s isobologram method (16), the isobologram method subsequently improved by Kano (17), the median effect principle according to Chou-Talalay (18), and others (19), are not suitable for drugs with a low cytotoxic effect or no dose-response curves, as in the case of MTA. Therefore, we used Kern’s method (20), subsequently...
were resuspended in ice-cold ethanol 70% and stored overnight 1% paraformaldehyde in PBS on ice for 15 min. Fixed cells drug washout, cells were collected by trypsinization and fixed in end of single or sequential drug treatments and a 24- or 48-h values representing the median from three experiments and the fractions of cells in the different cycle phases. The experimental Modfit (DNA Modeling System) software and expressed as /H9262 m filter assembly (RATCOM). Data were elaborated using

sequent analysis. They were then filtered through a disposable sample, 10,000 events were collected and stored for a subse-
sition was performed using CELLQuest software. For each

municated with avidin-biotin conjugate (LSAB kit; DAKO). The specimens were washed twice with PBS, incubated with biotinylated antimouse secondary antibody, rinsed in PBS, and in-

nonspecific binding with 3% BSA in PBS for 20 min and subsequently incubated for 1 h at room temperature with anti-TS antibody (clone TS106, dilution 1:100 in PBS). After this, the specimens were washed twice with PBS, incubated with bioti-

nated amphiphilic secondary antibody, rinsed in PBS, and in-

with avidin-biotin conjugate (LSAB+ kit; DAKO). Sections were then rinsed in PBS, and antibody binding was detected by staining with diaminobenzidine (DAB+ substrate chromogen system; DAKO). The cell nuclei were counter-

stained blue by Mayer’s Hemalum, and sections were mounted in Faramount (DAKO).

For all determinations, negative controls were obtained by omission of the primary antibody. Positive cells were quantified by evaluating at ≥3 × 10³ cells and were expressed as the percentage ratio over the total number of scored cells. All samples underwent blind evaluation at light microscope by two independent observers.

RESULTS
Colon cell lines showed a poor sensitivity to MTA. In LoVo cells, survival was inhibited by a maximum of 30% after a 48-h exposure at the highest concentration, and in LRWZ and WiDr lines, cell survival inhibition ranged from 2 to 12% at all drug concentrations used. GEM caused a strong dose-dependent cytotoxic effect in LoVo cells, and the IC₅₀ was reached at a concentration of 0.3 μg/ml. In contrast, IC₅₀ was never reached at any concentration in WiDr and LRWZ cell lines (Fig. 1).

The interaction between MTA and GEM activity was inves-
tigated using three different schemes: (a) a 48-h simultane-
ous exposure to both agents; (b) a 48-h exposure to GEM followed by a 48-h exposure to MTA (GEM → MTA); and (c) the inverse sequence (MTA → GEM; Fig. 1). The simultaneous exposure to MTA and GEM produced an antagonistic interaction in LRWZ and WiDr cells and an additive effect in LoVo cells. Conversely, the sequence MTA → GEM caused an additive effect in LRWZ and a synergistic effect in WiDr and LoVo cells, with an RI of 1.2 and 2.2, respectively (Table 1).

The inverse sequence, GEM → MTA, caused a synergistic interaction in all cell lines, with a modulation of RI ranging from

| Table I | Type of interaction on colon cell lines
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<td>GEM → MTA</td>
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*The two drugs were used at the following concentrations: 0.01, 0.1, 1, and 10 μg/ml.

modified by Romanelli (21). In brief, the expected cell survival (Sexp, defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) and the observed cell survival (Sobs) for the combination of A and B were used to construct an index (R): R = Sexp/Sobs. An RI of ≤1 (additive effect) indicated the absence of synergism or antagonism. Synergism was defined as any value of R greater than unity.

Flow Cytometric Analysis. For the analysis of cell cycle perturbations, exponentially growing cells were trypsinized, rinsed, and plated (2 × 10⁶ cells/dish) into 60-mm Petri dishes and incubated for 18–24 h at 37°C before drug exposure. Medium was removed and substituted with fresh medium containing MTA 0.1 μg/ml or GEM 0.1 μg/ml. Control samples were cultured under the same conditions, without drugs. After single or sequential drug treatments, and a 24- or 48-h washout, cells were collected by trypsinization and washed twice with PBS. DNA staining was performed with a solution containing RNase (10 KU/ml; Sigma), NP40 (0.01%; Sigma), and propidium iodide (1 μg/ml; Sigma). The samples were stored for 30–60 min before flow cytometric analysis.

All samples were analyzed using fluorescence-activated cell sorter vantage flow cytometer (Becton Dickinson, San Jose, CA) equipped with a water-cooled argon-ion laser. Data acquisition was performed using CELLQuest software. For each sample, 10,000 events were collected and stored for a subsequent analysis. They were then filtered through a disposable 40-μm filter assembly (RATCOM). Data were elaborated using Modfit (DNA Modeling System) software and expressed as fractions of cells in the different cycle phases. The experimental values representing the median from three experiments and the results were expressed as a percentage of control. Student’s t test for paired samples was performed.

Terminal Deoxynucleotidyltransferase Assay. At the end of single or sequential drug treatments and a 24- or 48-h drug washout, cells were collected by trypsinization and fixed in 1% paraformaldehyde in PBS on ice for 15 min. Fixed cells were resuspended in ice-cold ethanol 70% and stored overnight at −20°C. Cells were incubated in 50 μl of solution containing terminal deoxynucleotidyltransferase and FITC-conjugated dUTP deoxynucleotides 1:1 in reaction buffer (Boehringer Mannheim, Mannheim, Germany) for 60 min at 37°C in the dark. After washing in PBS containing 0.1% Triton X-100, the cells were stained with 5 μg of propidium iodide and 10 Ku of RNase A in 10 ml of PBS for 1 h at 4°C in the dark.

Flow cytometric analysis was performed on a fluorescence-activated cell sorter vantage flow cytometer (Becton Dickinson). The green (FL1) and orange (FL2) fluorescences of FITC and propidium iodide were collected with 530/30 and 585/42 band pass filters, respectively. Data acquisition and analysis were performed using CELLQuest software. For each sample, 10,000 events were recorded.

TS Expression. Cells were trypsinized, washed with PBS, and fixed in 10% formalin. Sections (3 μm) from paraffin-embedded blocks were mounted on positive-charged slides (BioOptica), deparaffinized in xylene, rehydrated, and treated with 3% hydrogen peroxide solution. TS antigen retrieval was performed by microwaving at 750 W in 10 mM citrate buffer (pH 6.0) for 15 min followed by cooling at room temperature for ≥20 min. The sections were then treated for nonspecific binding with 3% BSA in PBS for 20 min and subsequently incubated for 1 h at room temperature with anti-TS antibody (clone TS106, dilution 1:100 in PBS). After this, the specimens were washed twice with PBS, incubated with biotinylated antimouse secondary antibody, rinsed in PBS, and incubated with avidin-biotin conjugate (LSAB+ kit; DAKO). Sections were then rinsed in PBS, and antibody binding was detected by staining with diaminobenzidine (DAB+ substrate chromogen system; DAKO). The cell nuclei were counterstained blue by Mayer’s Hemalum, and sections were mounted in Faramount (DAKO).

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The inverse sequence, GEM → MTA, caused a synergistic interaction in all cell lines, with a modulation of RI ranging from
1.3 in WiDr to 6.7 in LoVo cells. The synergistic interaction progressively increased and plateaued at a concentration of 1 µg/ml each drug. The dose-response curves obtained showed a significantly higher activity of the sequential treatment GEM → MTA compared with the inverse sequence ($P < 0.05$) in LoVo and WiDr cells (Fig. 1). The two sequential schemes produced a similar synergistic activity with almost identical RI values (GEM → MTA = 1.3 and MTA → GEM = 1.2). The dose-response curves obtained were not significantly different ($P > 0.05$).

Flow cytometric analysis showed an S phase increase in all cell lines after a 48-h exposure to 0.1 µg/ml MTA and a decrease in G0-G1 and G2-M phase cells (Table 2). The accumulation reached a maximum after the end of treatment in LoVo (from 38 to 84%) and LRWZ (from 40 to 80%) cells and persisted up to the 48-h washout but decreased in LoVo cells and completely resolved in LRWZ after the 48-h washout. Conversely, in WiDr cell line, a block in S phase increased from 30 to 54% at the end of treatment and reached a maximum after the 48-h washout. After a 48-h exposure to 0.1 µg/ml GEM, a significant increase in S phase cells (from 38 to 86%) was observed in LoVo cell line, in concomitance with a total disappearance of cells with G0-G1 DNA content. The same type and degree of cell perturbations were still present 24 and 48 h after the end of treatment. In WiDr line, a slight increase in S phase was detected (from 30 to 47%) that was not longer present after the 48-h washout. In contrast, GEM did not cause cell cycle perturbations in LRWZ cells.

Only 1–3% of apoptotic cells were observed after GEM treatment, whereas a higher percentage (≤15%) was sometimes found after MTA exposure (Table 2).

Flow cytometric analysis was also performed at the end of the different drug sequences (Table 3). The profile observed in LoVo cells after MTA → GEM sequence was almost superimposable with that of untreated cultures. In contrast, post-treatment with MTA produced persistent cell cycle perturbations that consisted in a 63% increase in S phase cells and a 48% decrease in G0-G1 cells. In WiDr line, S phase cells more than doubled after either sequence, in parallel with a complete depletion of G0-G1 cells.

In LRWZ cells, statistically significant cell cycle perturbations were observed after either drug sequence. Apoptosis was only occasionally detected, with the exception of 18% apoptotic cells observed in WiDr line after the most cytotoxic drug sequence, GEM → MTA (Table 3).

TS protein expression was determined by immunohistochemistry (Fig. 2). TS was expressed in ~75% of LoVo and LRWZ cells but was totally absent in WiDr cells. In LoVo and LRWZ lines, a significant increase in TS-expressing cells was observed after MTA alone or followed by GEM but not after GEM exposure, either alone, or when followed by MTA. In the WiDr cell line, TS expression was induced in ~50% of cells after single or sequential drug treatments.

### DISCUSSION

MTA represents a new class of folate antimetabolite that inhibits multiple enzymes in purine and thymidine biosynthetic pathways.
In preclinical studies performed on cancer cell lines derived from hematological or solid tumors (22, 23), MTA has been shown to induce cell cycle perturbations and cytotoxic effects. In our experience also, MTA produced relevant cell cycle perturbations in all cell lines used. In particular, a transient accumulation of S phase cells was observed in LoVo and LRWZ, whereas WiDr cells showed a more persistent S phase block. However, the cell cycle perturbations, probably caused by an imbalance in the deoxynucleotide pools through the inhibition of target enzymes, did not result in a cytocidal effect in any cell line used, as confirmed by the negligible percentage of apoptotic cells detected at all treatment times. We can speculate that the nucleotide pools were not sufficiently depleted to induce persistent cytocidal damage and, thus, trigger apoptosis, as demonstrated by the total recovery of cells accumulated in S phase observed in LoVo and LRWZ cell lines 48 h after the end of the treatment. Furthermore, an increase in thymidylate synthase expression, which is known to be associated with inherent or acquired resistance to conventional antifolates (24–26), was observed in all cell lines, especially WiDr cells. It is possible that compensatory molecular mechanisms prevented the onset of apoptosis.

A generally lower sensitivity to MTA and higher sensitivity to GEM was observed in our study, although a modulation was seen on different cell lines. The modulation of cytotoxicity observed was an expected finding and can be attributed to the intrinsic different chemosensitivity of the cell lines, reflecting the behavior of clinical tumors.

Phase I and II trials with MTA have shown that the most common toxicities, i.e., myelosuppression and skin reactions, are generally tolerable and manageable. The most tolerable schedule resulting from Phase I clinical trials (Ref. 27; MTA 500 or 600 mg/m² every 21 days) has been widely used in Phase II studies and has proven active in breast, head and neck, lung, bladder, pancreatic, cervical, and colon cancers (28).

In oncology practice, the use of single cytotoxic drugs is unusual, and polychemotherapeutic regimens are almost always used. MTA in combination with different anticancer drugs, including GEM, has shown an antitumor activity in xenografts of breast and non-small cell lung cancer (29). The clinical efficacy of MTA and GEM combination has also been demonstrated in tumors resistant to conventional cytotoxic drugs, such as cholangiocarcinoma and mesothelioma (30).

In colon cancer, in vitro studies of MTA and GEM combination have yielded conflicting results. A recent study dem-

![Fig. 2](https://clincancerres.aacrjournals.org)
onstrated a synergistic cytotoxicity for MTA followed by GEM in HT29 colon cancer cell line in vitro or in xenografts (23). In contrast, a synergistic cytotoxicity has been reported in HCT8 human colon cancer cell line for the GEM → MTA sequence (30). This latter finding has been used as a rationale for a Phase I clinical study (30).

The present study aimed to define the optimal schedule of GEM and MTA combination by investigating the cytotoxic activity of simultaneous or different sequential exposures on a panel of colon cancer cell lines with different sensitivity to GEM and various antitumor agents (31) and a very low sensitivity to MTA. The simultaneous drug administration is the more frequently used and most practical clinical regimen. However, results from the present and previous in vitro studies show that the simultaneous exposure to these two antimetabolites does not significantly increase cell kill and probably does not improve the clinical therapeutic effect. Conversely, we observed that both drug sequences produced a greater cytotoxic effect than that exerted by single-agent use or simultaneous exposure. In particular, a higher synergistic interaction was produced by pretreatment with GEM followed by MTA exposure than by the opposite sequence (MTA → GEM) in LoVo and LRWZ cells, as shown by the RI values and by the results of the variance analysis. It is possible that MTA served as a modulator of GEM activity by depleting nucleotide pools required for DNA synthesis, thus preventing cells from repairing DNA damage caused by GEM. The weaker synergistic interaction observed after MTA → GEM sequence could be explained by the significant and sometimes dramatic increase in thymidylate synthase expression, which defends cells by reducing injury from MTA. Such an interpretation is supported by the inverse relation between the degree of TS increase and the RI value.

No relation was found between the presence of apoptotic cells or the degree of persistence of cell cycle perturbations and RI.

In conclusion, the present study further highlights the importance of treatment schedules and indicates that biochemical pathway targets may be important determinants of the antitumor activity of MTA and GEM. The highest cytotoxic activity observed after GEM → MTA exposure on colon cancer cell lines is in agreement with experimental and clinical results recently reported by Adjei et al. (30).

Despite the problems encountered in pharmacological translational research, additional preclinical and clinical studies are needed to define the most active treatment schedule of conventional and new drugs.

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