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Cell Cycle Modulation by a Multitargeted Antifolate, LY231514, Increases the Cytotoxicity and Antitumor Activity of Gemcitabine in HT29 Colon Carcinoma

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ABSTRACT

The proliferation rate of HT29 colon carcinoma cells was decreased by the multitargeted antifolate (MTA), LY231514. This effect correlated with a buildup of cells near the G_1-S interface after 24 h of incubation, and a synchronized progression of the population through S phase during the next 24 h. MTA treatment (0.03–3 μM) was minimally cytotoxic (20–30%) to HT29 cells after a 24-h exposure, and no dose response was observed. In contrast, the nucleoside analogue gemcitabine (GEM) was cytotoxic (IC_{50}, 0.071 ± 0.011 μM; IC_{90}, 0.648 ± 0.229 μM) after a 24-h exposure. We hypothesized that pretreatment of these cells with MTA would increase the potency of GEM by synchronizing the population for DNA synthesis. The cytotoxicity of GEM increased 2–7-fold when MTA was administered 24 h before GEM (IC_{50}, 0.032 ± 0.009 μM; IC_{90}, 0.094 ± 0.019 μM). In addition, an increase in cell kill for the combination compared with GEM alone (IC_{99}, 12 μM for GEM alone; IC_{99}, 0.331 μM for combination) was observed. No increase in potency or cell kill was observed when the two compounds were added simultaneously. MTA pretreatment also potentiated the cytotoxicity of a 1-h exposure to GEM. These cell-based observations were extended to evaluate the schedule-dependent interaction of these two agents *in vivo* using a nude mouse HT29 xenograft tumor model. At the doses tested, MTA alone (100 mg/kg) had a marginal effect on tumor growth delay, whereas GEM (80 mg/kg) produced a statistically significant tumor growth delay. In combination, the increase in tumor growth delay was greatest when MTA was administered before GEM, compared with simultaneous drug administration or the reverse sequence, *e.g.*, GEM followed by MTA. The effect of sequential administration of MTA followed by GEM was greater than additive, indicating synergistic interaction of these agents. Thus, *in vitro*, MTA induced cell cycle effects on HT29 cells that resulted in potentiation of the cytotoxicity of GEM. *In vivo*, combination of these two drugs also demonstrated a schedule-dependent synergy that was optimal when MTA treatment preceded GEM.

INTRODUCTION

The significant benefit of combination chemotherapy on cancer patient response rates has made the development of relevant preclinical models an active area of oncology research. Although useful combinations are usually discovered empirically, there have been many attempts to design models that rationally describe drug combinations (1–7). Often, drug combinations consist of two drugs with distinct but complementary biochemical mechanisms. Because of the defined nature of antifolate enzyme inhibition, antifolates have been the focus of numerous combination studies and modeling (1, 4, 6).

MTA[2] is an antifolate oncolytic currently in Phase III clinical trials. MTA inhibits the activity of several folate-requiring enzymes, including TS, DHFR, and GARFT (8). We have reported previously that MTA induces apoptosis in CCRF-CEM lymphocytic leukemia cells 48 h after addition (9). Prior to the apoptotic event, CEM cells accumulate at the G_1-S interface and progress synchronously into early S phase.

GEM is a difluoronucleoside used clinically for the treatment of pancreatic and non-small cell lung cancers (10–18). The cytotoxic activity of GEM is thought to occur, at least in part, through activation to the triphosphate and subsequent incorporation into nascent DNA (19, 20). This results in cessation of DNA synthesis and extensive DNA lesions, ultimately leading to apoptosis. The cytotoxicity of GEM may also stem from its inhibition of ribonucleotide reductase (19–21). Both of these mechanisms probably contribute to its cytotoxic action, although it is not presently known to what extent.

Here, we report on the antiproliferative and cytotoxic potential of MTA, GEM, and the combination in HT29 colon carcinoma cell cultures and the antitumor activity *in vivo* against HT29 xenograft tumors. Unlike drug combinations chosen for complementary mechanisms of cytotoxic action, we chose this combination based on cell cycle alterations induced by MTA in HT29 cultured cells. We found that by itself, MTA inhibited the growth of HT29 cells but was not cytotoxic. However, MTA did synchronize the population of cells in S phase. We hypothesized that GEM would be more effective if the entire population was entering S phase at the time of exposure to this drug. Increased incorporation of GEM could occur in an S phase-synchronized population relative to an asynchronous population.

Using cultured cells, we demonstrated that sequential exposure of MTA 24 h before the addition of GEM resulted in enhanced cytotoxic potency and efficacy of GEM. Because MTA did not exhibit a dose-response toxicity in these cells, the effect correlated only with cell cycle alterations. The enhanced activity of GEM was also observed *in vivo* against HT29 xenografts grown in nude mice. These observations have important implications regarding therapeutic dosing schedules in clinical studies using these two agents.

MATERIALS AND METHODS

Reagents and Chemicals. MTA and GEM were synthesized at Lilly Research Laboratories (Indianapolis, IN) as described previously (8, 10). All solutions were prepared daily in sterile PBS. PI was purchased from Molecular Probes (Eugene, OR); RNase, DNase Free was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Cell Culture and Proliferation Studies. HT29 colon carcinoma cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in McCoy's Modified 5A medium (Whitaker Bioproducts, Walkersville, MD) supplemented with 10% dialyzed fetal bovine serum (Life Technologies, Inc., Grand Island, NY). For all of these experiments, cells were plated at $20-40 \times 10^4$/cm^2 and allowed to grow for at least 36 h before treatment. At the indicated times after treatment, cells were trypsinized and counted by a Coulter ZM Cell Counter (Coulter Electronics, Hialeah, FL).

Analysis of DNA Content. All cells (attached and floating) from each treatment were harvested and fixed in 70% methanol (−20°C for 1–5 days). The fixed cells were collected by centrifugation and resuspended in Vindelov's PI stain [5 mM Tris (pH 7.4), 5 mM NaCl, 0.005% NP40, and 0.04 mg/ml PI] containing RNase, DNase-free (0.15 units/ml; Ref. 9). DNA content was

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[2] The abbreviations used are: MTA, LY231514 (*N*-[4-[2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)-ethyl]-benzoyl]-l-glutamic acid); TS, thymidylate synthase; DHFR, dihydrofolate reductase; GARFT, glycinamide ribonucleotide formyltransferase; GEM, gemcitabine (2′,2′-difluorodeoxycytidine); PI, propidium iodide; MTD, maximum tolerated dose; dNTP, deoxynucleotide triphosphate.

determined by flow cytometry on a Coulter Electronics XL analytical cytometer as described previously. Excitation of PI was accomplished with the 488 nm line of an argon laser (15 mW). For each experimental condition, 1×10^4 cells were analyzed.

Clonogenic Assays. Cytotoxicity was assayed by determining the ability of HT29 cells to form colonies after drug treatment. Cells were plated at a density of 40×10^4/ml ~36 h before the addition of compound. After the indicated treatments, medium was removed, and the cells were suspended by trypsinization and counted using a hemacytometer. Cells from each treatment were replated in fresh medium plus serum by serial dilution. Colonies were allowed to grow for 2 weeks at 37°C, 5% CO_2. The medium was then removed, and colonies were stained with 1% crystal violet (Sigma Chemical Co., St. Loius, MO) in 70% ethanol and then counted manually. Plating efficiency of the controls was ~65%. Cytotoxicity of each treatment was assessed by calculating the percentage of cells surviving relative to control (untreated) cells.

***In Vivo* Antitumor Studies.** The HT29 human colon xenograft tumor line was obtained from Dr. D. J. Dykes (Southern Research Institute, Birmingham, AL) and was carried by serial passage using trocar s.c. implantation in CD1 *nu/nu* immunodeficient mice (Charles River, Inc., Wilmington, MA). Each group consisted of 8–10 mice. After inoculation of mice with a tumor fragment, the tumors were allowed to grow for 12–19 days until the average calculated tumor size was ~300 mg. Drug treatment was initiated at this time (day 0). Tumors were allowed to grow until the average tumor size for a treatment group reached 1000 mg. The width and length of the tumors were measured twice per week with electronic calipers, and the tumor weights were calculated using the formula (width) × (width) × (length) × 0.5 (Ref. 22).

GEM (80 mg/kg) was prepared for administration using PBS and injected i.p. every third day for three total doses. MTA (100 mg/kg) was prepared in 0.9% saline and administered i.p. daily for 3 days. Previous *in vivo* studies had determined that the MTD for GEM was 240 mg/kg, when administered every third day for four total doses; the MTD for MTA was 300 mg/kg, when administered daily for 10 days.[3] In the studies reported here, compounds were administered three times at approximately one-third of the MTD. Each protocol had its own paired control (untreated group of tumor-bearing mice). Drug treatment was initiated according to the following schedules: Protocol 1: sequential administration of GEM followed by MTA (GEM days 0, 3, 6; MTA days 7, 8, 9), 10 mice in each group; Protocol 2: simultaneous administration of MTA and GEM (MTA days 0, 1, 2; GEM days 0, 3, 6), 10 mice in each group; Protocol 3: sequential administration of MTA followed by GEM [MTA days 0, 1, 2; GEM days 3, 6, 9 (study A: 8 mice/group; study B: 10 mice/group)]; Protocol 4: sequential administration of MTA, a 1-day drug holiday, and GEM [MTA days 0, 1, 2; 1-day drug holiday; GEM days 4, 7, 10 (study A: 8 mice/group; study B: 10 mice/group)]. In protocol 3, studies A and B were replicates, as were A and B in protocol 4; therefore, results of these experiments were pooled for statistical analysis. In protocol 4, a 1-day, drug-free holiday was introduced to reduce toxicity observed in animals on the combination arm of protocol 3.

Data Analysis. The time for tumor weights to reach 1000 mg was defined as the number of days elapsed since therapy began (day 0) until the average tumor weight for the treatment group was ≥1000 mg. Thus, for some mice within a group, the tumor weight may not have reached 1000 mg at the time mice within that group were sacrificed. In these instances, the time to reach 1000 mg was defined by the time to the termination of the experiment, *i.e.*, the time for the average weight of the group to reach 1000 mg. Animals that died during the studies due to toxicity were excluded from the analyses (protocols 1, 3, and 4; Table 3). The statistical package JMP, version 3.2.2 (SAS Institute, Inc., Cary, NC; 1997) was used for the analyses. ANOVA was performed to evaluate the results. Protocols 1 and 2 were analyzed using one-way ANOVA. Fisher's Least Significant Difference method (23) and Tukey-Kramer Honestly Significant Difference method (24)[4] were used for multiple comparison of treatment effects. Two-way ANOVA was performed on the combined data of Protocol 3, studies A and B, as well as on the combined data of Protocol 4, studies A and B. Contrasts were used to compare treatments within the combined data. The design of the experiments did not allow fair comparisons among different dosing schedules *i.e.*, protocols 1, 2, 3, and 4; therefore, dosing schedules were not compared. Synergy and additivity were assessed by evaluating within each protocol whether the effects were additive *versus* nonadditive (where synergy is $1 + 1 = >2$ or antagonism is $1 + 1 = <2$).

RESULTS

MTA-induced Antiproliferative, Cell Cycle, and Cytotoxic Effects. The growth of HT29 cells in the presence of MTA (0.2–1 μM) is shown in Fig. 1. All concentrations of MTA inhibited the growth of HT29 cells to a similar extent. During the first 48 h of the experiment, the treated populations underwent one doubling compared with two by the untreated group. After 48 h, the control cells continued to divide, whereas no further growth was observed in the MTA-treated cells. Although MTA had potent antiproliferative activity on these cells, cytotoxicity was not observed at any of the concentrations evaluated. Furthermore, over the range of concentrations examined, no dose-response relationship was observed.

The cell cycle effects of MTA were evaluated using flow cytometry. DNA content histograms of HT29 cells treated with MTA (Fig. 2) showed a buildup of cells at the G_1-S region of the histogram after 24 h of treatment with 0.2 or 1 μM MTA. By 48 h, both populations had entered early S phase and were capable of incorporating the nucleoside analogue 5-bromo-2′-deoxyuridine (data not shown). After 72 h of drug treatment, the populations progressed synchronously into mid S-phase. The presence of sub-G_1 debris at the 72-h time point indicated that some cell death had occurred; however, the majority of the cells maintained diploid DNA content. Although 0.1 μM MTA had antiproliferative activity, cells incubated at this concentration did not synchronize as was observed at concentrations of 0.2 μM and higher.

The cytotoxicity of MTA on HT29 cells was further examined by performing clonogenic assays. HT29 cells were plated as semiconfluent monolayers ~36 h prior to MTA addition. Cells were exposed to MTA according to two treatment schedules to evaluate its effects under conditions to be used for combination studies with GEM: (*a*) 24 h of continuous exposure followed by serial dilution and replating;

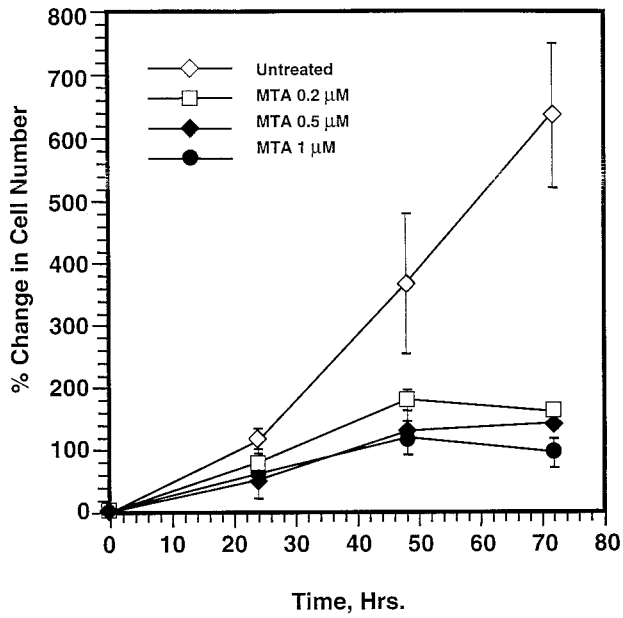

Fig. 1. The effect of MTA on the proliferation rate of HT29 cells in culture. HT29 cells were seeded in 24-well plates at a density of $20–40 \times 10^4/cm^2$. Cells were allowed to attach for ~36 h. The experiment was initiated by the addition of drug or control vehicle at this time (*Time* = 0). At the indicated times, cells from duplicate wells for each treatment were counted: ◇, untreated control; □, 0.2 μM MTA; ◆, 0.5 μM MTA; and ●, 1 μM MTA. Each point is the average of three independent experiments. *Bars*, SE.

[3] J. Worzalla, personal observations.

[4] J. W. Tukey, The problem of multiple comparisons. Unpublished notes, Princeton University, 1953.

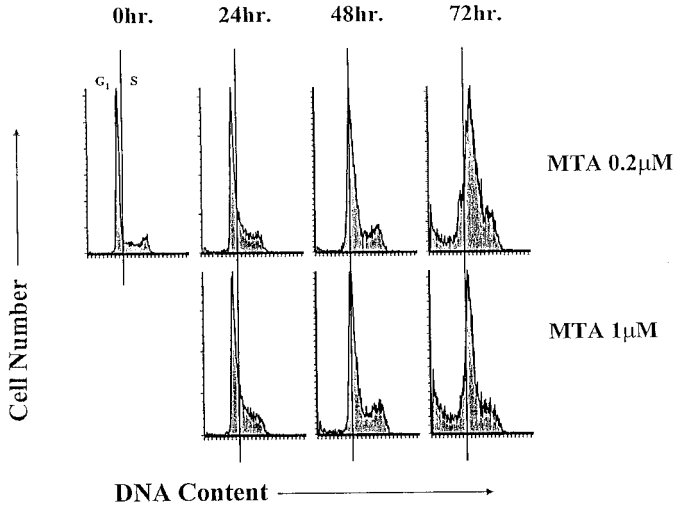

Fig. 2. DNA content histograms of HT29 cells treated with MTA. Cells were plated for ~36 h before the addition of drug. MTA was added (0.2 or 1.0 μM), and DNA content was measured at the indicated times as described in "Materials and Methods." Data from a representative experiment are shown here. A *vertical line* has been drawn through each histogram at the approximate resolution between G_1 and S-phase DNA content.

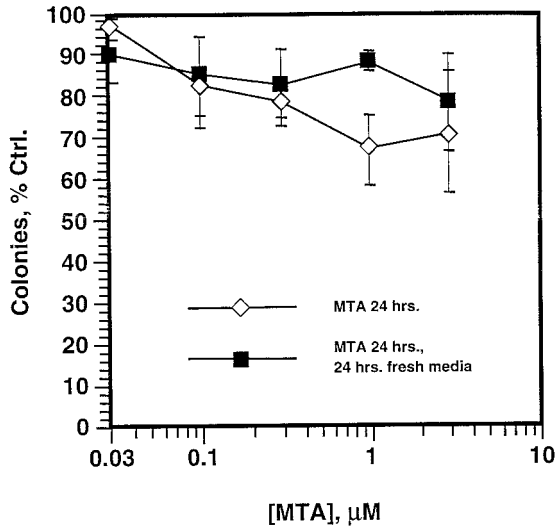

Fig. 3. Clonogenic assays of HT29 cells exposed to MTA. Subconfluent monolayers of HT29 cells were treated with MTA for 24 h and then cloned (◇) or treated for 24 h and then grown in fresh media for 24 h prior to cloning (■). Colonies were allowed to grow for 10–14 days, stained with crystal violet, and counted visually. Data are expressed as a percentage of the number of colonies in untreated samples. Each data point is the average of at least three independent experiments; *bars,* SE.

or (*b*) 24 h of treatment, followed by 24 h in fresh media, followed by serial dilution and replating. Both schedules resulted in similar survival profiles, with minimal cytotoxicity observed (Fig. 3). Maximal toxicity of 25–30% was achieved, with no discernible dose response.

***In Vitro* Combination Studies with GEM.** We hypothesized that synchronization of HT29 cells with MTA would prime the population for enhanced incorporation of a nucleoside analogue during progression into S-phase. To examine this hypothesis, three combination protocols were evaluated in cell culture: Protocol 1: sequential treatment with MTA followed by GEM [24 h of MTA (0.1 or 0.3 μM) followed by 24 h of GEM (3–300 μM)]; Protocol 2: simultaneous exposure (24 h of treatment with MTA and GEM); and Protocol 3: sequential treatment with abbreviated GEM exposure (24 h of treatment with MTA, followed by 1 h of GEM). At the completion of each treatment, the clonogenicity of the remaining cells was determined. Colonies were counted after 2 weeks, and the data were analyzed by median effect dose-plots (3).

The results from protocol 1 are shown in Fig. 4 and Table 1. GEM alone for 24 h was cytotoxic to HT29 cells. However, when treatment was preceded for 24 h with a minimally cytotoxic concentration of MTA (0.3 μM), a slight but reproducible increase in potency of GEM was observed, as shown by the 2-fold decrease in the IC_{50}, which was achieved experimentally (Fig. 4). The IC_{90} of GEM alone, extrapolated from median effect dose-plots was 0.648 μM. When treatment was preceded by MTA, this value fell to 0.094 μM, a concentration that was achieved experimentally. Perhaps most interesting was the observation that pretreatment with MTA also decreased the IC_{99} of GEM. The IC_{99} of GEM was calculated from median effect dose-plots to be 11.65 μM. Pretreatment with MTA increased the effect of GEM so that an IC_{99} was observed experimentally at 0.3 μM GEM, reflecting a 35-fold increase in efficacy of GEM-induced cytotoxicity.

It is important to note that 0.1 μM MTA did not significantly increase the potency and efficacy of GEM (Fig. 4 and Table 1). Alone, 0.1 and 0.3 μM MTA had similar antiproliferative activity (Fig. 1), and each was relatively noncytotoxic (Fig. 3). However, as noted previously, 0.1 μM MTA did not induce the cell cycle alterations caused by concentrations of 0.2 μM and higher. These data provide evidence that the increased potency observed by the drug combination was due to cell cycle alterations caused by MTA.

To assess the significance of the sequential schedule, compounds

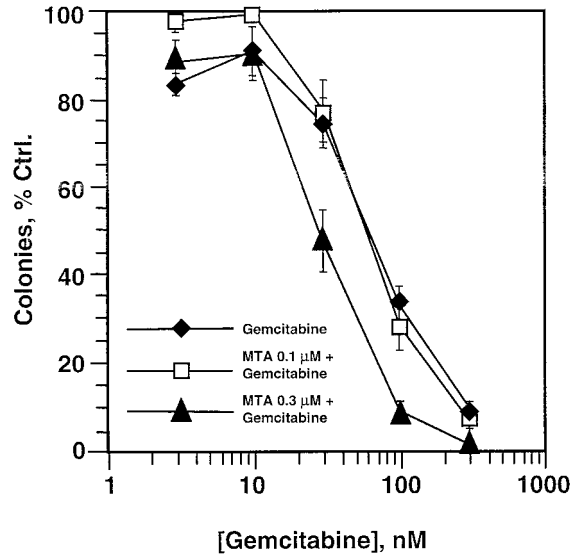

Fig. 4. The effect of sequential treatment with MTA followed by GEM (Protocol 1) on the cytotoxicity of GEM in HT29 cells. Subconfluent monolayers of HT29 cells were treated with MTA or vehicle for 24 h. The cells were washed, and fresh medium containing GEM was added to all wells for 24 h at the indicated concentrations. ♦, GEM controls (no MTA); □, MTA (0.1 μM); or ▲, MTA (0.3 μM). Cells were cloned as described in "Materials and Methods." Colonies were counted visually after 10–14 days. Each data point is the average of at least three independent experiments; *bars,* SE.

Table 1 *Protocol 1: Cytotoxicity of sequential treatment with MTA followed by GEM in vitro*

Cells were incubated with drug-vehicle or MTA 24 h before the addition of GEM for 24 h. Cells were cloned as described in "Materials and Methods." IC_{50}, IC_{90}, and IC_{99} values were calculated from median effect dose-plots of the data in Fig. 4 (3). The numbers in parentheses indicate the fold decrease in the corresponding value from GEM alone.

	GEM	MTA (0.1 μM) → GEM	MTA (0.3 μM) → GEM
IC_{50} (μM)	0.071 ± 0.011	0.074 ± 0.009	0.032 ± 0.009 (2.2)
IC_{90} (μM)	0.648 ± 0.229	0.205 ± 0.018 (3.2)	0.094 ± 0.019 (6.9)
IC_{99} (μM)	11.65 ± 5.93	0.638 ± 0.071 (18.3)	0.331 ± 0.070 (35.2)

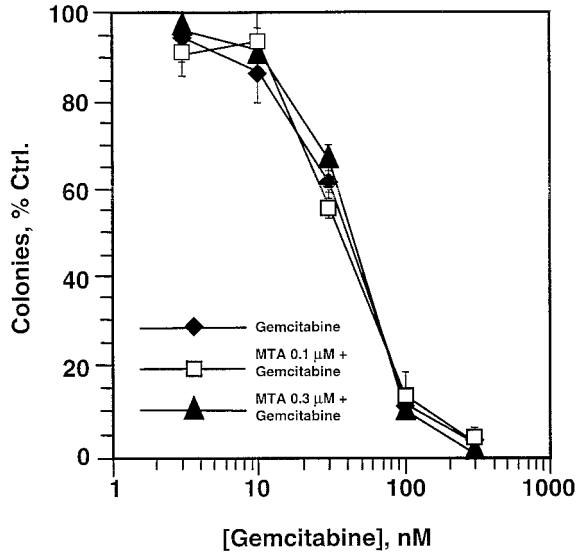

Fig. 5. The effect of simultaneous exposure to MTA and GEM on cytotoxicity to HT29 cells (Protocol 2). Subconfluent monolayers were treated for 24 h with GEM (♦), GEM plus MTA (0.1 μM; □), or GEM plus MTA (0.3 μM; ♦). After 24 h, the cells were cloned as described in "Materials and Methods." Colonies were counted visually after 10–14 days. Each data point is the average of at least three independent experiments; *bars*, SE.

Table 2 *Protocol 3: Cytotoxicity of the sequential treatment with MTA followed by 1 h of GEM in vitro*

Subconfluent monolayers were treated with MTA at 0, 0.3, or 1 μM for 24 h, followed by GEM for 1 h. Data are expressed as percentage of colonies formed relative to untreated samples. Values are averaged from three independent experiments ± SE.

	Colony formation, % untreated		
GEM, μM	No MTA	MTA (0.3 μM)	MTA (1 μM)
0	100	79 ± 5	68 ± 8
0.3	71 ± 7	61 ± 8	50 ± 8
1	71 ± 14	40 ± 4	45 ± 12
3	61 ± 6	37 ± 5	30 ± 6
10	51 ± 2	31 ± 4	21 ± 4
30	63 ± 3	38 ± 3	30 ± 8

were added simultaneously for 24 h (protocol 2, Fig. 5). In these experiments, MTA did not increase the potency of GEM. GEM alone was slightly more potent than observed in protocol 1 (Fig. 4). This may reflect the addition of GEM to the cells earlier in their growth curve during this combination study. In protocol 1, the cell population was well into log-phase growth prior to the addition of GEM, whereas in protocol 2, cells plated 36 h prior to drug addition were just emerging from stationary-lag phase growth (Fig. 1) at the time of GEM addition.

To reduce the variation in drug efficacy associated with time of drug addition relative to plating time, the effect of MTA pretreatment on the cytotoxicity of a 1-h GEM exposure was examined (protocol 3, Table 2). Under these conditions, GEM alone was 29–49% cytotoxic, and a shallow dose-response relationship was observed. Addition of MTA (0.3 or 1 μM) 24 h before GEM treatment (1 h) significantly increased the total cell kill to >60% at concentrations of GEM >1 μM. Moreover, the potency of the 1-h GEM exposure increased by 10–30-fold (estimated IC_{50} range, 0.3–1.0 μM) compared with control (estimated $IC_{50} \geq 10$ μM; no MTA treatment).

In Vivo Results. The effects of MTA, GEM, and several schedule-dependent combinations of these agents to inhibit HT29 tumor growth in nude mice were determined (Table 3). For each protocol, time for the HT29 tumors to reach 1000 mg was calculated as described in "Materials and Methods." Because these values are functions of the tumor doubling time and growth conditions within a particular experiment, statistical comparisons of tumor growth delays were made only among groups within a protocol using ANOVA, as described in "Materials and Methods."

In all protocols, MTA alone produced a minimal tumor growth delay ranging from 0.8 to 6.2 days. The median growth delay for all experiments was 2.6 days. The growth delay of 6.2 days (protocol 2) was significant ($P < 0.05$) in only one experiment. This weak activity was not unexpected, because an abbreviated dosing schedule for MTA was used (3 *versus* the usual 10 days), and the dose tested was approximately one-third the MTD.

GEM produced longer tumor growth delays of 3.5–15.1 days and had a median delay of 10.8 days. The effect of GEM alone was significant in all experiments ($P < 0.05$). In the combination arms of each protocol, MTA and GEM produced significant tumor growth delays (9.3–19.9 days) compared with vehicle-treated controls in all schedules.

Sequential administration of GEM followed by MTA (protocol 1) was equivalent to GEM alone, and both regimens produced significant decreases in tumor growth rates compared with vehicle-treated control mice. There was no difference between mice treated with MTA compared with controls, nor was there any difference between GEM and the combination.

In protocol 2, simultaneous administration of MTA and GEM was equivalent to GEM alone. The simultaneous combination of these drugs may, in fact, have antagonistic effects. MTA treatment resulted in a 6.2-day delay in tumor growth (Table 3), and GEM treatment produced a 15.1-day delay. Additive activity should have yielded 21.3 days of tumor growth delay; however, only 16.4 days of delay were observed (Table 3), ~5 days less than expected for additive activity. This antagonistic effect was significant ($P < 0.004$).

The effect of sequential administration of MTA followed by GEM was evaluated in replicate studies A and B, protocol 3. A similar schedule, protocol 4, was designed to include a 1-day, drug-free holiday between agents to reduce possible toxicity resulting from the rapid combination. A two-way ANOVA analysis was performed (Table 4). For both protocols 3 and 4, treatment with GEM resulted in a significant delay in tumor growth, whereas the effect of MTA

Table 3 *Time to reach 1000 mg tumor for xenograft HT29 colon tumors in nu/nu mice*

GEM was administered i.p. 80 mg/kg every third day for a total of three doses. MTA was administered i.p. 100 mg/kg daily for 3 days. The time to reach 1000 mg tumors was calculated in days, as described in "Materials and Methods."

Protocol	Days ± SE	Deaths
1. Sequential[a]		
Control	25.2 ± 2.0	0/10
MTA	26.0 ± 2.0	0/10
GEM	36.7 ± 2.0	0/10
GEM followed by MTA	34.5 ± 2.3	2/10
2. Simultaneous[b]		
Control	12.7 ± 0.8	0/10
MTA	18.9 ± 0.8	0/10
GEM	27.8 ± 0.8	0/10
Combination	29.1 ± 0.8	0/10
3. Sequential[c]		
Controls	16.09 ± 1.1	0/18
MTA	18.60 ± 1.1	0/18
GEM	27.00 ± 1.1	0/18
MTA followed by GEM	35.00 ± 1.6	5/18
4. Sequential: 1-day drug-free holiday[d]		
Controls	20.19 ± 1.1	0/18
MTA	22.97 ± 1.1	0/18
GEM	23.69 ± 1.1	0/18
MTA followed by GEM	40.04 ± 1.1	1/18

[a] Combination schedule: GEM days 0, 3, 6; MTA days 7, 8, 9.
[b] Combination schedule: MTA days 0, 1, 2; GEM days 0, 3, 6.
[c] Combination schedule: MTA days 0, 1, 2; GEM days 3, 6, 9.
[d] Combination schedule: MTA days 0, 1, 2; 1-day drug-free holiday; GEM days 4, 7, 10.

Table 4 *Comparative effect of MTA, GEM, and sequential combination schedules on HT29 tumor growth delay*

Two-way ANOVA was performed on the combined data of Protocol 3, studies A and B, as well as on the combined data of Protocol 4, studies A and B (Table 3). Contrasts were used to compare treatments within the combined data.

	Tumor growth delay			
	Protocol 3		Protocol 4	
Comparison	Days ± SE	*P*	Days ± SE	*P*
GEM *vs.* Control	10.91 ± 1.6	<0.0001	3.5 ± 1.51	0.023
MTA *vs.* Control	2.51 ± 1.6	0.12	2.78 ± 1.51	0.070
MTA → GEM *vs.* Control	18.91 ± 1.93	<0.0001	19.85 ± 1.53	<0.0001
MTA → GEM *vs.* MTA	16.4 ± 1.93	<0.0001	17.07 ± 1.53	<0.0001
MTA → GEM *vs.* GEM	8.0 ± 1.93	0.0001	16.35 ± 1.53	<0.0001

treatment was not significant. Compared to control tumors, sequential treatment of MTA followed by GEM produced a highly significant growth delay (18.9 days). The magnitude of this effect was similar, with or without the 1 day drug holiday before GEM administration.

The effect of sequential administration of MTA followed by GEM was compared with single-agent therapy. MTA followed by GEM was significantly more effective than MTA alone. Compared with GEM alone, the sequential therapy, MTA followed by GEM (protocol 3) produced a highly significant increase in tumor growth delay (8 days). This effect was even larger in studies that included the 1 day that was drug free prior to GEM administration (protocol 4, 16.4-day delay).

The tumor growth delay observed with sequential administration of MTA followed by GEM was synergistic because the increase in tumor growth delay was larger than the additive effects of each agent alone. In protocol 3, the additive effects of MTA (2.5-day growth delay) and GEM (10.9-day delay) would result in a 13.4-day total delay. The observed delay (18.9 days, Table 4) yielded a synergistic benefit of 6.5 days of tumor growth delay ($P < 0.033$). Similarly, in protocol 4, an additive effect would have resulted in a 6.3-day delay, whereas sequential administration resulted in 13.6 days of additional growth delay ($P < 0.001$).

DISCUSSION

MTA and GEM have independent biochemical mechanisms of action. MTA, an antifolate, depletes the intracellular supply of deoxynucleotides, limiting the ability of a cell to replicate its genome (8, 25). GEM is activated intracellularly to the triphosphate and is then incorporated into nascent DNA, resulting in cessation of DNA synthesis (20). Because of the disparate mechanisms of action, our decision to examine this combination was not predicated on the possibility of biochemical complementation. Rather, the observation that MTA induced cell cycle alterations in HT29 cells despite being only modestly cytotoxic in this cell line led us to hypothesize that MTA might synergize with GEM in a schedule-dependent manner.

MTA and other antifolates that inhibit TS have been shown to induce cell cycle alterations (9, 26). However, these changes usually occur as a prelude to apoptosis. In the experiments reported here, MTA fully inhibited proliferation at all concentrations after 48 h of exposure. However, under these conditions, there was little induction of apoptosis, suggesting that HT29 cells were resistant to the cytotoxic effects of MTA. In other cell types (*e.g.*, CCRF-CEM human leukemia cells), MTA is a potent cytotoxic agent inducing apoptosis with an IC_{50} ~25 nM (9). The synchronization of HT29 cells by MTA suggests that it was effecting a change in nucleotide pools through inhibition of its target enzymes TS, DHFR, and GARFT. However, it is possible that nucleotide pools were not depleted sufficiently to trigger apoptosis, or that compensatory mechanisms delayed the onset of these events. Investigation of these possibilities would provide insight into MTA resistance.

HT29 cells were sensitive to the cytotoxic action of GEM. Exposure times as short as 1 h resulted in significant inhibition of colony formation but with no concentration-dependent response. When exposure was increased to 24 h, the approximate doubling time of HT29 cells, a concentration-response was observed (IC_{50}, 0.071 μM; IC_{90}, 0.648 μM). This result may be related to the proposed mechanism of action of GEM, which depends on incorporation of GEM into DNA during S phase and completion of one growth cycle to induce apoptosis. The cytotoxicity of GEM at concentrations above 0.1 μM did not increase; thus, an IC_{99}, or complete cell kill, was never achieved in these experiments. By extrapolation of the median effect analysis, an IC_{99} of almost 12 μM was predicted.

Although we observed potentiated cytotoxicity from GEM when cells were pretreated with MTA, we were unable to demonstrate through classical isobol analysis that the two compounds were synergistic because treatment of HT29 cells with MTA did not result in dose-dependent toxicity. Isobol analysis for synergy is performed by comparing the dose-response of drug combinations with dose-response curves of the individual compounds. This method is very powerful at distinguishing additive effects from synergistic effects over an entire concentration range. Our purpose in combining MTA and GEM was to test whether a compound lacking cytotoxicity could potentiate the response of a toxic compound. We did in fact observe a modest yet reproducible effect on the potency of GEM and a more dramatic effect on total cell kill.

GEM is known to exert its cytotoxic effects at least partially through incorporation into nascent DNA (19, 20). We hypothesized that synchronization of the population in early S phase by MTA would prime cells for increased incorporation of GEM. The increased cytotoxicity observed using sequential combination, although modest, supports this hypothesis (IC_{50}, 0.032 μM; IC_{90}, 0.094 μM). In clonogenic assays, MTA alone modestly inhibited colony formation (~15%) at 0.1 and 0.3 μM. However, cell cycle alterations were observed only at concentrations >0.2 μM. Thus, potentiation of the activity of GEM correlated with MTA-induced cell cycle alterations and not its antiproliferative activity. When the two agents were administered simultaneously in cell culture, no increased toxicity was observed, consistent with the hypothesis that the mechanism of potentiation was dependent on temporally spaced events.

Because GEM is an analogue of deoxycytidine, the potentiation of its activity may have resulted from depletion of dCTP after MTA treatment. MTA is an inhibitor of multiple folate-requiring enzymes including TS, GARFT, and DHFR (8). In CEM lymphoma cells, MTA simultaneously reduced TTP, dGTP, and dCTP while increasing dATP. Similar effects have been reported for cells treated with pure TS inhibitors (25). In HT29 cells treated with MTA followed by GEM, depletion of dCTP induced by MTA may have increased GEM incorporation with commensurate enhancement of its cytotoxicity. Other investigators have reported potentiation and synergy of TS inhibitors when combined simultaneously with thymidine analogues, 3′-azido-3′deoxy-thymidine and idoxuridine (27–31). GEM is also known to inhibit ribonucleotide reductase (19–21). This mechanism, which also depletes dNTP pools may have contributed to its toxicity in HT29 cells. Thus, the correlation between cell cycle modulation by MTA and enhanced toxicity of GEM may be due to a general decrease in available dNTPs. In the present studies, the drug administration schedule was critical for observing potentiation. Thus, for this combination, it is unclear whether depletion of dCTP or other dNTP by MTA contributed to the observed effects. Studies to evaluate the metabolic effects of MTA, GEM, and their time and sequence-depen-

dent combination on nucleotide reductase and dNTP pools in the HT29 *in vitro* model are planned.

The effect of MTA to potentiate the cytotoxicity of GEM was explored using the HT29 tumor line *in vivo*. Combination protocols were investigated to explore the temporal effects of sequential administration of MTA and GEM on tumor growth delay. ANOVA analysis of these results showed that sequential administration of MTA, followed by GEM, was superior to simultaneous administration in this model. Sequential administration resulted in a synergistic effect of delayed tumor growth. Thus, the synergistic activity of these two agents in culture translated into enhanced antitumor activity *in vivo*.

Extrapolation of these conclusions to human clinical trials requires evaluation of these activities in additional preclinical models. However, the rationale for this drug combination, which is based on the temporal effects of these agents on cultured tumor cells, can be extended *in vitro* and in additional human tumor xenograft models to suggest appropriate clinical testing paradigms.

GEM has been approved as treatment for pancreatic cancer and lung cancer in a number of countries. MTA is presently in Phase III clinical trials. The results reported here suggest that sequential administration of these agents should be explored clinically.

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