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Activity of the multitargeted antifolate LY231514 in the human tumor cloning assay

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Abstract *Purpose*: This study was performed to evaluate the activity of the multitargeted antifolate (MTA or LY231514) against a broad range of human tumors taken directly from patients. *Materials and methods*: Human tumor colony-forming units were treated with MTA at concentrations of 0.1, 1.0, and 10 μg/ml in 1-h exposure studies. The responses of a limited number of specimens were also evaluated concurrently in 1-h exposures to cisplatin, fluorouracil, irinotecan, and/or paclitaxel. *Results*: Of 358 specimens plated in the 1-h exposure studies, 148 (41%) were evaluable. Overall, responses were observed in 3% of specimens (4/144) at 0.1 μg/ml, 11% (17/148) at 1.0 μg/ml, and 23% (33/141) at 10 μg/ml. In this range of concentrations achievable clinically, there was a significant concentration-response relationship. At 10 μg/ml in the 1-h exposure studies, the response rate in colorectal cancer specimens was 32% (9/28), and the response rate in non-small-cell lung cancer was 25% (6/24). Responses were also observed in several chemoresistant tumors, including renal cell carcinoma, hepatocellular carcinoma, mesothelioma, and pancreatic carcinoma. The activity of MTA was not completely cross-resistant with that of cisplatin, fluorouracil, irinotecan, and paclitaxel. *Conclusions*: MTA demonstrated in vitro activity against a spectrum of tumors, including several tumors generally considered chemoresistant.

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Introduction

The multitargeted antifolate (MTA or LY231514) is a novel folate antagonist with a pyrrole ring replacing the pyrazine ring in the pterine portion and a methylene group replacing the benzylic nitrogen in the bridge portion (Fig. 1) [20]. Following transport into the cell via the reduced folate carrier, MTA is rapidly polyglutamated [21, 23]. Compared to the parent compound, the polyglutamated metabolites reach higher intracellular concentrations and have increased affinity for the target enzymes [21]. The inhibition of multiple folate-requiring enzymes, including thymidylate synthase (TS), dihydrofolate reductase (DFHR), and glycinamide ribonucleotide formyltransferase (GARFT), results in the inhibition of cellular growth [21]. MTA has excellent cytotoxic activity against CCRF-CEM human leukemia cells, with a 50% inhibitory concentration (IC_{50}) of 0.007 μg/ml [5]. MTA also suppresses tumor growth in VRC5 and GC3 human colon cancer xenografts by more than 80% at doses of 25 and 50 mg/kg i.p. daily [23].

Clinical trials of MTA are currently in progress. The major toxicities include neutropenia, thrombocytopenia, anemia, nausea and vomiting, fatigue, reversible hepatic transaminase elevation, diminished renal function, anorexia, mucositis, and dermatitis [2, 3, 8, 10–16, 18]. To date, responses have been observed in patients with transitional cell carcinoma of the bladder [13], renal cell carcinoma [18], and carcinoma of the colon [3, 8, 10, 15], pancreas [12, 15], lung [2, 16], and breast [11]. Furthermore, complete responses have been achieved in patients with colorectal [3, 10], pancreatic [12], and breast [11] cancer. The most studied schedule to date consists of a bolus of MTA administered once every 3 weeks. The maximum tolerated dose (MTD) of 600 mg/m^2 once every 3 weeks achieves a maximum plasma concentration

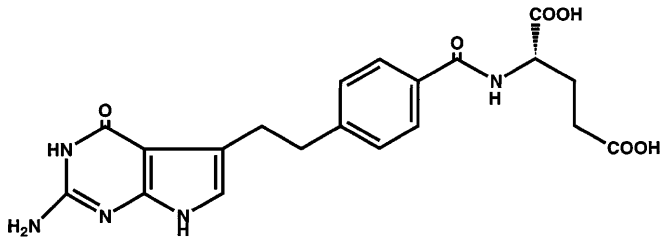

Fig. 1 Structure of multitargeted antifolate (MTA). *N*-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic acid

(C_{max}) of 142 μg/ml, and plasma concentrations of greater than 0.1 μg/ml are maintained for over 24 h postinfusion [15].

In an effort to further define the activity of MTA at biologically relevant concentrations, MTA was evaluated against a broad range of fresh human tumors using the human tumor cloning assay. In this assay, single-cell suspensions are prepared from fresh human tumors and cultured in a two-layer soft agar system [6, 7]. The use of a semisolid medium prevents the anchorage-dependent growth of nonmalignant bystander cells. Histologic studies have demonstrated that the cells grown in this system retain the morphology of the original tumor cells [6, 7]. Additionally, these cells are capable of self-renewal, consistent with the hypothesis that they are tumor stem cells [6]. This system was first proposed by Hamburger and Salmon in 1977 in an attempt to tailor chemotherapy for the individual patient [6, 7]. The assay has a sensitivity of 70% and a specificity of 86% for predicting tumor response [25]. Along with predicting the clinical activity of anticancer drugs on an individual patient basis, the assay may predict which tumor types will be the most responsive to a new drug [22]. In an effort to further define the activity of MTA at biologically relevant concentrations, MTA was evaluated against a broad range of fresh human tumors using the human tumor cloning assay.

Materials and methods

Tumor specimens

Specimens were collected with informed consent from patients undergoing routine diagnostic or therapeutic procedures. These specimens included fresh solid tumors, pleural effusions, ascites, and bone marrow aspirates. Solid samples were minced into fragments of 5 mm^3 or smaller and placed immediately in McCoy's medium 5A with 10% heat-inactivated newborn calf serum and 1% penicillin/streptomycin. Within 4 h, these solid tumors were forced through number 100 stainless steel mesh and needles of successively increasing gauge and then washed with McCoy's medium to generate single-cell suspensions [4, 7, 17, 22, 26, 28]. Marrow aspirates and pleural, pericardial, and ascitic fluids were placed in sterile containers with 10 units of preservative-free heparin per milliliter of fluid. The cells were harvested and washed with McCoy's medium plus 10% heat-inactivated fetal calf serum. The viability of cell suspensions was determined on a hemocytometer with trypan blue.

Test compounds

MTA was provided by Eli Lilly and Company (Indianapolis, Ind.). It was diluted to create concentrated stock solutions of 100 μg/ml. Aliquots of 0.5 ml of each stock solution were labeled and stored at −70 °C until thawed for use for each individual tumor sample. The final concentrations tested were 0.1, 1.0, and 10 μg/ml for 1-h exposures, and 0.001, 0.005, 0.01, and 0.1 μg/ml for continuous exposures of 14 days. The short-exposure concentrations were selected based on the phase I clinical trial of MTA given once every 3 weeks, which demonstrated that the plasma concentration is maintained above 0.1 μg/ml for over 24 h at the MTD [15]. Only a few specimens were tested in continuous exposure studies, as the long intracellular half-life of MTA polyglutamates allows the compound to be administered as a bolus in the clinic.

To compare the relative sensitivity of the specimens to MTA, concurrent analyses with MTA and cisplatin, fluorouracil, irinotecan, and paclitaxel were performed. Both cisplatin and paclitaxel were obtained from Bristol-Myers Squibb (Princeton, N.J.). Irinotecan was obtained from Pharmacia and Upjohn Company (Kalamazoo, Mich.), while fluorouracil was obtained from Solo-Pak Laboratories (Elk Grove Village, Ill.). Stock solutions of cisplatin, fluorouracil, irinotecan and paclitaxel were stored in the same manner as MTA. The final concentrations of these agents were based on the drug concentrations achievable in patients, usually in the range from the peak plasma level down to one-tenth the peak plasma level. Cisplatin and fluorouracil were tested in 1-h exposure studies at concentrations of 0.2 and 6 μg/ml, respectively. Irinotecan was tested in 1-h exposure studies at concentrations of 0.3, 1.5, and 3 μg/ml, while paclitaxel was tested at concentrations of 2.5 and 10 μg/ml.

Human tumor-cloning assay

For the short-exposure studies, cells were incubated with MTA in McCoy's medium for 1-h and then washed. Cells were then suspended in 0.3% agar in enriched Connaught Medical Research Laboratory (CMRL) medium 1066 with 15% heat-inactivated horse serum, penicillin (100 units/ml), streptomycin (2 mg/ml), glutamine (2 m*M*), insulin (3 units/ml), asparagine (0.6 mg/ml) and HEPES buffer (2 m*M*). Approximately 5×10^5 cells were plated in 1 ml of 0.3% agar over 1-ml base layers of 0.3% agar in 35-mm Petri dishes. Samples were plated in triplicate. For the 14-day exposure studies, cells were combined with MTA in the CMRL medium, then plated as for the 1-h exposure. Since the compound was not washed from the culture during the 14-day incubation period, these studies are referred to as continuous exposure studies.

All assessments were performed following incubation at 37 °C for 14 days. The number of colonies formed in the three MTA-treated plates were compared with the number of colonies formed in the untreated controls. The percentage of colonies surviving at each concentration was calculated. For each tumor tissue sample tested, three positive control plates were also set up, containing the nonspecific cellular toxin orthosodium vanadate at a concentration of 200 μg/ml which completely inhibits colony growth. An evaluable experiment was defined as one averaging at least 20 colonies on day 14 on the untreated control plates and less than 30% survival in the positive control (orthosodium vanadate) plates when compared with untreated control plates. The plating efficiency was calculated for the evaluable untreated controls by dividing the number of colonies grown by the number of cells plated (5×10^5).

The cisplatin, fluorouracil, irinotecan, and paclitaxel studies were performed concurrently with the MTA studies, employing the same techniques.

Analysis

The Spearman's rank correlation coefficient was used to determine if there was a relationship between the number of colonies in control cultures and the response to MTA. The sensitivities of the human tumor colonies to MTA at different concentrations were

compared using McNemar's test. Comparisons of sensitivities between agents were also performed using McNemar's test. In addition, scatter plots were used to demonstrate the relative antiproliferative effects of MTA and the other agents. Points for the scatter plots were generated by calculating the percentage survival in individual specimens exposed to MTA at 10 μg/ml (*x* axis) and cisplatin, fluorouracil, irinotecan, or paclitaxel at clinically relevant concentrations (*y* axis). All of these analyses were performed using SAS version 6.11 (SAS Institute, Cary, N.C.).

Results

For the MTA 1-h exposure studies, a total of 358 specimens were plated and 148 (41%) were evaluable. For the continuous exposure studies, only 25 specimens were plated, and 8 (32%) were evaluable. The number of tumor colonies on the evaluable untreated control plates ranged from 20 to 736, yielding a plating efficiency of 0.004% to 0.15%. There was a very weak positive relationship between the number of colonies on the evaluable control plates and the percentage of colonies surviving treatment with MTA (Spearman's rho = 0.16 to 0.20, $P = 0.013$ to 0.055).

The activity of MTA is summarized in Table 1. The overall in vitro response rates in the 1-h exposure studies were 3% (4/144) at 0.1 μg/ml, 11% (17/148) at 1.0 μg/ml, and 23% (33/141) at 10 μg/ml. In the short exposure studies, the sensitivity to the drug was significantly greater at 10 μg/ml than at both 1.0 μg/ml ($P = 0.0001$) and 0.1 μg/ml ($P < 0.0001$). The sensitivity rate at 1.0 μg/ml was also, significantly greater than that at 0.1 μg/ml ($P = 0.0033$). These results suggest that there is a relationship between MTA concentration and inhibition of colony formation.

Specimens were procured from a wide variety of malignancies, although the most frequently evaluated tumor types were colorectal, non-small cell lung, and ovarian cancers. The responses to individual tumor types are displayed in Table 1. Of note, in colorectal cancer there was a 13% (4/31) in vitro response rate at 1.0 μg/ml and a 32% (9/28) in vitro response rate at 10 μg/ml. Also, in non-small-cell lung cancer, there was a 12% (3/25) in vitro response rate at 1.0 μg/ml and a 25% (6/24) in vitro response rate at 10 μg/ml. There were also in vitro responses demonstrated in several chemoresistant tumor types, including renal cell carcinoma, hepatocellular carcinoma, mesothelioma, and pancreatic cancer.

The growth of fresh human tumor specimens was concurrently evaluated in the presence of MTA, cisplatin, fluorouracil, irinotecan, and paclitaxel in 1-h exposure studies. In Table 2, the activity of MTA at 10 μg/ml is compared with the activity of the other agents in 1-h exposure studies. Among the 76 tumors whose growth was studied in parallel in the presence of MTA at 10 μg/ml and cisplatin at 0.2 μg/ml, 17% of tumors (10/60) (including two mesotheliomas and two non-small-cell lung cancers) resistant to cisplatin were sensitive to MTA. Synchronous evaluations of MTA at 10 μg/ml and fluorouracil at 6 μg/ml were performed in 42 specimens, and 19% of tumors (7/36) (including three colon cancers) resistant to fluorouracil were sensitive to MTA. In a comparison between MTA at 10 μg/ml and irinotecan at 3 μg/ml in 25 specimens, 14% of tumors

Table 1 Tumor-specific in vitro responses to MTA in a human tumor cloning system (response was defined as ≤50% survival of tumor colony-forming units)

Tumor type	1-h exposure Concentration in μg/ml			Continuous exposure Concentration in μg/ml			
	0.1	1.0	10.0	0.001	0.005	0.01	0.1
Bladder	0/2	0/2	0/2	–	–	–	–
Brain	0/2	0/2	0/2	–	–	–	–
Breast	0/9	0/9	0/9	–	–	–	–
Carcinoid	0/2	1/2	2/2	–	–	–	–
Colon	0/30	4/31	9/28	–	–	–	–
Gallbladder	0/2	1/2	0/2	–	–	–	–
Head and Neck	0/3	0/3	0/3	–	–	–	–
Kidney	0/10	1/10	1/10	0/2	0/2	0/2	1/2
Liver	0/4	0/4	1/4	–	0/2	0/2	0/2
Lung, non-small-cell	1/24	3/25	6/24	0/1	0/2	0/2	0/2
Lung, small-cell	0/4	1/4	3/4	–	–	–	–
Melanoma	0/7	2/8	1/7	–	–	–	–
Mesothelioma	0/3	2/3	2/3	–	–	–	–
Ovarian	1/22	1/22	3/21	–	–	–	–
Pancreas	0/4	0/5	2/4	–	–	–	–
Sarcoma	1/1	1/1	1/1	0/1	1/1	0/1	0/1
Skin, not melanoma	0/1	0/1	0/1	–	–	–	–
Stomach	1/5	0/5	1/5	–	–	–	–
Uterus	0/2	0/2	0/2	–	0/1	0/1	1/1
Unknown primary	0/7	0/7	1/7	–	–	–	–
Total	4/144 (3%)	17/1488 (11%)	33/141 (23%)	0/4 (0%)	1/8 (13%)	0/8 (0%)	2/8 (25%)

Table 2 Comparison of MTA (at 10 μg/ml) in vitro activity with that of other agents (S/S sensitive to both MTA and the agent, S/R sensitive to MTA but resistant to the agent, R/S resistant to MTA but sensitive to the agent, R/R resistant to both MTA and the agent, N/A not applicable; sensitive was defined as ≤50% survival of tumor colony-forming units)

Agent	Concentration (μg/ml)	S/S	S/R	R/S	R/R	Total	*P*-value using McNemar's test
Cisplatin	0.2	8	10	8	50	76	0.64
Fluorouracil	6.0	2	7	4	29	42	0.37
Irinotecan	0.3	0	5	0	20	25	N/A
	1.5	0	5	0	20	25	N/A
	3.0	2	3	2	18	25	0.66
Paclitaxel	2.5	1	8	3	23	35	0.13
	10.0	6	3	10	14	33	0.052

(3/21) (two colon cancers, one mesothelioma) resistant to irinotecan were sensitive to MTA. Finally, MTA at 10 μg/ml and paclitaxel at 10 μg/ml were compared in 33 specimens, and 18% of tumors (3/17) (including one non-small-cell lung cancer) resistant to paclitaxel were sensitive to MTA, while 42% of tumors (10/24) (including five ovary and three non-small-cell lung cancers) resistant to MTA were sensitive to paclitaxel. In this categorical analysis, there was no statistically significant difference in the sensitivities of the specimens to MTA when compared to the other anticancer drugs ($P = 0.052$ to 0.66).

In an effort to further compare the relative antiproliferative effects of MTA with those of other agents, a scatterplot analysis was performed. Figure 2 shows the percentage of surviving colonies for each specimen exposed to MTA and cisplatin (Fig. 2A), fluorouracil (Fig. 2B), irinotecan (Fig. 2C,D), or paclitaxel (Fig. 2-E,F). These graphs demonstrate that resistance to the anticancer agents was not necessarily complete. In some 'resistant' specimens, the percentage of surviving colonies was between 50 and 100, demonstrating that some 'resistant' specimens were partially sensitive to the effects of the drugs.

Discussion

MTA is a novel compound representative of the newest generation of folate antimetabolites. Like the classic antifolates, MTA requires energy-dependent membrane transport and polyglutamation for activation and retention [21]. MTA and its polyglutamates affect folate metabolism predominantly through blockade at TS, DHFR, and GARFT [21]. This inhibition of folate-dependent enzymes results in a broad spectrum of cytotoxicity.

In this study, the activity of MTA was further explored utilizing the human tumor cloning assay. Consistent with the previous experience with this assay, the evaluability of the tumor samples ranged from 32% to 41%, and the plating efficiency ranged from 0.004% to 0.15% [6, 7, 9, 25, 27]. In vitro responses were observed following 1 h exposures to 0.1, 1, and 10 μg/ml of MTA. There was a significant concentration-response relationship, with a 3% response rate at 0.1 μg/ml, and a 23% response rate at 10 μg/ml. These concentrations were comparable to the plasma concentrations achieved in the phase I clinical trial of MTA administered once every 21 days. In patients, the peak plasma concentration (C_{max}) was 142 μg/ml following a 600 mg/m^2 bolus of MTA [15]. Plasma concentrations were maintained above 10 μg/ml for over 4 h, and above 0.1 μg/ml for over 24 h [15]. The concentrations applied in the 1-h exposure studies of MTA in the human tumor cloning assay were thus clinically relevant.

MTA demonstrated activity against a spectrum of tumors, including several chemoresistant tumors. The in vitro activity of MTA against two mesothelioma specimens was consistent with the four partial responses achieved following administration of MTA and cisplatin to patients with mesothelioma [24]. The in vitro activity of MTA against colorectal and non-small cell lung cancer specimens was also encouraging, and preliminary results from phase II clinical trials of single-agent MTA have demonstrated responses in these tumor types [2, 3, 8, 10, 15, 16]. However, the relationship between the response rate in the human tumor cloning assay and in the clinic is not completely understood. The human tumor cloning assay appears to predict for activity against some tumor types better than others [9]. Also, it may be difficult to extrapolate results with antimetabolites in the cloning assay to humans as low concentrations of animal serum are used in the cloning assay. Nevertheless, the activity of MTA in the human tumor cloning assay supports further clinical testing in chemoresistant tumors such as mesothelioma, as well as in colorectal and non-small-cell lung cancer.

A limited number of fresh tumor specimens were tested separately with clinically achievable concentrations of both MTA and another TS inhibitor, fluorouracil. Of 36 tumors (including three colon cancers) resistant to fluorouracil, 7 (19%) were sensitive to MTA. This is consistent with a study in which H630 human colon carcinoma cells resistant to fluorouracil were only modestly resistant to MTA [19]. Potential mechanisms of resistance to fluorouracil include decreased metabolic activation, decreased incorporation into RNA or DNA, and upregulation of TS [1]. Although augmented TS activity could diminish both fluoropyrimidine and antifolate cytotoxicity, MTA could overcome this through the inhibition of other folate-dependent enzymes.

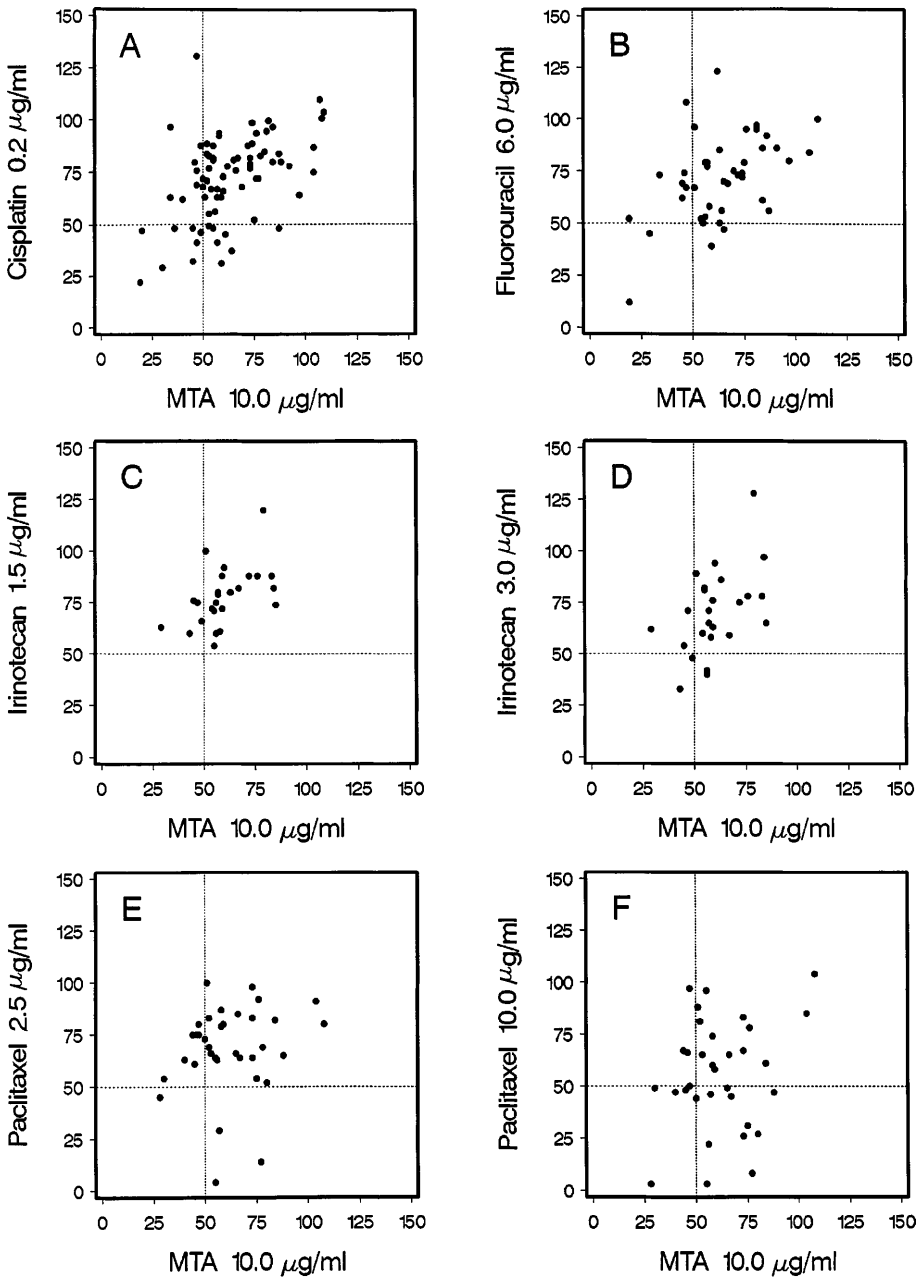

Fig. 2A–F Scatter plots comparing survival following 1-h exposures to 10 μg/ml MTA with that of 0.2 μg/ml cisplatin (**A**), 6 μg/ml fluorouracil (**B**), 1.5 μg/ml irinotecan (**C**), 3 μg/ml irinotecan (**D**), 2.5 μg/ml paclitaxel (**E**), and 10 μg/ml paclitaxel. (**F**) Points were generated by calculating the percentage survival in individual specimens exposed to MTA (*x* axis) and other compounds (*y* axis), such as cisplatin (**A**), fluorouracil (**B**), irinotecan (**C**,**D**), and paclitaxel (**E**,**F**). Graphs are divided into four quadrants by dashed lines. Points in the right upper quadrant of each graph represent specimens resistant to both MTA and the alternative compound, while points in the left lower quadrant of each graph represent specimens sensitive to both MTA and the alternative compound. (Two specimens were excluded from **A** because greater than 150% of colonies survived treatment. One specimen was resistant to both MTA and cisplatin, while the other specimen was resistant to MTA and sensitive to cisplatin.)

Synchronous evaluations were also performed using clinically relevant concentrations of MTA and other agents, including cisplatin, irinotecan, and paclitaxel. In the overall population of specimens, the activity of MTA was similar to that of the alternative agents. When individual specimens were considered, however, there were some notable exceptions. Two mesothelioma specimens and two non-small-cell lung cancer specimens which were resistant to cisplatin were sensitive to MTA, and two colon cancer specimens which were resistant to irinotecan were sensitive to MTA. This suggests that the activity of MTA was not completely cross-resistant with that of the other agents evaluated.

This study utilized the human tumor cloning assay to demonstrate the activity of MTA against a variety of tumors at clinically relevant concentrations. Activity was observed against tumors generally considered chemoresistant, such as renal cell carcinoma, hepatocellular carcinoma, mesothelioma, and pancreatic cancer. Additionally, there was promising activity against more common malignancies, including non-small-cell lung cancer and colorectal cancer. The results of this in vitro study await further confirmation in clinical trials.

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