# MTA (LY231514) in Combination Treatment Regimens Using Human Tumor Xenografts and the EMT-6 Murine Mammary Carcinoma

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**An important component in the development of a new anticancer drug is an understanding of its potential for inclusion in combination treatment regimens. LY231514, a multitargeted antifolate (MTA), was tested in combination with cisplatin, methotrexate, 5-fluorouracil, paclitaxel, docetaxel, doxorubicin, LY329201 (a glycinamide ribonucleotide formyltransferase [GARFT] inhibitor), and fractionated radiation therapy in vivo using EMT-6 mammary carcinoma, human HCT 116 colon carcinoma, and human H460 non–small cell lung carcinoma grown as xenografts in nude mice. Isobologram methodology was used to determine the additivity or synergy of the combination regimens. MTA administered with cisplatin, paclitaxel, docetaxel, or fractionated radiation therapy produced additive to greater than additive tumor response by tumor cell survival assay and tumor growth delay. While an additive tumor response was observed when MTA was administered with methotrexate, synergistic tumor responses were seen when MTA was administered with the GARFT inhibitor, LY329201, or with the topoisomerase I inhibitor, irinotecan. MTA was administered in combination with full doses of each anticancer agent studied, with no evidence of increased toxicity resulting from the combination.**

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N-[4-[2-(2-AMINO-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]-pyrimidin-5-yl)ethyl]-benzo-yl]-L-glutamic acid, LY231514 (MTA), was discovered through structure activity relationship studies based on the novel antipurine antifolate lometrexol.[1] MTA contains a pyrrole moiety in the place of the tetrahydropyridine ring of lometrexol, which results in a major loss of activity in the inhibition of purine biosynthesis and a shift to the inhibition of pyrimidine biosynthesis (thymidylate cycle).[2-4] MTA is a substrate for mammalian folylpolyglutamate synthase[5] and is a potent inhibitor, especially as the triglutamate, of the enzymes thymidylate synthase, dihydrofolate reductase, glycinamide ribonucleotide formyltransferase (GARFT), and aminoimidazole carboxamide ribonucleotide formyltransferase.[6]

In 1984, Jackman et al[7] reported that relatively high concentrations of circulating thymidine have been found in human plasma. Thus, those concentrations found in mice may tend to underpredict both the antitumor activity and toxicity of drugs that inhibit thymidylate synthase compared withwhat may be expected in humans.[8] MTA was a very active antitumor agent against the thymidine kinase-negative/hypoxanthine-negative murine lymphoma L51784/TK-/HX.[9] MTA was also found to be an effective antitumor agent against several human tumor xenografts with normal thymidine kinase levels, including the VRC5 colon carcinoma, the GC3 colon carcinoma, the BXPC3 pancreatic carcinoma, the LX-1 non–small cell lung carcinoma, and MX-1 breast carcinoma.[1] In several studies, the folate levels in mice were modulated by feeding a low-folate diet then repleting the animals by administration of specific doses of folic acid.[10] Both the antitumor activity and toxicity of MTA could be modulated in this manner, and at certain folate levels antitumor activity toward specific tumors could be optimized.

In the current studies, MTA was administered alone, in combination with standard chemotherapeutic agents, or with radiation therapy to tumor-bearing mice to explore the potential interaction of MTA in combination anticancer treatment regimens.

## MATERIALS AND METHODS

### *Drugs*

MTA and LY329201 (a GARFT inhibitor) were prepared according to published methods and procedures.[2,11,12] Cisplatin, methotrexate, 5-fluorouracil, paclitaxel, and doxorubicin were purchased from Sigma Chemical Co (St Louis, MO). Irinotecan (CPT-11) was purchased from the Indiana University Medical School Pharmacy, Indianapolis, IN.

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### Tumors

The EMT-6 murine mammary carcinoma was an in vivo–in vitro tumor system. The EMT-6 tumor was carried in BALB/c mice (Taconic Farms, Germantown, NY). For the experiments, $2 \times 10^6$ tumor cells prepared from a brei of several stock tumors were implanted subcutaneously into the hind legs of the BALB/c mice at 8 to 10 weeks of age.[13,14]

HCT 116 human colon carcinoma originated from a male patient in 1979.[15] The HCT 116 cell line is tumorigenic in nude mice. HCT 116 cells were purchased from ATCC (Rockville, MD). H460 human non–small cell lung carcinoma was obtained from the National Cancer Institute (Bethesda, MD). The H460 cell line is tumorigenic in nude mice.

Nude mice were purchased from Charles River Laboratories (Wilmington, MA) at 5 to 6 weeks of age. When the animals were 7 to 8 weeks of age they were exposed to 4.5 Gy total body radiation delivered using a GammaCell 40 irradiator (Nordion, Inc, Ottowa, Ontario, Canada). Twenty-four hours later, HCT 116 or H460 tumor cells ($5 \times 10^6$) prepared from a brei of several stock tumors were implanted subcutaneously in a 1:1 mixture of RPMI tissue culture media and Matrigel (Collaborative Biomedical Products, Inc, Bedford, MA). HCT 116 tumors grow to 500 $mm^3$ in 19.7 ± 1.5 days and H460 tumors grow to 500 $mm^3$ in 14.0 ± 0.8 days.

### Tumor Excision Assay

When the EMT-6 tumors were approximately 100 $mm^3$ in volume (8 days after tumor cell implantation), the animals were given intraperitoneal injections of various doses of MTA (50, 100, 150, or 200 mg/kg) by intraperitoneal injection four times (AM and PM) over 48 hours alone or in combination with cisplatin (10, 20, or 30 mg/kg), methotrexate (1, 5, or 10 mg/kg), or LY329201 (1, 5, or 10 mg/kg), or with radiation therapy (5, 10, 15, or 20 Gy). The second chemotherapeutic agents and the radiation therapy were administered with the third dose of MTA. Mice were killed 24 hours after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage. The tumors were excised, and single cell suspensions were prepared as described previously.[13,14,16] The untreated tumor cell suspensions had a plating efficacy of 10% to 16%. The results are expressed as the surviving fraction ± SE of cells from treated groups compared with that of cells from untreated controls.[13,16]

### Bone Marrow Toxicity

Bone marrow cells were taken from the same animals used for the tumor excision assay, and the assay for granulocyte/macrophage colony-forming units (CFU-GM) was carried out as described previously.[13,16] Colonies of at least 50 cells were scored on a Acculite colony counter (Fisher, Springfield, NJ). The results from three experiments, in which each group was measured in triplicate, were averaged. The results are expressed as the surviving fraction ± SE of cells isolated from treated animals compared with that of cells isolated from untreated animals.

### Tumor Growth Delay Experiments

*HCT 116.* Treatment was initiated on day 7 after tumor cell implantation, when the HCT 116 tumors were approximately 150 $mm^3$ in volume. Animals were treated by intraperitoneal injection with MTA (100 or 125 mg/kg) on days 7 through 11 and days 14 through 18, alone or with 5-fluorouracil (7.5, 15, or 30 mg/kg) on days 7 through 11, irinotecan (7.5, 15, or 30 mg/kg) on days 7 through 11, or fractionated radiation therapy (2, 3, or 4 Gy/fraction) on days 7 through 11 and days 14 through 18 after tumor cell implantation.

*H460.* Treatment was initiated on day 8 after tumor cell implantation, when the H460 tumors were approximately 200 $mm^3$ in volume. Animals were treated by intraperitoneal injection with MTA (100 mg/kg) on days 8 through 12 and days 15 through 19 alone or with 5-fluorouracil (30 mg/kg) by intraperitoneal injection on days 8 through 12; cisplatin (10 mg/kg) by intraperitoneal injection on day 8 or day 15; docetaxel (22 mg/kg) by intravenous injection on days 8, 10, 12, and 15; or doxorubicin (1.75 mg/kg) by intraperitoneal injection on days 8 through 12 after tumor cell implantation. In another experiment, MTA (100 mg/kg) was administered by intraperitoneal injection on days 8 through 12 alone or with 5-fluorouracil (30 mg/kg) by intraperitoneal injection on days 16 through 20 after tumor cell implantation.

The progress of each tumor was measured three times per week until it reached a volume of 2,000 $mm^3$. Tumor growth delay (TGD) was calculated as the time taken by each individual tumor to reach 500 $mm^3$ compared with the time in the untreated controls. Each treatment group included five animals, and each experiment was repeated three times. Tumor growth delay times (days) are the mean values ± SE for the treatment group compared with those for the control group.

### Data Analysis

Using the method of Deen and Williams,[17] isobolograms were generated for the special case in which the dose of one agent is held constant. This method produced envelopes of additive effect for different levels of the variable agent and is conceptually identical to generating a series of isobolograms and replotting the results at a constant dose of one agent on a log effect by the dose of the second-agent coordinate system.

Dose response curves for each agent were first generated. The envelopes of additivity shown were generated from a series of isoeffect curves derived from the complete dose response curves for each agent. Overall, combinations that produced the desired effect and that were within the envelope boundaries were considered additive. Those displaced to the left were considered to be superadditive, while those displaced to the right were considered to be subadditive.[18,19] This general approach can be extrapolated to the special case in which the level of an agent is held constant. Under these conditions, an isobologram can be derived that plots the expected effect for any level of the variable agent, plus the constant agent combinations.[20] Experimentally, this approach is far simpler than classical isobologram methodology and readily facilitates determination of additive and nonadditive combinations.[13]

Statistical comparisons for the TGD assays were conducted with the Dunnett multiple comparisons test after a significant effect was found by ANOVA.[21,22]

## RESULTS

To examine potential interactions with other anticancer chemotherapeutic agents and radiation

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therapy, various doses of MTA were administered four times over 48 hours to mice bearing the EMT-6 mammary carcinoma. Twenty-four hours later the tumors were excised and suspensions of known numbers of single cells were plated for colony formation (Fig 1). MTA killed 50% of the EMT-6 tumor cells when the animals received 150 mg/kg or 200 mg/kg four times. There was no toxicity toward the bone marrow CFU-GM with these regimens of MTA alone.

Cisplatin was administered to EMT-6 tumor-bearing mice as a single dose of 10, 20, or 30 mg/kg in a log-linear manner with increasing dose of the drug (Fig 2). One log of tumor cell killing was achieved with 17 mg/kg of cisplatin. The combination of MTA and cisplatin was additive to greater than additive. The synergy of the combination treatment increased with increasing dose of cisplatin. Cisplatin is not very cytotoxic toward the bone marrow CFU-GM and combining treatment of MTA with cisplatin did not increase the toxicity toward the bone marrow CFU-GM.

When administered as a single dose, methotrexate kills EMT-6 tumor cells in a shallow log-linear manner (Fig 3). A single dose of 6 mg/kg of methotrexate killed 50% of the EMT-6 tumor cells. The combination of MTA with methotrexate resulted in additive to greater than additive tumor cell killing. With the combination treatment regimen, 50% of EMT-6 tumor cells were killed by 2 mg/kg of methotrexate and 90% (one log) of EMT-6 tumor cells were killed by 8.5 mg/kg of methotrexate. The addition of MTA to treatment with methotrexate did not increase the cytotoxicity of methotrexate toward the bone marrow CFU-GM.

LY329201 is a potent inhibitor of GARFT. A single dose of 1 mg/kg of LY329201 killed approximately 1 log of EMT-6 tumor cells, but there were only small increases in the tumor cell killing at the higher doses of 5 and 10 mg/kg of LY329201 (Fig 4). The combination of MTA with LY329201 produced markedly greater than additive (synergistic) EMT-6 tumor cell killing across the dosage range of LY329201 studied. There was a greater

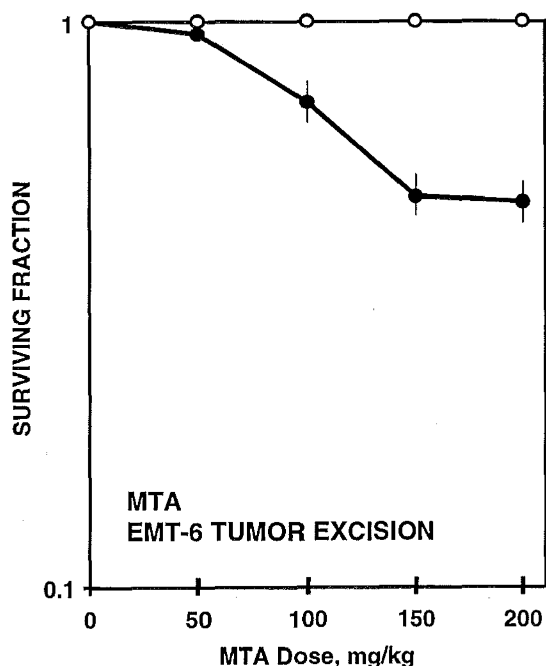


**Fig 1. Survival of EMT-6 murine mammary tumor cells from tumors (●) and bone marrow CFU-GM (○) from animals treated with MTA (50, 100, 150, or 200 mg/kg) intraperitoneally four times over 48 hours. The points are the mean values of two independent experiments; the bars indicate the SEM.**

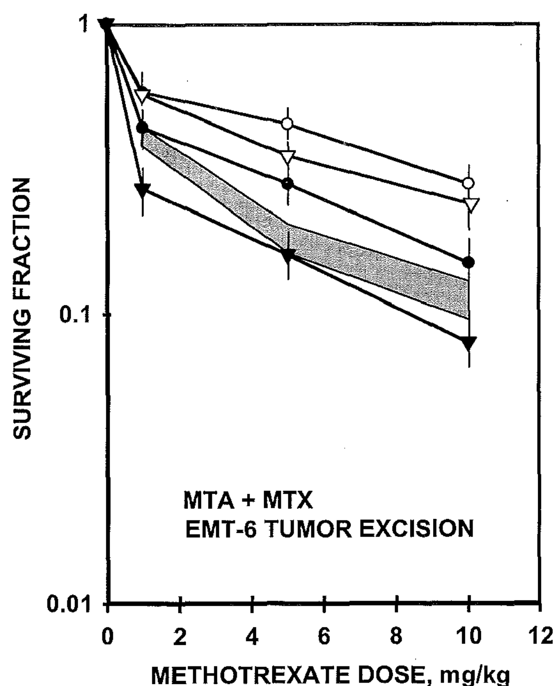


**Fig 2. Survival of EMT-6 murine mammary tumor cells from tumors (●, ▼) and bone marrow CFU-GM (○, ▽) from animals treated with cisplatin (10, 20, or 30 mg/kg) intraperitoneally (●, ○) or with MTA (100 mg/kg) intraperitoneally four times over 48 hours and cisplatin (10, 20, or 30 mg/kg) intraperitoneally along with the third MTA dose (▼, ▽). The points are the mean values of two independent experiments; the bars indicate the SEM. The shaded area represents the envelope of additivity by isobologram analysis.**

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than 1 log increase in EMT-6 tumor cell killing when MTA was combined with LY329201 (5 mg/kg) and a greater than 2 log increase in EMT-6 tumor cell killing when MTA was combined with LY329201 (10 mg/kg). Neither LY329201 nor the combination of MTA and LY329201 was cytotoxic toward bone marrow CFU-GM.

Radiation therapy was administered to EMT-6 tumor-bearing mice in single doses between 5 and 20 Gy. Radiation killed EMT-6 tumor cells in a log-linear dose-dependent manner (Fig 5). Treatment of the animals with MTA and radiation therapy produced tumor cell killing that was additive for the two treatments.

The human HCT 116 colon carcinoma was selected for the initial study of MTA in combination treatments because the HCT 116 tumor is

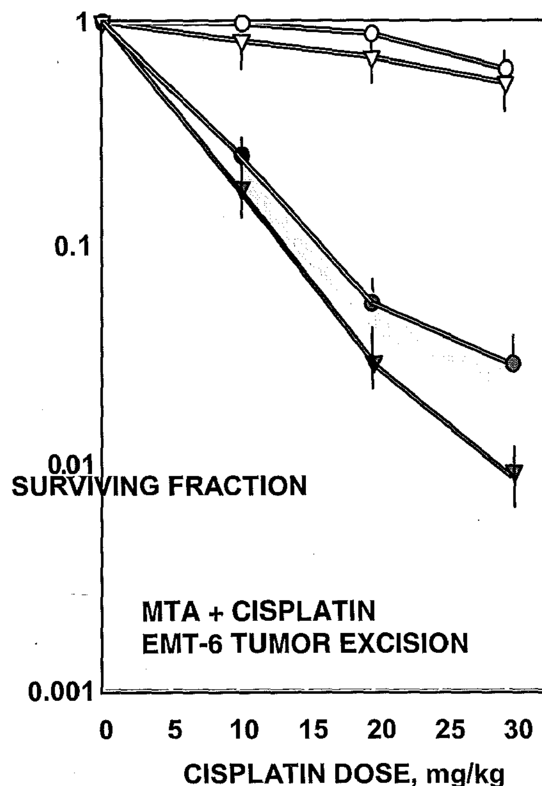


**Fig 3.** Survival of EMT-6 murine mammary tumor cells from tumors (●, ▼) and bone marrow CFU-GM (○, ▽) from animals treated with methotrexate (1, 5, or 10 mg/kg) intraperitoneally (●, ○) or with MTA (100 mg/kg) intraperitoneally four times over 48 hours and methotrexate (1, 5, or 10 mg/kg) intraperitoneally along with the third MTA dose (▼, ▽). The points are the mean values of two independent experiments; the bars indicate the SEM. The shaded area represents the envelope of additivity by isobologram analysis.

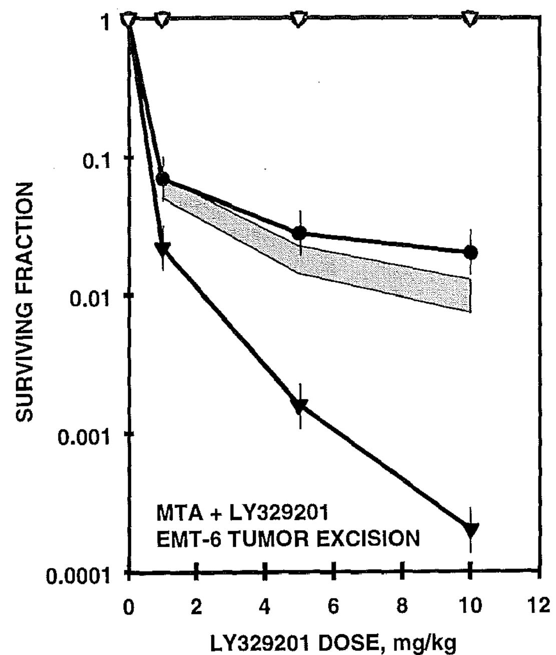


**Fig 4.** Survival of EMT-6 murine mammary tumor cells from tumors (●, ▼) and bone marrow CFU-GM (○, ▽) from animals treated with the GARFT inhibitor (LY329201) (1, 5, or 10 mg/kg) intraperitoneally (●, ○) or with MTA (100 mg/kg) intraperitoneally four times over 48 hours and the GARFT inhibitor (1, 5, or 10 mg/kg) intraperitoneally along with the third MTA dose (▼, ▽). The points are the mean values of two independent experiments; the bars indicate the SEM. The shaded area represents the envelope of additivity by isobologram analysis.

responsive to MTA and because antitumor activity of MTA has been observed in patients with colon cancer.[1,3,4,23,24] Treatment of nude mice bearing subcutaneously implanted HCT 116 colon tumors with MTA (100 mg/kg) twice daily for 5 days produced a TGD of 2.7 ± 0.3 days. 5-Fluorouracil administered daily for 5 days produced increasing TGD with increasing dose of the drug (Fig 6). Treatment with the combination of MTA and 5-fluorouracil produced TGD that was additive. No toxicity was observed when a full standard dose of MTA was administered with a full standard dose of 5-fluorouracil.

Irinotecan administered daily for 5 days produced increasing TGD with increasing dose of the drug (Fig 7). Treatment of HCT 116 tumor-bearing animals with MTA and irinotecan resulted in greater than additive tumor growth for the two drugs, reaching 27 days when the irinotecan dose was 30 mg/kg. No toxicity was observed when a

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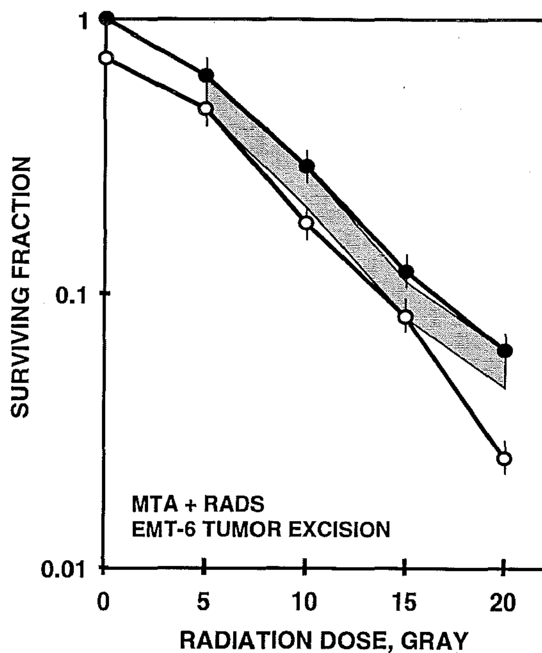


**Fig 5. Survival of EMT-6 murine mammary tumor cells from tumors from animals treated with radiation therapy (5, 10, 15, or 20 Gy) alone (●) or with MTA (100 mg/kg) intraperitoneally four times over 48 hours and radiation therapy along with the third MTA dose (○). The points are the mean values of two independent experiments; the bars indicate the SEM. The shaded area represents the envelope of additivity by isobologram analysis.**

full standard dose of MTA was administered with a full standard dose of irinotecan.

Fractionated radiation therapy was administered locally to the tumor-bearing limb of the nude mice carrying human HCT 116 colon carcinoma xenografts twice daily for 5 days. Radiation therapy delivered in fractions of 2, 3, or 4 Gy produced increasing TGD with increasing radiation dose (Fig 8). Administration of MTA (100 mg/kg) with fractionated radiation resulted in additive TGD and administration of MTA (125 mg/kg) with fractionated radiation resulted in additive to greater than additive TGD. The largest increase in TGD observed with the combination of MTA and fractionated radiation occurred at the radiation dose of 2 Gy, the most clinically relevant dose.

Combination treatment regimens including MTA were also used in nude mice bearing subcutaneously implanted human H460 non–small cell lung carcinoma (Table 1). Administration of MTA (100 mg/kg) twice daily for 5 days to animals bearing the H460 tumor produced a TGD of 2.8 ± 0.3 days. When cisplatin (10 mg/kg) was administered as a single dose on the first day of treatment with MTA, there was a greater than additive TGD; however, when administration of cisplatin was delayed to midway through the MTA treatment, subadditive TGD resulted. The combination of docetaxel and MTA produced greater than additive TGD, while the combination of paclitaxel and MTA resulted in additive TGD. Doxorubicin was not a very active antitumor agent against the H460 non–small cell lung carcinoma and the combination of doxorubicin and MTA produced additive TGD.

Administration of MTA (100 mg/kg) daily for 14 days to animals bearing the H460 tumor resulted in a TGD of 3.7 days (Table 1). Treatment with 5-fluorouracil during the last 5 days of an MTA treatment regimen produced additive to greater than additive TGD.

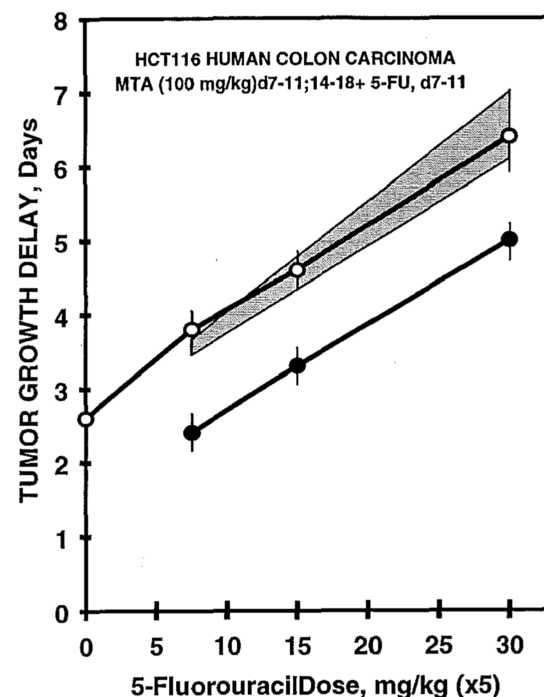


**Fig 6. Growth delay of human HCT 116 colon carcinoma grown as a xenograft in nude mice after treatment with 5-fluorouracil (7.5, 15, or 30 mg/kg) intraperitoneally on days 7 to 11 after tumor cell implantation alone (●) or along with MTA (100 mg/kg) intraperitoneally on days 7 to 11 and days 14 to 18 (○). The points are the mean values of two experiments with five animals per group per experiment; the bars indicate the SEM. The shaded area represents the envelope of additivity by isobologram analysis.**

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