

Efficacy of Recombinant Methioninase in Combination with Cisplatin on Human Colon Tumors in Nude Mice

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ABSTRACT

The present treatment of colon cancer is based on 5-fluorouracil (5-FU). Despite promising results of combining leucovorin or levamisole with 5-FU, the 5-year survival rate of patients with advanced colon cancer has not increased significantly. Colon tumors *in vitro* have been shown previously to have an elevated requirement for methionine, suggesting a new therapeutic target. In this study, targeting the methionine dependence of colon tumors is effected by recombinant methioninase (rMETase), alone and in combination with cisplatin (CDDP). *In vitro* results demonstrated that CDDP and rMETase act synergistically on the human colon cancer cell line SW 620, with a combination index (CI) of 0.45, as well as on the human colon cancer cell line Colo 205 with a CI of 0.7. Human colon cancer lines HCT 15, HT 29, Colo 205, and SW 620 growing in nude mice were treated with rMETase to determine an effective dose for depletion of tumor methionine. rMETase at 15 units/g/day for 5 days depleted tumor methionine in all four tumor types to ~30% of untreated control. rMETase alone arrested growth of HCT 15 and HT29 in nude mice for 1 week after treatment termination. Colo 205 and SW 620 were partially arrested by rMETase. However, CDDP in combination with rMETase resulted in tumor regression of Colo 205 and growth arrest of SW 620 in nude mice. The ratio of the treated:control group (T:C) tumor weights for Colo 205 was 8% when CDDP was given on day-5, followed by treatment on days 5–9 with rMETase. This treatment schedule resulted in two of the six animals having no detectable tumor when the experiment was terminated on day 16. SW620 was resistant to CDDP alone and only partially sensitive to rMETase alone. However, when SW 620 was treated with rMETase from days-5 to -9 and CDDP on day-5, tumor growth was arrested. The results demonstrate that rMETase used simultaneously in combination with CDDP had signif-

icant antitumor efficacy in colon cancer *in vitro* and *in vivo*. The data suggest a novel and promising therapeutic approach by targeting the elevated methionine dependence of colon cancer.

INTRODUCTION

Colon cancer accounts for 10% of the total mortality of cancer in the United States in 1996 (1). At the time of diagnosis of colon cancer, 38% of the patients have regional spread of disease and 21% have distant spread (2). After the cancer has spread regionally to involve adjacent organs or lymph nodes, the 5-year survival rate drops to 63%, and for patients with distant metastases, the 5-year survival rate is <7% (1, 3, 4). Surgery combined with radiation is presently the most effective method of treating colorectal cancer but has no efficacy on advanced colon cancer. Presently, the chemotherapy of advanced colorectal cancer is still based on 5-FU.² Modulation of 5-FU by leucovorin as well as by methotrexate provides better tolerated regimens and may result in increased response rates (1, 3, 4). However, survival rates from cancer of the colon and rectum have not significantly improved (2). New treatment protocols for patients with advanced colorectal cancer and for those at high risk of developing metastatic cancer are thus urgently needed.

The elevated minimal methionine requirement of tumor cells for proliferation relative to normal cells, which we have termed methionine dependence (5, 6), is present in all colon cancer cell lines tested thus far (7–9). The elevated methionine dependence of colon cancer is thus a potential new therapeutic target.

Goseki *et al.* (10) have demonstrated the clinical efficacy of targeting the elevated methionine dependence of advanced gastric cancer in Phase II clinical trials using a methionine-depleted TPN. The methionine-depleted TPN solution doubled the response and survival rate of advanced gastric cancer patients in combination with 5-FU and mitomycin C compared with the two drugs given with MET-replete TPN (10). Goseki *et al.* (11) also reported that the combination of a methionine-free TPN with 5-FU reduced the metastatic potential of the Yoshida sarcoma growing in Donryu rats. Methionine-free TPN combined with doxorubicin and vincristine also extended survival of Yoshida sarcoma-bearing rats compared with methionine-replete TPN in combination with these two drugs (12). Methionine-free TPN only partially lowered serum methionine levels, demonstrating that even partial methionine depletion has anti-tumor efficacy.

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² The abbreviations used are: 5-FU, 5-fluorouracil; TPN, total parenteral nutrition; rMETase, recombinant methioninase; CDDP, cisplatin; OPA, ophthalmaldehyde; CI, combination index; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Efficacy studies *in vitro* and *in vivo* have suggested that rMETase, which rapidly degrades methionine *in vitro* and *in vivo*, has potential as an effective, broad spectrum, tumor-selective agent (7, 13, 14). A tumor-selective cell cycle block at the S-G₂ phase (15) and induction of apoptosis in tumor cells (9) are among the effects of rMETase. All types of human cancers tested thus far, including colon, lung, prostate, kidney, brain, and melanoma, are more methionine-dependent than various types of normal cells tested (7–9, 13, 16).

Scanlon *et al.* (17, 18) and Mineura *et al.* (19) demonstrated that cisplatin affected methionine metabolism in tumor cells. We have observed that a methionine-free diet in combination with CDDP slowed the growth of the human MX-1 breast tumor in nude mice (20). These studies provided the background to determine the efficacy of rMETase in combination with CDDP, which is described in the present report.

MATERIALS AND METHODS

Production of rMETase

The pAC-1 rMETase high expression clone with a T7 promoter, which was constructed in our laboratory, was used for the production of rMETase in *Escherichia coli*. The crude cell lysate, which contained ~40% rMETase, was purified with a DEAE Sepharose FF column. Endotoxin was removed with an Acticlean Etox column (14). The purified rMETase was 98% pure by HPLC and a single band of M_r 43,000 by SDS-PAGE. The specific activity of rMETase used in this study was ~20 units/mg protein, and the endotoxin level was <0.2 EU/mg (14, 21).

Human Cancer Cell Lines

Human colon cancer cell lines HCT 15, HT 29, Colo 205, and SW 620 were obtained from the United States National Cancer Institute (22).

Growth Inhibition of Human Colon Cancer Cell Lines by the Combination of rMETase and CDDP *in Vitro*

The *in vitro* growth inhibition experiments of Colo 205 and SW 620 human colon cancer cells with rMETase and CDDP were carried out in 96-well plates. Twenty-four h after the cells were seeded at a density of 3000 cells/well, 0.3 μ g/ml CDDP and 0.125 units/ml rMETase were added alone or in combination to the wells. After 72 h of exposure, the medium was discarded, and 200 μ l of MTT solution (0.5 mg/ml) were added to each well. After incubation at 37°C for 3 h, the solution was discarded, and 200 μ l/well isopropanol were added to each well. The resulting absorbance was read at 570 nm. The percentage of cell proliferation of the treated cells was calculated by defining the MTT value of untreated cells as 100%. All determinations were repeated three times.

Antitumor Efficacy of rMETase on Human Colon Cancer Xenografts in Nude Mice

Animals. Four-week-old outbred female BALB/c-*nu/nu* mice, with an average body weight of 20 g, were used for tumor transplantation. All nude mice were bred and maintained in an isolated specific pathogen-free facility at AntiCancer, Inc. with a controlled light/dark cycle, temperature, and humidity. Cages,

bedding, food, and water were all autoclaved. All animal procedures were carried out under NIH guidelines under assurance number A3873-1.

Human Tumor Models. The doubling time of human cancer cell lines *in vivo* (from 100 to 200 mg) was 4–6 days for HT 29 and HCT 15 and 5–7 days for Colo 205 and SW 620. The cell lines were initially transplanted s.c. in nude mice. The tumor specimens were inspected, and grossly necrotic and suspected necrotic tissue was removed. The tumor tissue was subsequently cut into small pieces of approximately 1 mm³ and placed in Hanks' medium. Three of the tumor pieces were then transplanted s.c. with a trocar on the right and left flank of each nude mouse used for efficacy testing.

Doses and Schedules. The date of tumor transplantation was defined as day-0. rMETase was given i.p. three times per day at doses of 5–10 units/g body weight. Treatment was started on day-5 for Colo 205 and SW 620 or on day-2 for HCT 15 and HT 29 and continued for 5 days. CDDP was given i.p. at a dose of 7 mg/kg body weight once on day-5 or day-10 to Colo 205 and SW 620.

Efficacy Evaluation. Tumor size and body weight were measured every three days until the experiments were terminated. Tumor weight was calculated as (length \times width²) \div 2. Tumor growth inhibition was determined as the ratio of treated:control (T:C) tumor weight (23). T:C was calculated as mean tumor weight of the treated animals (T) \div mean tumor weight of the control animals (C) \times 100. A T:C value of <42% is the minimum level for determining that a treatment regimen has activity. Complete response of a tumor was scored when any residual tumor was below the limit of palpation. Partial response was scored as a 50% or more reduction in tumor mass (23).

Tumor Methionine Measurement. Tumor methionine levels were determined with an HPLC (Hitachi L-6200A Intelligent pump; Hitachi, Ltd., Tokyo, Japan) after derivitization of serum amino acids with the fluoroldehyde reagent OPA as described previously (24, 25). Supernatants were prepared from tumor tissue after sonication for 30 s and subsequent centrifugation at 13,000 rpm for 10 min. Tumor supernatant samples (25 μ l) were precipitated by acetonitrile (75 μ l). Ten μ l of supernatant were mixed with 5 μ l of OPA. After 1 min, 50 μ l of 0.1 M sodium acetate (pH 7.0) were added, and a 20- μ l sample was loaded on a reversed-phase Supelcosil LC-18-DB column (particle size, 5 μ m, 25 cm \times 4.8 mm) at room temperature. The column was eluted with solution A [tetrahydrofuran:methanol: 0.1 M sodium acetate (pH 7.2); 5:95:900] and solution B (methanol). A gradient from 20–60% of solution B, run at a flow rate of 1.5 ml/min, resolved the amino acids. The eluate was read with a fluorescence spectrophotometer (Hitachi, F1000) at a wavelength of 350–450 nm. The limit of detection was ~0.1 μ M methionine.

Statistical Methods

Differences in the size of the primary tumors between the groups at defined time points were assessed for significance using the Student's *t* test.

Table 1 Efficacy of rMETase and CDDP on human colon cancer *in vitro*

Treatment	Growth rate as % of control		
	Observed (%)	Expected (%)	Observed/Expected
A. Efficacy of rMETase and CDDP on human colon cancer Colo 205 <i>in vitro</i>			
CDDP (0.3 µg/ml)	88.2 ± 6		
rMETase (0.125 units/ml)	72.4 ± 4		
CDDP + rMETase	44.8 ± 4	63.9	0.7
B. Efficacy of rMETase and CDDP on human colon cancer SW 620 <i>in vitro</i>			
CDDP (0.3 µg/ml)	83.4 ± 4		
rMETase (0.125 units/ml)	88.8 ± 5		
CDDP + rMETase	33 ± 3	74	0.45

The *in vitro* growth inhibition efficacy of rMETase and CDDP was carried out as described in "Materials and Methods;" 3000 cells/well were seeded in 96-well plates, and 24 h later, CDDP (0.3 µg/ml) and rMETase (0.125 unit/ml) were added alone or in combination to the wells. After 72 h of exposure, the medium was discarded, and 200 µl of MTT solution (0.5 mg/ml) were added to each well. After incubation at 37°C for 3 h, the solution was discarded, and 200 µl/well of isopropanol were added. The resulting absorbance was read at 570 nm. The percentage of cell proliferation in the treated cultures was calculated by defining the MTT value of the untreated cells as 100%. All determinations were made three times. The combination results were evaluated by the method of Benz *et al.* (26) and Tan *et al.* (27). The expected growth rate of the combination of drug A and drug B = growth rate of drug A × growth rate of drug B. The CI = observed growth rate/expected growth rate. CI of <0.8 was synergistic, 0.8–1.2 was additive, and >1.2 was antagonistic.

RESULTS AND DISCUSSION

Efficacy of rMETase and CDDP on Human Colon Cancers *in Vitro*. The growth rate relative to control of Colo 205 cancer cells treated with CDDP alone was 88%; rMETase treatment alone resulted in a 73% growth rate; and the combination of CDDP and rMETase resulted in a growth rate of 56% of control. The growth rate relative to control of SW 620 cancer cells treated with CDDP alone was 82%; treatment with rMETase alone resulted in a growth rate of 88%; and the combination treatment of CDDP and rMETase resulted in a growth rate of 31% of control. The combination results were evaluated for synergy by the method of Benz *et al.* (26) and Tan *et al.* (27). The growth inhibition by the combination treatment of CDDP and rMETase on Colo 205 and SW 620 was synergistic (CI = observed growth rate/expected growth rate = 0.7 and 0.45, respectively; Table 1).

Depletion of Tumor Methionine by rMETase. The free-methionine levels of the four human colon cancers in nude mice were depleted by rMETase treatment (i.p., day-5 to day-9 at a dose of 15 units/g/day) from a range of 22–34 nmol/mg protein to a range of 6–12 nmol/mg by treatment day-9 (Fig. 1). The differences between the treated groups and control groups were significant ($P < 0.01$). These results demonstrated that rMETase could deplete tumor methionine levels despite the multiple sources of methionine for the tumor including the diet, methionine biosynthesis, and necrosis-related proteolysis.

Efficacy of rMETase Alone on Human Colon Tumors in Nude Mice. Five or 10 units/g of rMETase were administered by i.p. injection every 8 h in nude mice with human colon tumors HCT 15 and HT 29 growing s.c. There were four mice with bilateral tumors in each group. Treatment started 2 days after transplantation and continued from day-2 to day-6. Tumor growth was arrested through day-12 (Fig. 2). For HCT 15, the treated:control ratio of tumor weight (T:C) was 23% ($P < 0.001$) after rMETase treatment at 15 units/g/day; and 20% ($P < 0.001$) with rMETase given at 30 units/g/day. For HT 29, the T/C was 31% for rMETase given at 15 units/g/day; and 27%

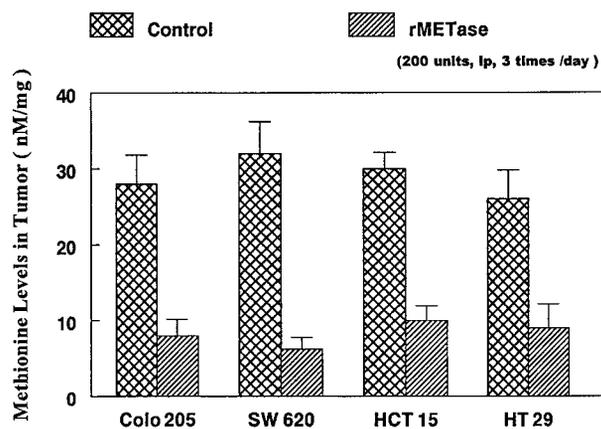


Fig. 1 Depletion of tumor methionine by rMETase. Tumor methionine levels were determined with an HPLC as described in "Materials and Methods." Supernatants were prepared from tumor tissue after sonication. Amino acids were derivitized with the fluoraldehyde reagent, OPA. Tumor supernatant samples (25 µl) were precipitated by acetonitrile (75 µl). Ten µl of supernatant were mixed with 5 µl of OPA. After 1 min, 50 µl of 0.1 M sodium acetate (pH 7.0) were added, and a 20-µl sample was loaded on a reversed-phase Supelcosil LC-18-DB column at room temperature. The column was eluted at room temperature with solution A [tetrahydrofuran:methanol:0.1 M sodium acetate (pH 7.2); 5:95:900] and solution B (methanol). A gradient from 20 to 60% of solution B, pumped at a flow rate of 1.5 ml/min, resolved the amino acids. The eluate was read with a fluorescence spectrophotometer at a wavelength of 350–450 nm. The limit of detection was ~0.1 µM methionine. Bars, SD.

($P < 0.001$) for rMETase given at 30 units/g/day ($P < 0.005$). No significant body weight loss occurred. The results suggest that rMETase alone has significant efficacy against the HCT 15 and HT 29 human colon cancers (Table 2).

Five units/g of rMETase were administered by i.p. injection every 8 h in nude mice with human colon tumors Colo 205 and SW 620 growing s.c. There were six mice with bilateral

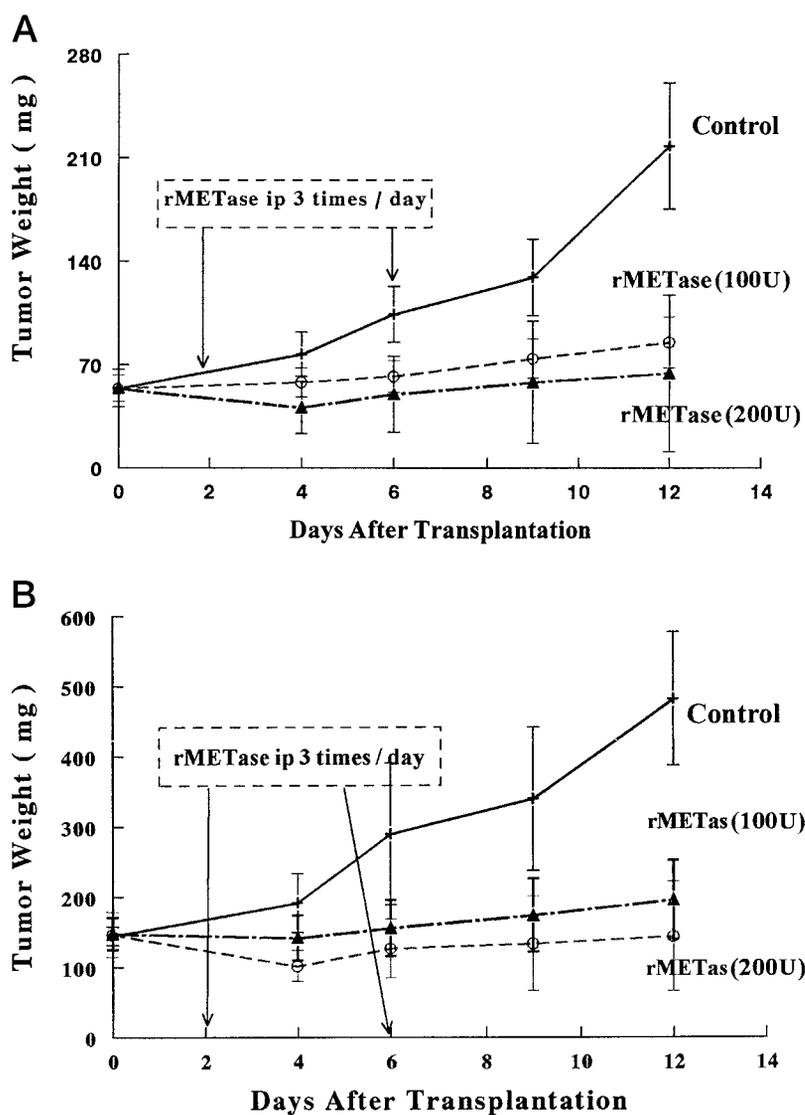


Fig. 2 Efficacy of rMETase on growth of human colon tumors HCT 15 and HT 29 in nude mice. rMETase (5 or 10 units/g every 8 h) was administered by i.p. injection in nude mice with human colon tumor HCT 15 (A) and HT 29 (B) growing s.c. There were four mice with bilateral tumors in each group. Treatment started 2 days after transplantation and was given from day-2 to day-6. The tumor size and body weight were determined every 3 days. The efficacy was evaluated by tumor weight. Bars, SD.

tumors in each group. Treatment started 5 days after transplantation and continued from day-5 to day-9. The tumor growth was slowed through day-16. The T:C values of the rMETase treatment group compared with control groups were 40% for Colo 205 ($P < 0.05$); and 56% for SW 620 ($P < 0.01$). No significant body weight loss occurred (Table 3). The results suggested that rMETase alone was partially effective against Colo 205 and SW 620 (Table 3).

Efficacy of rMETase and CDDP on Human Colon Tumors Colo 205 and SW 620 in Nude Mice. CDDP was chosen to be given in combination with rMETase because it has been shown to affect methionine metabolism (17–19). As stated above, for Colo 205, rMETase given alone from days-5 to -9 resulted in a T:C value of 40%. For CDDP given alone at 7 mg/kg on day-5, the T:C was 44% ($P < 0.05$); and when CDDP was given on day-10, the T:C was 42% ($P < 0.05$). The combination of rMETase and CDDP at the same doses as given alone resulted in a T:C of 19% ($P < 0.01$) when

rMETase was given from days-5 to -9 and CDDP at day-10; and a T:C value of 8% ($P < 0.001$) when CDDP was given at day-5, with rMETase given from days-5 to -9. The latter schedule resulted in two of the six animals having no detectable tumor on day-16, which was 7 days after treatment stopped (Fig. 3A; Table 3A).

SW620 was not sensitive to CDDP alone. Treatment of CDDP at 7 mg/kg at day-5, resulted in a T:C of 80% ($P > 0.05$); and when administered at day-9, a T:C of 78% ($P > 0.05$). SW620 was only partially sensitive to rMETase alone at a dose of 15 units/g/day (T:C, 56%). However, when SW 620 was treated with rMETase on days 5–9 at a dose of 15 units/g/day and CDDP was given on day-5 or day-10 at a dose of 7 mg/kg, the T:C values were 25% ($P < 0.01$) and 47% ($P < 0.05$), respectively (Fig. 2B; Table 3B).

The results suggest that rMETase used in combination with CDDP could produce enhanced antitumor efficacy compared with either of these agents alone. CDDP appeared to be more

Table 2 Efficacy of rMETase on growth of human colon tumors in nude mice

rMETase (5 or 10 units/g every 8 h) was administered by i.p. injection in nude mice with human colon tumor HCT 15 or HT 29 growing s.c. There were four mice with bilateral tumors in each group. Treatment started 2 days after transplantation and was given from days-2 to -6. Tumor size and body weight were measured every 3 days. Body weight (%) was compared to day-0. The efficacy of rMETase was calculated by the tumor weight ratio of the treated group to the control group (T:C). *P* was calculated using the Student's *t* test.

Group	Agent	Treatment			Results (day-12)		<i>P</i> (comparison to control)
		Route	Dose	Schedule ^a	T:C (%)	BW (%)	
A. Efficacy of rMETase on growth of human colon tumor HCT 15 in nude mice							
A	PBS buffer	i.p. q 8 h	0.5 ml	Days 2–6	100	120	
B	rMETase		5 units/g		23	113	<0.001
C	rMETase		10 units/g		20	110	<0.001
B. Efficacy of rMETase on growth of human colon tumor HT 29 in nude mice							
A	Buffer	i.p. q 8 h	0.5 ml	Days 2–6	100	106	
B	rMETase		5 units/g		31	92	<0.001
C	rMETase		10 units/g		27	90	<0.001

^a Day-0 was the day of tumor transplantation.

Table 3 Efficacy of the combination of rMETase and CDDP on growth of human colon tumors in nude mice

Human colon tumors Colo 205 and SW 620 were transplanted s.c. bilaterally to nude mice. Five days after transplantation, the mice were randomized to six per group. The mice were treated with rMETase (5 units/g every 8 h, i.p., days 5–9) and CDDP (7 mg/kg, once on day-5 or day-10, i.p.). Tumor size and body weight were measured every 3 days until day-16, which was 7 days after the last treatment. The T:C value and *P* (Student's *t* test) were then calculated from these data.

Group	Agent	Treatment			Results (day-12)		<i>P</i> (comparison to control)
		Route	Dose	Schedule ^a	T:C (%)	BW (%)	
A. Efficacy of the combination of rMETase and CDDP on growth of human colon tumor Colo 205 in nude mice							
A	Buffer	i.p. q 8 h	0.5 ml	Days 5–9	100	120	
B	rMETase	i.p. q 8 h	5 units/g	Days 5–9	40	113	<0.05
C	CDDP	i.p. once	7 mg/kg	Day-5	44	120	<0.05
D	CDDP	i.p. once	7 mg/kg	Day-10	42	105	<0.05
E	rMETase + CDDP	i.p. q 8 h	5 units/g	Days 5–9			
		i.p. once	7 mg/kg	Day-5	8	104	<0.001
F	rMETase + CDDP	i.p. q 8 h	5 units/g	Days 5–9			
		i.p. once	7 mg/kg	Day-10	19	110	<0.01
B. Efficacy of the combination of rMETase and CDDP on growth of human colon tumor SW 620 in nude mice							
A	Buffer	i.p. q 8 h	0.5 ml	Days 5–9	100	105	
B	rMETase	i.p. q 8 h	5 units/g	Days 5–9	56	98	<0.05
C	CDDP	i.p. once	7 mg/kg	Day-5	80	110	>0.05
D	CDDP	i.p. once	7 mg/kg	Day-10	72	115	>0.05
E	rMETase + CDDP	i.p. q 8 h	5 units/g	Days 5–9			
		i.p. once	7 mg/kg	Day-5	25	96	<0.01
F	rMETase + CDDP	i.p. q 8 h	5 units/g	Days 5–9			
		i.p. once	7 mg/kg	Day-10	47	80	<0.05

^a Day-0 was the day of tumor transplantation.

efficacious when given on day-5 at the start of rMETase treatment.

The potential of combination chemotherapy with methionine depletion was first indicated >10 years ago when cocultures of human normal and tumor cells were treated with methionine-depleted medium and doxorubicin and subsequently with the anti-mitotic agent vinblastine. This treatment resulted in the selective elimination of the tumor cells, whereas the normal cells remained vigorous (28). Goseki's group (12) confirmed this result *in vivo* demonstrating that methionine-free TPN plus doxorubicin followed by methionine-replete TPN and vincristine significantly

extended the life span of Yoshida sarcoma-bearing rats. Clinical studies with methionine-free TPN, 5-FU, and mitomycin C were shown to be effective in advanced gastric cancer (10).

Recently, Kokkinakis *et al.* (29) observed in brain cancer and non-small cell lung cancer cells that when these tumor cells were deprived of methionine *in vitro*, their *O*⁶-methylguanine DNA methyltransferase was markedly down-regulated. This made these tumor cells ~10 times more sensitive to the alkylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea, suggesting very effective synergy of methionine depletion and alkylating agents. Antitumor efficacy was observed by Kokkinakis *et al.* (29) *in vivo* after long-term depletion of methionine on human brain tumor xenografts in athymic mice (30). We have reported

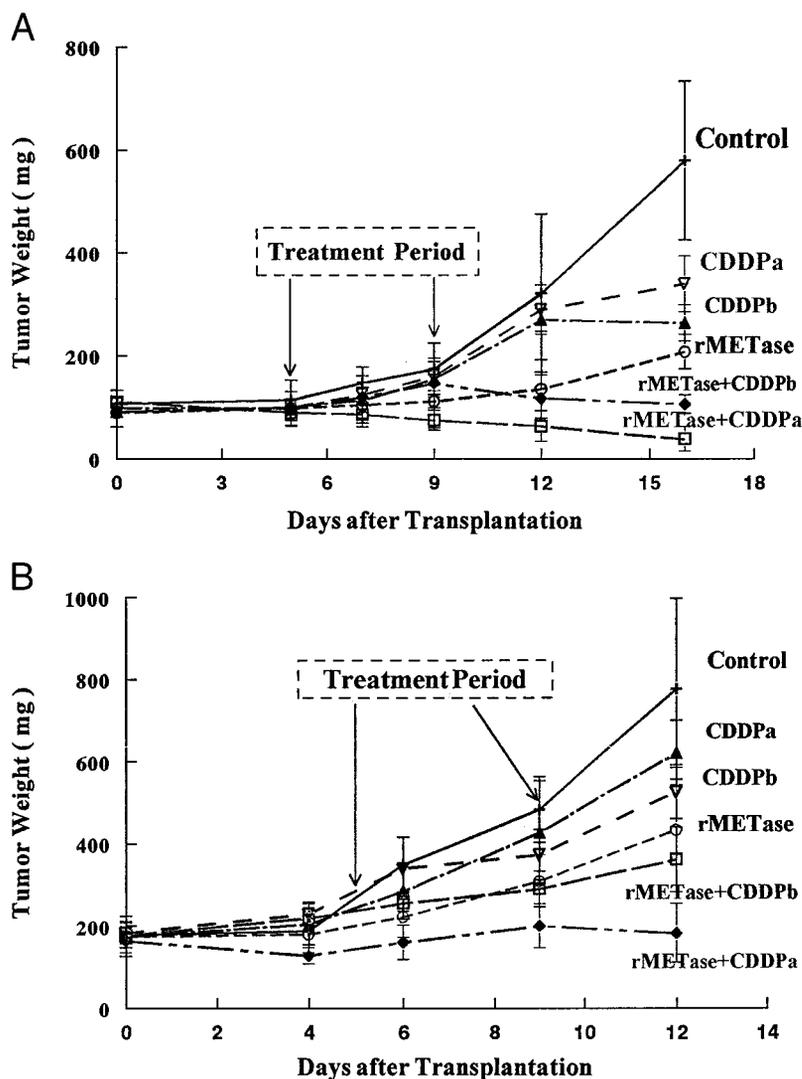


Fig. 3 Efficacy of the combination of rMETase and CDDP on growth of human colon tumors Colo 205 and SW 620 in nude mice. Human colon tumor Colo 205 (A) and SW 620 (B) cells were transplanted s.c. bilaterally to nude mice. Five days after transplantation, the mice were randomized into six per group. The mice were treated with rMETase (5 units/g every 8 h, i.p., days 5–9) and CDDP (7 mg/kg, once on day-5 or day-10, i.p.). Tumor size and body weight were measured every 3 days until day-16, which was 7 days after the last treatment. The efficacy was evaluated by tumor weight. Bars, SD.

that methionine depletion modulates the efficacy of 5-FU in human gastric cancer in nude mice (31). We have shown recently that rMETase and 5-FU had combined efficacy against the Lewis Lung carcinoma *in vivo* (32).

We have shown previously that a methionine-depleted diet enhanced the efficacy of CDDP against the human MX-1 breast carcinoma in nude mice (20). The possibility of enhanced efficacy of CDDP combined with methionine depletion was first suggested by the observations of Scanlon *et al.* (17, 18) and Mineura *et al.* (19). These studies set the precedent for the studies described in the present report.

In conclusion, human colon cancers were shown to be sensitive to the combination of rMETase and CDDP *in vitro* and *in vivo*. rMETase alone inhibited human colon cancers HCT 15 and HT 29 tumor growth in nude mice. Although human colon cancers Colo 205 and SW 620 were only partially sensitive to either rMETase alone or CDDP alone, when rMETase was administered with CDDP in combination, tumor growth arrested or regressed. Similar results were obtained *in vitro*, suggesting

the direct-acting effect of rMETase *in vivo* which is also suggested by the ability of rMETase to deplete tumor methionine. The results suggest that rMETase has promise as a novel cancer therapeutic in combination with CDDP for human colon cancer. Future studies will test this and other combinations (32–35) against additional important tumor types.

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