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Anticancer Efficacy *in Vivo* and *in Vitro*, Synergy with 5-Fluorouracil, and Safety of Recombinant Methioninase

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ABSTRACT

The elevated exogenous-methionine dependency of tumors for growth has been observed in all major cancer cell types. We have previously cloned a methioninase (rMETase) from *Pseudomonas putida* to deplete methionine. Growth inhibition followed by apoptotic cell death was induced by treatment of tumor cells with rMETase *in vitro*. A single i.p. injection of 300 units of rMETase can lower the serum methionine level in the mice from 70 μM to less than 1 μM within 2 h and maintain this depleted level for 8 h. Repeated dosing of rMETase of tumor-bearing mice could be administered without acute immune-hypersensitivity. rMETase treatment demonstrated growth inhibitory activity against human tumors in nude mice, including those which were multiple drug-resistant. No body weight loss or hematotoxicity, except a slight anemia, was found throughout the therapy. The combined treatment of the Lewis lung carcinoma with a fixed rMETase dose and increasing doses of 5-fluorouracil (5-FU) resulted in a dose-dependent enhanced antitumor efficacy for survival as well as tumor growth inhibition. Thus, methionine depletion by rMETase potentiates the antitumor efficacy of 5-FU. The data presented in this report thus indicate that rMETase is active alone, is synergistic in combination with 5-FU, and has negligible toxicity suggesting a novel clinical approach for effective cancer therapy.

INTRODUCTION

It has been shown that methionine dependence, the elevated minimal level of methionine necessary for cell growth, is a tumor-specific metabolic defect found in human cancer cell lines of all major types as well as in fresh human tumor specimens (1–5). Although methionine starvation by means of methionine-depleted diet or methionine-free TPN[2] had been used for *in vivo* therapeutic experiments, this was insufficient to prevent metastasis and tumor growth (6–9).

A purified methionine cleaving enzyme, METase, from *Pseudomonas putida* has been found previously to be an effective antitumor agent *in vitro* as well as *in vivo* (10–14). For the large-scale production of METase, the gene from *P. putida* has been cloned in *Escherichia coli* and a purification protocol for rMETase has been established with high purity and low endotoxin for preclinical and clinical studies (15–17). It has been demonstrated that methionine starvation induces a tumor-selective G_2 cell cycle arrest of tumor cells (18–21).

Methionine starvation in cancer cells mobilizes folate metabolism due to elevated methionine synthesis from homocysteine via methionine synthase (22, 23). This suggests that rMETase could act as a biochemical modulator of 5-FU. In the present study, we report induction of apoptosis in tumor cells by rMETase, human tumor growth inhibition in nude mice, and a significant increase in survival of Lewis lung carcinoma-bearing animals treated with the combination of rMETase and 5-FU. The data in this report suggest that rMETase is a novel agent for effective cancer therapy.

MATERIALS AND METHODS

Animals. BDF1 and athymic BALB/c-nu/nu mice (female, 8–10 weeks of age) were purchased from Japan SLC Inc. (Shizuoka, Japan) and CLEA Japan Inc. (Tokyo, Japan), respectively. C57BL/6 mice (female, 7–9 weeks of age) were produced in a breeding colony maintained in a barrier facility at the Aburahi Laboratory, Shionogi and Co., Ltd.

Tumor Cells. HT-1080 (human fibrosarcoma), HCT-116 (human colon carcinoma), and NCI-H460 (human large-cell-lung carcinoma) were obtained from ATCC (Manassas, VA). KB3–1 and KB8–5 (human squamous cell carcinoma) were kindly provided by Dr. M. Akiyama (Kagoshima University Medical School, Kagoshima, Japan). Ma44 (human squamous lung cancer) was kindly provided by Dr. T. Komiya (Habikino Hospital, Osaka, Japan). All cell lines were maintained by *in vitro* passage using EMEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FCS (Life Technologies, Inc.: Rockville, MD). The Lewis murine lung carcinoma was obtained from the National Cancer Institute (Bethesda, MD) and was maintained by serial s.c. transplantation as tumor fragments in C57BL/6 mice.

Preparation of rMETase. The gene encoding METase from *P. putida*, ICR 3460, was kindly provided by Dr. K. Soda (Okayama University, Okayama, Japan). The gene was inserted into the expression vector pMGL1204 with the tetracycline resistance (Tc^r gene). pMGL1240 was introduced into *E. coli* JM109 competent cells.

rMETase production reached approximately 2.5 g/l after 24 h in a jar fermenter at 28°C, which corresponded to approximately 40% of total soluble protein of the recombinant *E. coli*. rMETase in extracts of the fermented *E. coli* was purified to electrophoretic homogeneity by a procedure that consisted of heat treatment, two anion-exchange chromatographies and gel-filtration. The enzyme was purified with a yield of about 40% from the cell extracts. The specific activity of the final preparation was about 45 units/mg with L-methionine as the substrate.

Enzyme activity was assayed using the method of Tanaka *et al.* (24). Protein was determined according to the method of Bradford (25), using a prepared reagent (Bio-Rad Laboratories, Richmond, CA) and BSA as the standard. The endotoxin level of the purified enzyme was between 0.1 and 4 Eunits/mg protein.

***In Vitro* Cell Culture.** For determination of *in vitro* growth inhibition of tumor cells by rMETase, the MTT assay was used as described previously (26). Cells (2000 cells/well) were plated in a 96-well culture plate (Sumitomo Bakelite Co., Tokyo, Japan) in 100 μl of culture medium.

Twenty-four hours later, a serial 2-fold dilution of rMETase (100 μl) was added to each well. After 96 h incubation in a CO_2 incubator (Tabai Espec Corp., Osaka, Japan), cytotoxicity was determined with the MTT assay to obtain an IC_{50} value.

Assay for DNA Fragmentation. DNA was prepared by phenol-chloroform extraction (27). DNA (5 μg) was separated by 2% agarose gels electrophoresis.

Measurement of Serum Methionine Concentration. The concentration of methionine in serum was determined by a previously described method (28) with slight modification. A plasma sample was diluted with equal volume of 0.1N HCl and 100 μl of diluted plasma was mixed with 700 μl of CH_3CN. The mixture was then centrifuged and a 700 μl aliquot of the supernatant was evaporated in a Speedvac concentrator. Amino acid derivatization was carried out in 30 μl of ethanol/water/triethylamine/phenylisothiocyanate mixture (7:

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[2] The abbreviations used are: TPN, total parenteral nutrition; rMETase, recombinant methioninase; 5-FU, 5-fluorouracil; EMEM, Eagle's minimum essential medium; MTT, 8–4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; ILS, increased life span; BMC, bone marrow cell; CI, combination index; METase, L-methionine α-deamino-γ-mercaptomethane lyase.

Table 1 *In vitro tumor growth inhibition by rMETase*

The growth inhibitory activity of rMETase was analyzed by MTT assay as described in "Materials and Methods."

	Tumor cells	IC_{50} (units/ml)
KB3-1	human squamous (head and neck)	1.90
KB8-5	human squamous (MDR[a])	1.70
PC-9	human lung	0.73
PC-9/CDDP	human lung (cisplatin-resistant)	0.75
Ma44	human lung	0.74
Lewis	murine lung	0.43
HCT-116	human colon	0.30
HT-1080	human fibrosarcoma	0.20

[a] MDR, multidrug-resistant.

1:1:1, v/v). The sample was incubated for 5 min at room temperature and then evaporated to dryness. The precipitate was dissolved in 300 μl of 20% acetonitrile. A 100 μl aliquot was analyzed by high-pressure liquid chromatography with a COSMOSIL (Code No. 392–65, Nacalai Tesque, Kyoto, Japan) column. The elution buffer consisted of solvent A, containing 25 mM ammonium acetate, pH 6.8, and solvent B, containing 70% acetonitrile in 50 mM ammonium acetate, pH 6.8.

***In Vivo* Therapeutic Experiments.** All experiments consisted of 6–10 mice/group. For treatment with rMETase alone, tumor cells (10^5~10^6 cells) were implanted intradermally in the back of nude mice (day 0). rMETase was injected i.p. in the mice twice a day with 8-h intervals beginning from day 1. For combination therapy of 5-FU and rMETase, a tumor fragment (8 mm^3) of the Lewis lung carcinoma was implanted s.c. in the back of BDF1 mice on day 0. 5-FU (3.2, 6.5, or 13 mg/kg) was injected i.v. into the mice daily for 5 days from day 7, and rMETase was injected i.p. into the mice twice a day with 8-h intervals from day 0-day 11. All studies were performed under the guidelines and with the approval of the Shionogi Animal Care and Use Committee (29, 30).

Evaluation of Antitumor Efficacy. Tumor size, body weight, and survival were scored throughout each experiment. Growth inhibitory efficacy and prolonged survival were estimated by percentage inhibition and ILS percentage, respectively. For the evaluation of combination therapy, the CI was used (30).

The CI was calculated using the ILS percentage value as follows:

CI = ILS percentage of drug A with drug B/(ILS percentage of drug A + ILS percentage of drug B).

A CI of more than one indicated synergy, a CI equal to one indicated additivity, and a CI of less than one indicated antagonism. The statistical significance in the present experiments was evaluated with the Dunnet's test (31).

Hematotoxicity Study. Blood (0.5 ml) was collected from the portal vein of anesthetized nude mice. Nucleated BMCs were collected from the right femur. The number of WBCs, platelets, RBCs, and BMCs were counted with an automatic cell counter (K-1000 and CDA-500, Sysmex, Kobe, Japan).

RESULTS

Methionine-dependent Growth of Tumor Cells and Growth Inhibition by rMETase *in Vitro*. It has been shown previously in methionine-depleted cell culture that the growth of tumor cells, but not normal diploid cells, was methionine-dependent (1–5). As shown in Table 1, tumor growth was inhibited by adding 1 unit/ml rMETase into the culture medium. A fibrosarcoma was the most sensitive (IC_{50} of 0.2 units/ml) followed by colon cancer, 0.3 units/ml, and human lung cancer, 0.73–0.75 units/ml).

Induction of Apoptotic Cell Death by rMETase. We observed that the number of viable tumor cells was immediately decreased by rMETase treatment, suggesting that rMETase induced not only cell cycle arrest but also cell death. Thus, we investigated whether rMETase can induce apoptosis with representative results shown in Fig. 1. HCT-116 human colon carcinoma cells were cultured *in vitro* to allow colony formation. Tumor colony growth was progressive in the culture medium without rMETase, whereas in the rMETase-containing medium the tumor cells in the colony died and the colony was eventually diminished within approximately 2 days (Fig. 1). To clarify whether this cell death induced by rMETase was apoptosis, DNA fragmentation in the rMETase-treated cells was analyzed. The

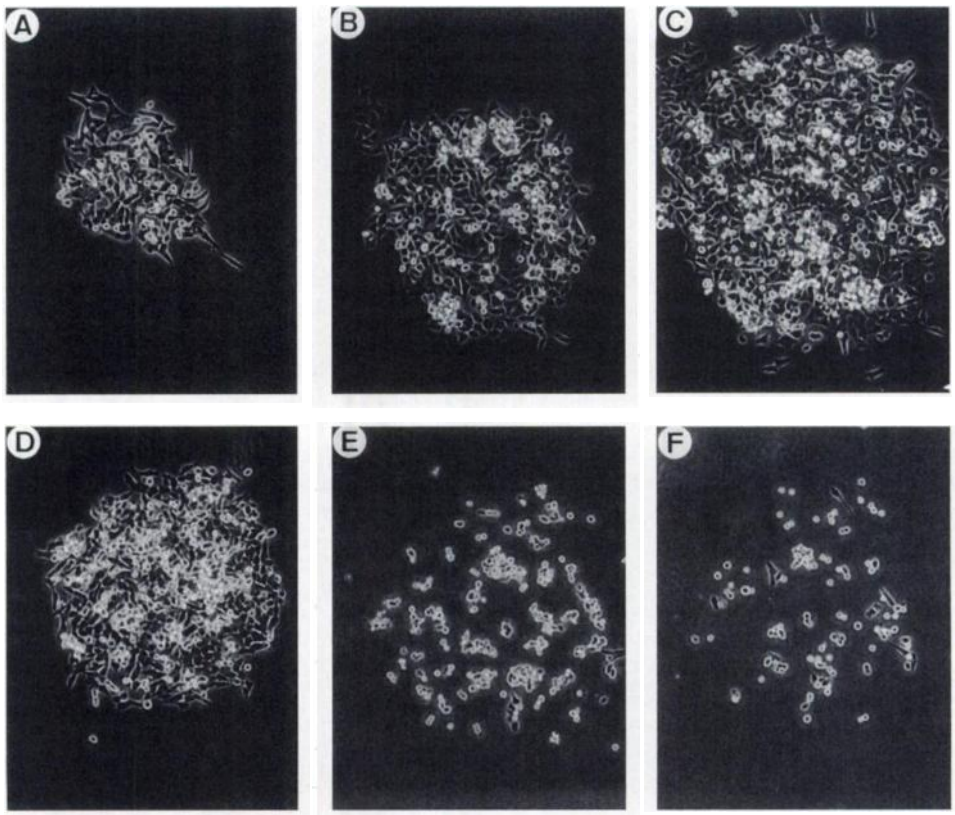

Fig. 1. Induction of tumor cell death by *in vitro* treatment with rMETase. Colonies of HCT-116 human colon cancer cells were cultured in either EMEM (*A-C*) or in EMEM containing 2 units/ml rMETase (*D-F*). The same colonies on day 0 (*A* and *D*), day 2 (*B* and *E*), and day 4 (*C* and *F*) in culture are shown.

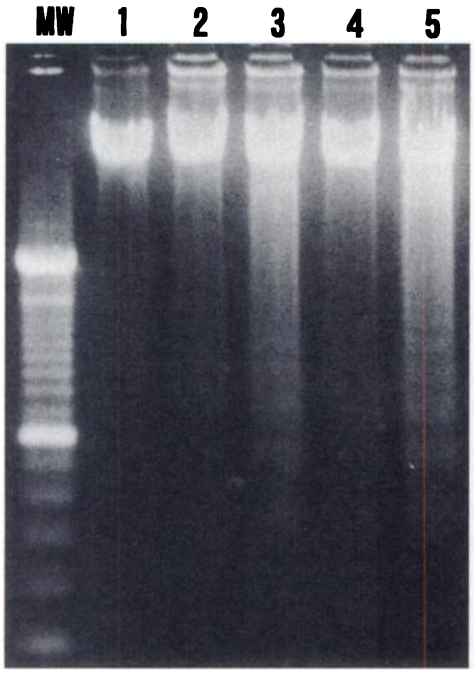

Fig. 2. Induction of DNA fragmentation by *in vitro* treatment with rMETase. DNA (5 μg) isolated from rMETase-treated HCT-116 cells was separated by 2% agarose gel electrophoresis. MW, molecular weight markers; *Lane 1*, no treatment; *Lane 2*, 0.5 units/ml for 24 h; *Lane 3*, 2 units/ml for 24 h; *Lane 4*, 0.5 units/ml for 48 h; *Lane 5*, 2 units/ml for 48 h.

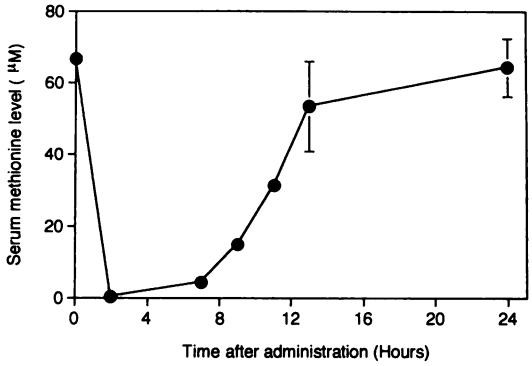

Fig. 3. Serum methionine depletion by rMETase in mice. Three-hundred units of rMETase were injected into the tail veins of BDF1 mice ($n = 3$). The blood was collected before injection and at 2, 7, 9, 11, 13, and 24 h after injection. Serum methionine levels were measured as described in "Materials and Methods."

results shown in Fig. 2 demonstrate that time-dependent and dose-dependent DNA fragmentation was induced in rMETase-treated HCT-116 cells, indicating that methionine depletion by rMETase caused apoptotic cell death of the tumor cells. The evidence for apoptotic DNA strand breaks was also obtained using the terminal deoxynucleotidyl transferase mediated dUTP nick and labeling technique (data not shown; 32).

Serum Methionine Depletion by rMETase in the Experimental Animals. The serum-depletion efficacy of a single i.p. injection of 300 units of rMETase *in vivo* on methionine levels in serum was determined. The depletion of serum methionine began immediately and reached undetectable levels (less than 1 μM) by 2 h. The depleted methionine level was maintained for 8 h after injection (Fig. 3).

***In Vivo* Antitumor Efficacy and Toxicity of rMETase.** On the basis of the pharmacokinetic data of methionine depletion by rMETase, 100–600 units of rMETase were administered twice a day with 8-h intervals in the mice with s.c. growing human tumors. In all cell lines used, the administration of rMETase resulted in dose-dependent, significant ($P < 0.01$) inhibition of tumor growth including those which are multidrug resistant *in vivo* (Table 2).

To evaluate the hematotoxicity of rMETase, nontumor-bearing mice were treated with 600 units of rMETase twice a day for 21 days. In the course of the treatment, no significant body weight loss was observed (Fig. 4*A*). After completion of the treatment, blood samples were collected and blood cells were counted. Although no difference in the number of WBCs, platelets, and BMCs were detected between control and rMETase-treated groups, the number of RBCs was slightly, but significantly, decreased in the rMETase-treated group (85% of the control, $P < 0.01$, Fig. 4*B*). These results demonstrated that long-term treatment of rMETase caused a slight anemia but no other hematotoxicity.

Antigenicity of rMETase. Because rMETase is bacterial-derived protein, one should consider its antigenicity especially in the case of repeated treatment. Immune-competent BDF1 mice were treated i.p. with 100 units of rMETase twice a day for 10 days. One and one-half months after the previous administration, the mice were again treated i.p. with 100 units of rMETase. In addition, the mice received injections i.v. of rMETase on the next day. All mice survived without piloerection, hypothermia, and linanimation after boosting. These results demonstrated that at least in the murine model, the antigenicity of rMETase is minimal.

Antitumor Efficacy of the Combination of rMETase and 5-FU. Methionine starvation of cells accelerates the rate of conversion of 5-methyltetrahydrofolate into tetrahydrofolate coupled with methionine synthesis from homocysteine via methionine synthase (19, 20). Thus, it was thought that rMETase could act as a biochemical modulator of 5-FU against tumor cells *in vivo* due to elevated levels of methylene tetrahydrofolate, which enhances binding of 5-FU to thymidylate synthetase (33). Other synergistic effects are also possible because 5-FU also interferes with RNA and DNA synthesis (34). These possible mechanisms of synergy will be investigated in future studies.

We, therefore, combined rMETase with 5-FU using the murine Lewis lung carcinoma model. Tumor-bearing mice were treated with 600 units of rMETase twice a day with 8-h intervals from day 0-day 11 and with 5-FU from day 7-day 11. Fig. 5*A* demonstrated that growth inhibition of tumor cells was enhanced by the combined treatment of rMETase and 5-FU. As shown in Fig. 5*B*, the combination of rMETase and 5-FU conferred a significant increase in survival. The ILS percentage of rMETase alone, 5-FU alone, and a combination of both drugs were 17, 30, and 78%, respectively.

The interaction of the efficacy of the two drugs on survival was

Table 2 *Antitumor efficacy of rMETase against human cancers*

Tumor cells	rMETase[a] (unit/shot)	Treatment period (day)	No. of mice	Tumor volume[b] (Mean ± SD)	Tumor volume[b] Inhibition (%)
KB3-1 (squamous)	buffer		8	3265 ± 434	
	100	10	8	1814 ± 284[c]	56
KB8-5 (squamous)	buffer		6	1946 ± 649	
	100	21	6	953 ± 383[c]	51
	300	21	6	814 ± 197[c]	64
HT-1080 (fibrosarcoma)	buffer		5	4003 ± 1394	
	100	14	5	921 ± 255[c]	77
	300	14	5	640 ± 457[c]	84
Ma44 (squamous)	buffer		7	5004 ± 1224	
	100	29	4	3062 ± 371[c]	39
	300	29	6	2996 ± 682[c]	40

[a] i.p. injected twice a day.
[b] mm^3, on day 17 for KB3-1; on day 22 for KB8-5; on day 24 for HT-1080; and on day 20 for Ma44.
[c] $P < 0.01$ by Dunnet's test.

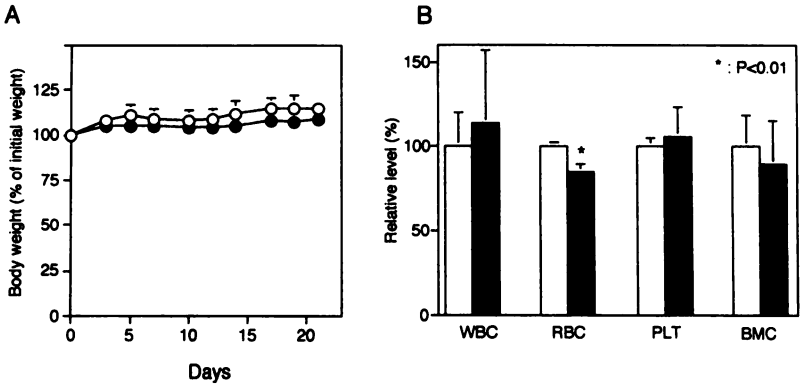

Fig. 4. Body weight change and hematotoxicity due to rMETase treatment. BALB/c nude mice were treated with 600 units of rMETase twice a day for 21 days. *A*, change of body weight during the treatment with vehicle control (○) or rMETase (●) are shown. *B*, after the treatment, the blood was analyzed as described in "Materials and Methods." *Open bar*, vehicle control; *Solid bar*, rMETase.

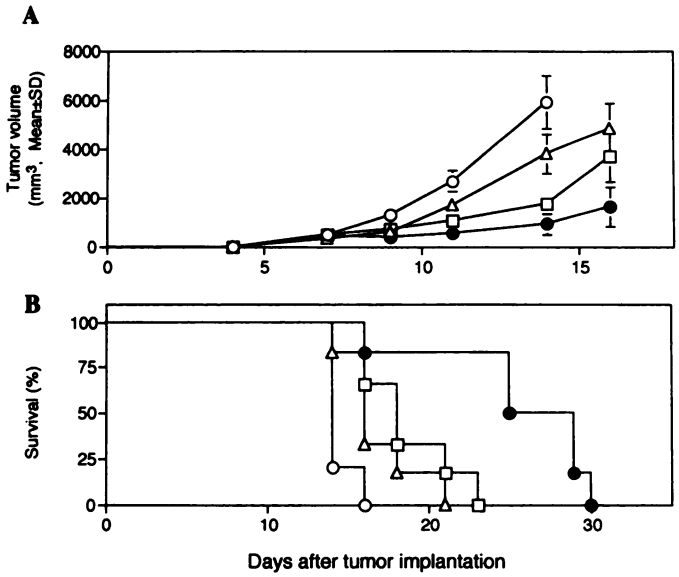

Fig. 5. Antitumor efficacy of the combination of rMETase and 5-FU on the Lewis lung carcinoma. BDF1 mice bearing the Lewis lung carcinoma were treated with vehicle control (○), rMETase (600 units × 2/day × 12; △), 5-FU (13.2 mg/mg × 5; □), or the combination of rMETase and 5-FU (●) as described in "Materials and Methods." The tumor growth inhibition (*A*) and survival rate (*B*) are shown.

analyzed by calculating the CI, which indicated that the survival effect was synergistic in combination therapy of rMETase and 5-FU (CI = 1.5). A strong dose response for survival was demonstrated for 5-FU in combination with rMETase (Fig. 6).

These results demonstrate that rMETase exerted antitumor efficacy, synergy, and safety in experimental therapeutic models.

DISCUSSION

To attack the methionine dependence target of tumors, methionine-free diets or TPN alone has been shown to be insufficient to prevent initial growth of the tumor (6–9). This is probably due to the fact methionine-free diets or TPN alone could not completely deplete serum methionine.

In vitro analysis has demonstrated that sufficiently-depleted methionine levels are necessary for antitumor efficacy. A methionine concentration lower than 10 μM is necessary for inhibition of tumor-cell growth accompanied by cell death. This level of methionine depletion can be rapidly attained at 2 units/ml rMETase *in vitro* (data not shown). The results presented in this study demonstrate that rMETase treatment could induce tumor cell death via apoptosis.

We have also demonstrated that the administration of 300 units of rMETase *in vivo* was able to deplete the serum methionine level to less than 1 μM and maintain this level for at least 8 h. These results suggested that rMETase therapy had the possibility to induce tumor cell death that could lead to solid tumor regression *in vivo*.

The status of the p53 gene may determine the sensitivity or efficacy of currently-used cancer therapy (35, 36). Methionine starvation also induced cell death in the HCT-116 colon carcinoma used in this study, which has been shown to have the wild-type p53 gene (37). In tumor cells with a mutated p53 such as the HT29 colon carcinoma (38) methionine starvation also induced cell death (data not shown). These results demonstrate that the apoptotic cell death induced by rMETase may be p53-independent.

rMETase therapy was effective on multidrug resistant cells (Tables 1 and 2). rMETase also inhibited the growth of cisplatin-resistant tumor cells (Table 1). Because there is no evidence of resistance to methionine starvation thus far, this might be a significant advantage of rMETase therapy for clinical use.

It has been reported that CDDP inhibits methionine uptake into tumor cells, which perturbs folate metabolism and makes tumor cells more sensitive to 5-FU (22). Given the superior efficiency of rMETase to deplete cellular methionine, it is expected that the combination of rMETase and 5-FU would enhance antitumor efficacy. The results presented in this study indicated that rMETase synergistically potentiated the antitumor efficacy of 5-FU *in vivo*, including a significant extension of survival that was dose-dependent with respect to 5-FU. A precise understanding of the mechanisms of the synergistic interaction of rMETase and 5-FU will be important for the appropriate use of rMETase in clinical studies.

The preclinical efficacy of rMETase, its novel mechanisms, lack of toxicity, and high-level recombinant expression and production demonstrated in this report suggest strong clinical potential.

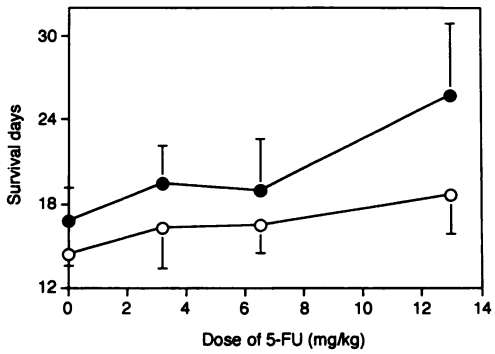

Fig. 6. Dose-response survival effect of 5-FU in the combination of rMETase with 5-FU on the Lewis lung carcinoma. BDF1 mice bearing Lewis lung carcinoma were treated with the indicated doses of 5-FU with (●) or without (○) rMETase (600 units × 2/day × 12).

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