Pharmacokinetics, Methionine Depletion, and Antigenicity of Recombinant Methioninase in Primates

Zhijian Yang,[1] Junhua Wang,[2] Takayuki Yoshioka,[3] Baoqiu Li,[4] Quan Lu,[2] Shukuan Li,[1] Xinghua Sun,[1] Yuying Tan,[1] Shigeo Yagi,[1] Eugene P. Frenkel,[5] and Robert M. Hoffman[1]

[1]AntiCancer, Inc., San Diego, California; [2]Kingsley Pharmaceutical Inc., Nanjing, People's Republic of China; [3]Shionogi and Co., Ltd., Osaka, Japan; [4]Jinan Persun Pharmaceutical Technology Co., Ltd., Jinan, People's Republic of China; and [5]University of Texas, Southwestern Medical School, Dallas, Texas

ABSTRACT

Pharmacokinetics, methionine depletion, antigenicity, and toxicity of recombinant methioninase (rMETase), which has shown efficacy in achieving cell kill in a broad range of human tumor models, were examined in macaque monkeys. Dose-ranging studies at 1000, 2000, and 4000 units/kg i.v. identified the 4000 units/kg dose as able to reduce plasma methionine to an undetectable level (less than 0.5 μM) by 30 min, and the level so remained for 8 h. Pharmacokinetic analysis showed that rMETase was eliminated with a $T_{1/2}$ of 2.49 h. A 2-week i.v. administration of 4000 units/kg every 8 h/day for 2 weeks resulted in a steady-state depletion of plasma methionine to less than 2 μM. The only manifest toxicity was decreased food intake and slight weight loss. Serum albumin and red cell values declined transiently during treatment, which may be related to extensive blood sampling. Re-challenge on day 28 resulted in anaphylactic shock and death in one animal. Subsequent pretreatment with hydrocortisone prevented the anaphylactic reaction, although vomiting was frequently observed. Re-challenge was carried out at days 66, 86, and 116. Anti-rMETase antibodies (at 10^{-3}) were found after the first challenge, and these increased to 10^{-6} after the fourth challenge and decreased to 10^{-2} by 2 months post therapy. The main rMETase antibody was IgG, and although it has some *in vitro* features of being a neutralizing antibody, each challenge dose was effective in depleting plasma methionine levels. Thus, rMETase was able to effectively deplete plasma methionine levels with minimal toxicity in a primate model. These data provide the bases for alteration by polyethyleneglycol conjugation (PEGylation) of the enzyme to increase its duration of effect and reduce its immunogenicity.

INTRODUCTION

Methionine dependence, the elevated minimal methionine requirement for cell growth relative to normal cells, has been observed in many human cancer cell lines and cancer xenografts in animal models (1–5). Methionine dependence is a metabolic defect seen only in cancer cells and precludes the cells from growing in media in which methionine is depleted. Methionine dependence is thought to be due to increased rates of transmethylation in cancer cells compared with normal cells (6, 7). It has been demonstrated that methionine deprivation inhibits the growth of cancer cells by causing them to arrest predominantly in the G_2 phase of the cell cycle and to eventually undergo apoptosis (8–12).

These intriguing observations led to an examination of the effect of dietary methionine restriction, including in combination with chemotherapy, on tumor growth in xenograft models of human cancer (13, 14). Efficacy was shown in these models, and synergism was produced by adding chemotherapy.

A much more powerful approach to methionine depletion is the use of L-methionine α-deamino-γ-mercaptomethane-lyase (methioninase), a methionine-cleaving enzyme from *Pseudomonas putida* (15, 16). The enzyme has been cloned and produced in *Escherichia coli* [Refs. 17 and 18; recombinant methioninase (rMETase)] and thereby affords a more tightly controlled means to methionine depletion.

rMETase alone arrested growth of colon tumors HCT 15 and HT29 and partially arrested Colo 205 and SW 620 in nude mice. Cisplatin (CDDP) in combination with rMETase resulted in tumor regression of Colo 205 and growth arrest of SW 620. SW 620 was resistant to CDDP alone and only partially sensitive to rMETase alone. However, when SW 620 was treated with rMETase and CDDP, tumor growth was arrested. The results demonstrate that rMETase used simultaneously in combination with CDDP had significant efficacy (19).

Combination treatment of the Lewis lung carcinoma with a fixed rMETase dose and increasing doses of 5-fluorouracil resulted in a dose-dependent increase in survival as well as tumor growth inhibition. rMETase potentiated the efficacy of 5-fluorouracil (20).

The growth of Daoy, SWB77, and D-54 glioblastoma xenografts in athymic mice was arrested after the depletion of mouse plasma methionine with a combination of an rMETase and methionine-free diet. Methionine depletion for 10–12 days induced mitotic and cell cycle arrest, apoptotic death, and widespread necrosis in tumors but did not prevent tumor regrowth after cessation of the regimen. However, when a single dose of 35 mg/kg *N,N′*-bis (2-chloroethyl)-*N*-nitrosourea (BCNU), which was otherwise ineffective as a single therapy in these

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tumors, was given at the end of the methionine depletion regimen, a more than 80-day growth delay was observed for Daoy and D-54 (21). An interesting correlation to these studies is that methionine depletion was more effective against high-grade, more aggressive gliomas than those more differentiated (astrocytic) tumors (22).

The rMETase gene has been shown to be active as an antitumor agent including its use with selenomethionine as a pro-drug (23–25).

We have previously carried out a pilot Phase I clinical trial of rMETase. Patients with advanced breast cancer, lung cancer, renal cancer, and lymphoma were given a single rMETase treatment at doses ranging from 5,000 units (0.25 g) to 20,000 units (1.0 g) by i.v. infusion over 6–24 h. The preliminary finding from this clinical trial showed that rMETase depleted plasma methionine levels without observed clinical toxicity in patients with very advanced cancers (26, 27).

The previous pilot Phase I study was conducted mainly with a single dosing regimen. To investigate the toxic effects, antigenicity, and toxicity of rMETase requires repeated dosing. We chose primates for this study, in particular, to answer the question of antigenicity of rMETase.

The current study investigated the pharmacokinetics, methionine depletion, antigenicity, and systemic toxicity of repeated administration of rMETase with dose escalation in macaque monkeys.

MATERIALS AND METHODS

Drugs. rMETase was manufactured by Shionogi & Co. Ltd. (Osaka, Japan). The rMETase was more then 95% pure by high-performance liquid chromatography and a single band of M_r 43,000 on SDS-PAGE with a tetramer:oligomer ratio of 96.7:3.3. The specific enzyme activity of rMETase was 55 units/mg. The endotoxin level was 0.06 EU/mg. Frozen rMETase was thawed and warmed to 37°C just before use. The vehicle solution was constituted with normal physiological saline containing 0.1 mmol/liter pyridoxal 5′-phosphate.

Animals. Male *Macaca fascicularis* monkeys, ages 5–7 years and weighing 5–8 kg, were obtained from the Xishungbanna Monkey Station of the National Laboratory Primate Center (Yunnan, China). The monkeys were individually housed in stainless steel cages in a controlled environment (13 h/day artificial lighting; 15 times/h ventilation; 20–25°C room temperature; 40–60% humidity). The monkeys were fed regular monkey chow purchased from the Experimental Animal Center (Basic Medical Science Institute, Beijing, China), fruit, and water at scheduled times. All monkeys were acclimated in the same facility for a 2-week period before the initiation of treatment. During this period, they were observed for clinical signs and examined for food consumption and body weight. Blood studies that included complete blood counts and a broad chemical panel as well as measurement of plasma methionine concentration were done.

Dose Ranging. Six monkeys were randomly assigned to three dose levels of 1000 units/kg (monkeys 950111 and 960383), 2000 units/kg (monkeys 962147 and 960427), and 4000 units/kg (monkeys 970475 and 970985). Single doses of 1000, 2000, or 4000 units/kg rMETase solution were administered to groups of three groups via the saphenous veins of the hind limbs under anesthesia consisting of 15 mg/kg ketamine hydrochloride administered i.m. All monkeys were fasted 8 h before and after dosing. rMETase was administered by bolus injection over 1–2 min.

Repeated Dose Administrations. Based on the dose-ranging study, a dose of 4000 units/kg rMETase was administered to each of four naive monkeys (monkeys 970475, 960267, 960553, and 950945) three times per day at 8-h intervals for 14 consecutive days. As controls, two monkeys (monkeys 960235 and 962423) received the same volume of vehicle solution in the same manner as the rMETase-treated monkeys. All animals were fasted 2 h before and after each injection.

Challenge Doses. On day 28 after administration of rMETase, two rMETase-treated monkeys (monkeys 960553 and 950945) were given challenge injections of rMETase at the same dose used in the repeated-dose studies. Another two rMETase-treated monkeys (monkeys 970475 and 960267) received monthly challenge injections of rMETase for 4 months with hydrocortisone (50 mg) pretreatment i.v. 10 min before each challenge injection.

Determination of Plasma rMETase Activity. To determine plasma rMETase activity and methionine levels after a single rMETase administration, blood samples were collected before rMETase injection and 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h post injection. For determination of plasma enzyme activity and methionine levels during repeated dosing of rMETase, blood samples were drawn on days 1, 5, 9, 14, 28, and 66 at time points before the first rMETase injection of the day and 1, 2, 4, and 8 h post injection on each sampling day. All blood samples were collected in EDTA-coated tubes and centrifuged at 13,000 rpm for 2 min at 4°C to obtain plasma. Plasma samples were aliquoted and frozen at −70°C until analysis. We used primate plasma with known METase activity as a positive control and METase-untreated primate plasma as a negative control, because no METase exists in primates.

rMETase enzyme activity was determined from α-ketobutyrate produced from L-methionine according to the method of Esaki and Soda (28) with slight modification. The assay was carried out in triplicate. Change in absorbance (ΔE) was calculated by subtracting average absorbance of blanks from average absorbance of the reaction mixture. The enzyme activity was calculated by the following equation: Activity (units/ml) = $0.548(1.07 + 2.2\Delta E)\ \Delta E$. One unit of enzyme is defined as the amount of enzyme that produced 1 μM α-ketobutyrate/min at infinite concentration of L-methionine.

Determination of Plasma Methionine. The plasma methionine concentration was measured using a precolumn derivatization, followed by high-performance liquid chromatography separation based on a previously described method with modification (29). A 10-μl plasma sample or methionine standard was used. The plasma methionine was identified by the retention time of a methionine standard solution and quantitated according to a methionine standard curve. The limit of detection was 0.5 μM methionine. The upper limit of detection for methionine assay is 100 μM. Coefficient of variations for the inter- and intra-day assays were 5.7% and 2.8%, respectively.

Pharmacokinetic Analysis. The concentration-time curves were generated as the plasma enzyme activity (units/ml) *versus*

time after single rMETase administration. Pharmacokinetic parameters including initial drug concentration, elimination constant, apparent volume of distribution, $T_{1/2}$, area under (concentration-time) curve, and total body clearance were calculated based on the noncompartment model using pharmacokinetic software 3P97 (Mathematics Institute, Academy of Science, Beijing, China).

Systemic Toxicity Studies. Clinical parameters including vital signs, gastrointestinal symptoms, infections, and overall activity and behavior were observed daily. Body weights were recorded weekly during the 2-week treatment period as well as on day 21. Food consumption values were recorded daily until day 28, and average daily food consumption of each week was calculated weekly.

Hematology and Blood Chemistry. For hematology and blood chemistry examinations, 2 ml of blood samples were collected via the saphenous vein from each animal anesthetized by an i.m. injection of ketamine. Collections were made on days 5, 9, 14, 26, and 86 during the experimental period. Approximately 1 ml of blood was collected into EDTA-coated tubes. Blood was analyzed by an automated hematology analyzer (CELL-DYN 1700) for the following hematological parameters: RBC; WBC; platelet; hemoglobin; hematocrit; mean corpuscular hemoglobin; mean corpuscular volume; and mean corpuscular hemoglobin concentration. Approximately 1 ml of blood was collected into serum separator tubes and centrifuged. The resulting sera samples were analyzed by an automated chemistry analyzer (Synchron Clinical System CX(R)5; Beckman) for the following blood chemistry parameters: alanine aminotransferase; γ-glutamyl transpeptidase; alkaline phosphatase; total protein; albumin; globulin; albumin/globulin; total bilirubin; direct bilirubin; indirect bilirubin; cholesterol; triglycerides; glucose; blood urea nitrogen; creatinine; aspartate aminotransferase; creatine phosphokinase; lactate dehydrogenase; Ca^{2+}; Na^{+}; K^{+}; and Cl^{-}.

Tissue Studies. One rMETase-treated monkey died after the first challenge injection of rMETase on day 28. Gross necropsy was performed. Brain, lung, liver, adrenals, kidneys, and spleen were weighed. The tissues from heart, aorta, lungs, liver, and kidneys were immediately collected and fixed in 10% neutral buffered formalin for histopathology examinations.

Determination of Plasma Anti-rMETase Antibody. Blood samples were collected on days 5, 9, 14, and 26 after administration of rMETase. During the challenge injection period, blood samples were collected every 2 weeks. Plasma was prepared and stored at −70°C. Plasma anti-rMETase antibody measurement and identification of immunoglobulin class were performed using a Sandwich ELISA. One hundred μl of rMETase (200 μg/ml) in 0.1 M carbonate coating buffer (pH 9.5) were added to each well of a 96-well microplate and incubated at 4°C overnight. The plate was washed three times with PBS (pH 7.4) containing 0.05% Tween-20 and blocked for 2 h at room temperature with 200 μl of PBS assay buffer (pH 7.4) containing 10% fetal bovine serum. After washing three times, 100 μl of 10-fold serial dilutions of the plasma samples in PBS were added to appropriate wells and incubated for 2 h at room temperature, followed by washing again. One hundred μl of optimally diluted antimonkey polyvalent immunoglobulins, horseradish peroxidase conjugate (obtained from the Basic Medical Science Institute, Beijing, China), or antihuman IgG, IgM, and IgA subtype-horseradish peroxidase conjugates (Sigma) were added to each well. The plate was incubated for 1 h at room temperature and washed three times. One hundred μl of substrate solution containing *O*-phenylenediamine dihydrochloride and hydrogen peroxide (Sigma) were added to each well, followed by 30 min of incubation at room temperature. Fifty μl of 2 N sulfuric acid were added to each well to stop the color reaction, and the absorbance of each well was measured at 492 nm.

The presence of neutralizing antibody was examined in plasma (0.2 ml) obtained from rMETase-treated monkeys. This plasma was mixed with 0.2 ml of various concentrations of rMETase solution. The saline and plasma obtained from a vehicle-treated monkey were used as blank and negative control, respectively. After incubation at 37°C in a water bath for 30 min, the reaction mixtures were measured for rMETase enzyme activity.

RESULTS

Dose-Ranging Study. Plasma methionine depletion in the monkeys after three single doses of rMETase is shown in Table 1. Plasma methionine concentration fell from pretreatment levels of 11–26 μM to the limit of detection level by 30 min and remained at the limit of detection for 4 h in all monkeys receiving three single doses of rMETase. It was found that plasma methionine could be depleted below 5 μM for 8 h using

Table 1 Plasma methionine concentration (μM) after single escalating doses of rMETase

Animals were dosed at time 0 with the single dose of rMETase listed below. See "Materials and Methods" for details.

	1000 units/kg		2000 units/kg		4000 units/kg	
Time points (h)	Monkey 950111	Monkey 960383	Monkey 960427	Monkey 962147	Monkey 970475	Monkey 970985
0	17.4	14.08	12.66	11.08	15.45	26.46
0.5	0	0	0	0	0	0
1.0	0	0	0	0	0	0
2.0	0	0	0	0	0	0
4.0	0	0	0	0	0	0
8.0	5.93	4.14	3.24	4.62	0.99	1.85
12.0	8.91	5.53	5.28	7.22	5.61	6.12
24.0	10.40	13.27	8.97	8.07	12.27	24.73

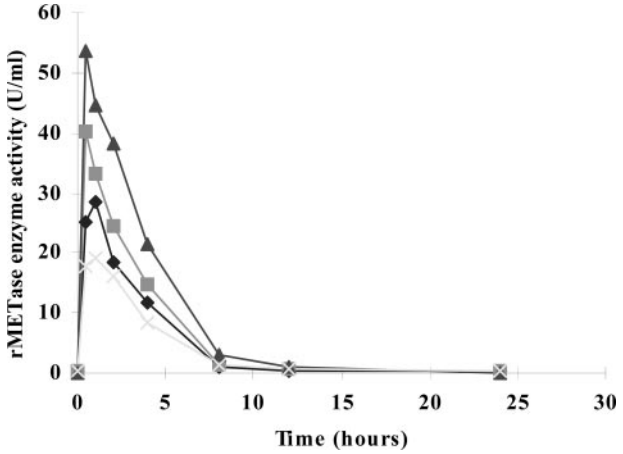

Fig. 1 Plasma rMETase concentration-time profiles in monkeys after 2000 and 4000 units/kg doses of rMETase at time 0. Monkeys 962147 and 960427 were dosed with 2000 units/kg. Monkeys 970475 and 970985 were dosed with 4000 units/kg. Blood was sampled at the times indicated. rMETase activity was measured as described in "Materials and Methods." A $T_{1/2}$ of 2.49 h for rMETase was calculated from these data. ◆, 962147; ■, 960427; ▲, 970475; ✕, 970985.

rMETase doses of 1000, 2000, and 4000 units/kg. Four thousand units/kg rMETase were found to be sufficient to deplete plasma methionine to approximately 1 μM for 8 h.

Pharmacokinetics. Pharmacokinetics was examined in the monkeys receiving a single i.v. injection of rMETase at a dose of 2000 or 4000 units/kg. The plasma rMETase concentration-time profiles of the monkeys are presented in Fig. 1. The calculated pharmacokinetic parameter values are shown in Table 2. The i.v. injection of rMETase showed a rapid reduction of plasma enzyme activity with a $T_{1/2}$ of 2.49 h. Total systemic body clearances of rMETase were 11.28 ml/h and 30.77 ml/h for the 2000 and 4000 units/kg doses, respectively.

Plasma rMETase Activity in Monkeys Receiving Repeated Doses of rMETase. In all four monkeys treated with 4000 units/kg rMETase three times per day for 2 weeks, plasma rMETase enzyme activity could be continually maintained at greater than 18.3 ± 10.6 units/ml, with highest observed plasma enzyme activity of 125.5 ± 54.7 units/ml during the 2-week treatment period. The plasma 8-h rMETase concentration-time profiles showed no statistically significant differences on days 1, 5, 9, 14, and 66 (Fig. 2).

Methionine Depletion in Monkeys Receiving Repeated Doses of rMETase. During the 2-week, three-times-per-day treatment period, plasma methionine in the rMETase-treated monkeys was depleted effectively by repeated doses of rMETase. The plasma methionine was maintained at the limit of detection for at least 4 h after each i.v. injection of rMETase. At 8 h after each injection, the plasma methionine level ranged from 0.9 ± 0.6 to 2.2 ± 1.2 μM (Table 3). By day 66, the methionine levels returned to day-1 pretreatment levels.

Effect of rMETase on Food Intake and Body Weight. All monkeys treated with rMETase or vehicle tolerated the 2-week treatment period. Two monkeys had a mild vomiting reaction after each injection of rMETase within the first 6 days of treatment. From days 7 to 14, vomiting and faintness was observed in all rMETase-treated monkeys. Mild diarrhea occurred in two rMETase-treated monkeys on days 11 and 12. These reactions disappeared after the termination of the 2-week treatment.

Reduced food intake and body weight loss occurred in all rMETase-treated monkeys during the 2-week treatment period. The mean daily feed consumption value (g/day) in rMETase-treated monkeys was reduced from the pretreatment level of 265 ± 26 g/day to 168 ± 15 g/day in the 1st week and 1.8 ± 3.6 g/day in the 2nd week of treatment. The food consumption recovered after the 2-week treatment (38 ± 15 g/day in 3rd week) and returned to normal in the 4th week. The mean body weight loss in the rMETase-treated monkeys was 9.3 ± 3.3% (range from 4.7 to 12.5%) in the 1st week of treatment and 18.2 ± 5.2% (range from 11.8 to 25%) in the 2nd week of treatment. Body weight gain after the 2-week treatment period was found to be slow (16.3 ± 3.8% in the 4th week).

Immunological Reactions to rMETase. Anaphylactic shock occurred in two monkeys immediately after the first challenge injection of rMETase on day 28. Both monkeys were immediately given i.v. injections of hydrocortisone at a dose of 50 mg/monkey. One monkey recovered 1 h later and survived, whereas the other monkey did not recover and was sacrificed for necropsy. To prevent anaphylactic shock in the remaining two monkeys, hydrocortisone was given 10 min before each challenge injection of rMETase. A total of four challenge injections were given to these two monkeys at an interval of 1 month. Only

Table 2 Pharmacokinetic parameters of rMETase in monkeys

Monkeys were treated with the single dose of rMETase listed below. Blood was drawn, analyzed for plasma enzyme activity, and evaluated for the parameters listed below as described in "Materials and Methods."

Monkey	Co[a] (units/ml)	Ke (liters/h)	Vc × 10^3 (units/ml)	$T_{1/2}$ (h)	AUC (units/ml/h)	CLs (ml/h)
2000 units/kg dose						
962147	32.51	0.28	0.03	2.49	116.69	8.75
960427	48.67	0.32	0.04	2.15	144.81	13.81
4000 units/kg dose						
970985	22.64	0.24	0.04	2.84	92.67	43.13
970475	61.83	0.28	0.06	2.49	221.76	18.4

[a] Co, initial drug concentration; Ke, elimination constant; Vc, apparent volume of distribution; $T_{1/2}$, biological half-life; AUC, area under (concentration-time) curve; CLs, total body clearance.

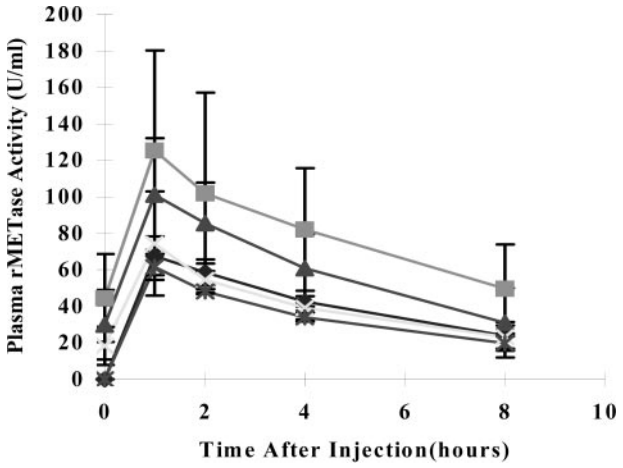

Fig. 2 Plasma rMETase enzyme activity in the monkeys receiving repeated treatment with rMETase ($n = 4$). Monkeys were treated with 4000 units/kg rMETase. Blood was sampled at the indicated times. rMETase was measured as described in "Materials and Methods." ◆, day 1; ■, day 5; ▲, day 9; ✕, day 14; ∗, day 66.

vomiting, face flushing, and piloerection were observed after each challenge injection. Anaphylactic shock did not occur in any other animal regardless of challenges given.

Effect of rMETase on Hematological and Blood Chemical Parameters. Hematology and blood chemistry examinations results are shown in Table 4. All hematology parameters were normal during the 2-week treatment except RBC and Hgb, which were found to decrease from day 9 of rMETase treatment. WBC and Hgb were reduced to 34% and 40% of pretreatment level, respectively, by the end of the treatment period, indicating a mild anemia. The blood chemistry examinations revealed no abnormalities in most liver function tests, renal function, glucose, lipids, and electrolytes in the rMETase-treated monkeys compared with the controls and pretreatment values. However, decreased albumin/globulin and higher total bilirubin and indirect bilirubin values were noted from day 5 of rMETase treatment.

Histopathological Effects of rMETase. There were no obvious gross lesions observed in the major organs of the rMETase-treated monkey at necropsy except for ascites with a light yellow color. The organ weights, including heart, liver, kidney, lung, spleen, brain, and adrenal glands were in the normal range. No histological changes were observed in the heart, kidney, trachea, lung, and thoracic aorta. In the kidney, a small fraction of epithelial cells of the proximal convoluted tubules showed slight fine granular vacuolization. In the liver, the structures of the lobules, sinusoids, hypatocyte plates, and hepatocytes were normal. However, scattered spotty necrosis infiltrated with acute and chronic inflammatory cells was observed. Some of the hepatocytes showed cloudy degeneration and fatty metamorphosis.

Determination of Plasma Anti-rMETase Antibodies. Plasma-specific anti-rMETase antibodies were not detected until treatment day 26. A 10^{-3} plasma antibody titer was detected on day 26 in four rMETase-treated monkeys. The challenge injections of rMETase (which followed the treatment period) resulted in increased plasma antibody titers up to 10^{-6} after the fourth challenge injection of rMETase. The plasma anti-rMETase antibody decreased after termination of rMETase challenge injections, with only a 10^{-2} plasma antibody titer remaining 2 months after the last challenge.

Antibody subtypes in rMETase-treated monkeys on day 65 included anti-rMETase IgG as the dominant antibody (10^{-4} titer) with a low titer of IgM antibody (10^{-2} titer) found. No IgA antibody was detected in the plasma of the rMETase-treated monkeys.

Plasma anti-rMETase antibody appeared to have neutralizing potential. rMETase lost 80% of its original enzyme activity when 2 units/ml rMETase were incubated *in vitro* with anti-rMETase antibody-positive serum obtained from an rMETase-treated monkey. However, no significant enzyme activity losses were observed when 100 units/ml rMETase concentration was used in the above reaction. Importantly, *in vivo* the enzyme administration for the challenge doses resulted in effective depletion of plasma methionine levels despite the presence of antibodies.

DISCUSSION

The efficacy of methionine depletion by rMETase to reduce tumor growth has now been documented with a variety of tumors that include Lewis lung carcinoma (20), several human colon cancer lines (19), and glioblastoma (21, 22, 30). An even more important observation is the growing evidence that a variety of chemotherapeutic agents appear to be synergistically enhanced by plasma methionine depletion; these include doxirubicin (11), 5-fluorouracil and mitomycin C (13, 23), CDDP (19), and nitrosoureas (21, 22, 30).

The effectiveness of antitumor activity of rMETase is

Table 3 Plasma methionine concentration (μM)[a] in monkeys during repeated rMETase treatment ($n = 4$)

Four monkeys were treated with 4000 units/kg rMETase for 14 days three times per day. Blood was sampled on the indicated days and measured for methionine by HPLC as described in "Materials and Methods."

Time points (h)	Day 1	Day 5	Day 9	Day 14	Day 66
0	11.3 ± 3.96	1.90 ± 1.12	2.17 ± 1.24	1.35 ± 1.09	12.56 ± 0.75
1	0	0	0	0	0
2	0	0	0	0	0
4	0	0	0	0	0
8	1.33 ± 0.60	1.68 ± 0.76	1.37 ± 0.61	0.92 ± 0.62	0.98 ± 0.21

[a] Limit of detection, <0.5 μM.

Table 4 Hematology and blood chemistry analysis in rMETase-treated monkeys ($n = 4$)

Monkeys were treated with rMETase at 4000 units/kg for 14 days three times a day. Blood was sampled at the times indicated with the listed analytes tested as described in "Materials and Methods."

	Time (days after administration of rMETase)					
Laboratory test	Pre	Day 5	Day 9	Day 14	Day 26	Day 86
WBC ($\times 10^9$/liter)	13.0 ± 2.1	11.2 ± 5.6	6.3 ± 2.4	12.0 ± 4.0	17.3 ± 6.9	15.4 ± 1.1
RBC ($\times 10^{12}$/liter)	6.28 ± 0.47	6.66 ± 1.17	4.31 ± 1.28	5.24 ± 1.54	4.12 ± 0.17	5.40 ± 0.49
Hgb (g/liter)	141 ± 18	134 ± 18	102 ± 24	113 ± 34	85 ± 14	121 ± 8
HCT	0.49 ± 0.04	0.54 ± 0.10	0.33 ± 0.10	0.45 ± 0.12	0.39 ± 0.02	0.43 ± 0.04
PLT ($\times 10^9$/liter)	366 ± 131	283 ± 50	324 ± 111	475 ± 262	617 ± 316	371 ± 76
MCH (pg)	22.5 ± 2.1	20.3 ± 0.9	21.6 ± 1.2	21.6 ± 1.4	20.7 ± 2.6	22.6 ± 0.6
MCV (fl)	78.8 ± 5.7	81.6 ± 4.3	77.7 ± 4.9	85.3 ± 1.1	94.9 ± 1.8	79.3 ± 0.4
MCHC (g/liter)	286 ± 20	249 ± 16	271 ± 21	260 ± 7	217 ± 24	284 ± 9
ALT (units/liter)	19 ± 10	23 ± 11	13 ± 3	9 ± 4	12 ± 6	81 ± 45
GGT (units/liter)	74 ± 10	57 ± 20	62 ± 17	50 ± 12	42 ± 19	55 ± 18
AKP (units/liter)	383 ± 261	423 ± 309	374 ± 118	351 ± 95	351 ± 188	207 ± 157
TP (g/liter)	73.6 ± 2.6	50.5 ± 23.4	58.1 ± 1.7	58.8 ± 0.8	68 ± 1.2	73.7 ± 1.6
ALB (g/liter)	47.2 ± 4.8	39.0 ± 8.4	29.9 ± 1.1	27.3 ± 2.0	29.3 ± 4.8	43.9 ± 4.5
GLB (g/liter)	26.4 ± 3.0	29.9 ± 1.0	28.2 ± 2.6	32.1 ± 1.4	38.7 ± 3.7	29.8 ± 2.8
A/G	1.8 ± 0.7	1.1 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	1.4 ± 0.3
TBIL (μmol/liter)	6.3 ± 0.4	9.3 ± 4.5	14.2 ± 7.7	5.7 ± 1.5	8.5 ± 1.5	8.7 ± 1.5
DBIL (μmol/liter)	5.5 ± 0.5	3.8 ± 1.5	5.1 ± 2.9	3.8 ± 0.8	6.7 ± 1.1	5.9 ± 0.8
IBIL (μmol/liter)	0.9 ± 0.3	5.5 ± 3.5	9.2 ± 4.9	1.9 ± 0.8	1.8 ± 1.1	2.8 ± 0.7
CHOL (mmol/liter)	3.14 ± 0.88	2.79 ± 1.02	1.58 ± 0.68	2.04 ± 0.84	2.83 ± 0.20	3.13 ± 0.37
TG (mmol/liter)	0.53 ± 0.05	0.66 ± 0.17	0.74 ± 0.08	0.67 ± 0.33	0.65 ± 0.22	0.43 ± 0.13
GLU (mmol/liter)	3.37 ± 0.85	3.91 ± 2.30	3.25 ± 0.55	4.14 ± 1.06	3.28 ± 0.92	2.33 ± 0.34
BUN (mmol/liter)	4.96 ± 0.98	13.67 ± 5.61	10.71 ± 0.47	8.70 ± 1.20	7.37 ± 1.48	7.16 ± 1.28
CR (μmol/liter)	141.3 ± 21.3	123.1 ± 22.8	95.4 ± 7.5	89.0 ± 11.0	83.3 ± 7.4	106.9 ± 9.1
AST (units/liter)	30 ± 1	52 ± 29	28 ± 10	39 ± 10	46 ± 6	56 ± 1
CK (units/liter)	136 ± 49	305 ± 120	177 ± 175	67 ± 40	89 ± 21	138 ± 27
LDH (units/liter)	454 ± 86	786 ± 179	445 ± 69	643 ± 128	596 ± 84	520 ± 31
Ca^{2+} (mmol/liter)	2.99 ± 0.41	2.09 ± 0.96	2.26 ± 0.34	2.31 ± 0.11	2.54 ± 0.42	2.45 ± 0.18
Na^+ (mmol/liter)	169.2 ± 8.6	158.7 ± 5.0	155.4 ± 7.5	151.3 ± 5.1	145.6 ± 1.0	157.0 ± 9.9
K^+ (mmol/liter)	5.27 ± 1.09	4.57 ± 0.52	5.05 ± 0.75	5.39 ± 1.01	6.06 ± 1.34	4.25 ± 0.21
Cl^- (mmol/liter)	123.7 ± 4.2	114.9 ± 3.7	112.5 ± 6.7	109.5 ± 4.7	106.7 ± 5.3	118.5 ± 6.4

[a] HCT, hematocrit; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; AKP, alkaline phosphatase; TP, total protein; ALB, albumin; GLB, globulin; A/G, albumin/globulin; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; CHOL, cholesterol; TG, triglycerides; GLU, glucose; BUN, blood urea nitrogen; CR, creatinine; AST, aspartate aminotransferase; CK, creatine phosphokinase; LDH, lactate dehydrogenase; Hgb, hemoglobin; PLT, platelets.

dependent on its ability to deplete methionine in the plasma and subsequently in the tumor. Previous preclinical studies in mice have demonstrated that rMETase is an effective antitumor agent without significant systemic toxicities (20, 30). Lack of systemic toxicity was additionally supported by limited treatment of cancer patients with rMETase in a pilot Phase I clinical trial (26, 27).

In the current study, we extended the preclinical study of rMETase to include pharmacokinetics, antigenicity, and systemic toxicity in primates. Three dose levels of rMETase were administrated i.v. to determine an appropriate dose and dosing schedule. We found that plasma methionine was efficiently depleted by all three single doses of rMETase to less than 5 μM at 8 h, which has been demonstrated to be sufficient to arrest human tumor xenograft growth in athymic mice (21). A plasma methionine level of approximately 1 μM at 8 h could be achieved by 4000 units/kg dose (Table 1). Therefore, 4000 units/kg doses were used to achieve a constantly lowered plasma methionine level and for antigenicity and toxicity determination.

rMETase pharmacokinetic studies were carried out using a single i.v. administration of 2,000 or 4,000 units/kg (Fig. 1). We found the plasma concentration-time profiles for both dosages were similar to our prior study in mice (18). After i.v. injection, rMETase is rapidly eliminated with a mean plasma half-life of 2.49 h. The monkeys receiving 4,000 units/kg rMETase, iv. three times per day for 2 weeks showed a steady state of high rMETase activity and low methionine levels in the plasma (Fig. 2; Table 3). Previous pharmacokinetic studies in mice showed that a single injection of rMETase at doses ranging from 5,000–15,000 units/kg could deplete plasma methionine levels to less than 5 μM for 8 h.[6] The dose range and schedule for this primate study were initially determined from these doses used in mice. Therefore, we started with 1,000 units/kg for the primate dose-ranging study.

Although no toxic deaths were seen during the 2-week rMETase treatment, we found gastrointestinal reactions such as vomiting and significantly decreased food consumption in all

[6] Z. Yang, S. Li, X. Sun, Y. Tan, S. Yagi, R. M. Hoffman, unpublished data.

rMETase-treated monkeys. The body weights were reduced up to 19% by the end of the treatment period. The body weight loss was in part due to the gastrointestinal side effects. However, an important factor in the weight loss was related to the preplanned fasting for 2 h before and after the injections. The observed weight loss may be able to be countered by various approaches to hyperalimentation in future clinical studies.

The hematology studies demonstrated that mild anemia occurred in rMETase-treated monkeys, which is consistent with our previous report in mice (20). The anemia may partly result from decreased protein intake caused by reduced food consumption, which also was suggested by the findings of lower albumin and albumin/globulin ratios in the blood chemistry analysis. Careful calculations of the volumes of blood removed for the extended battery of tests did help explain the decreased RBC values during the prolonged treatment. Although the very transient increase in bilirubin suggested a hemolytic episode, we were unable to document this nor define a mechanism for increased red cell destruction. Furthermore, the changes were transient, and values returned toward normal despite continued treatment. Nevertheless, these changes will be more extensively evaluated in our next series of studies.

In the present study, we evaluated the antigenicity of rMETase because of potential anaphylactic reactions on challenge. In contrast to previous antigenicity results in mice in which no anaphylactic reactions were observed (23), the monkeys receiving the 2-week rMETase treatment at a dose of 4000 units/kg had a significant anaphylactic reaction at the first challenge. Autopsy examination of the one monkey that died showed findings consistent with anaphylaxis that included ascites and scattered foci of necrosis in the liver. It is of particular importance that subsequent pretreatment with hydrocortisone completely eliminated the anaphylactic reactions.

The measurement of plasma antibody during rMETase treatment period demonstrated that anti-rMETase antibodies were induced. The anti-rMETase antibodies were detected 26 days after the initiation of the 2-week rMETase treatment period and increased with each monthly challenge injection (Fig. 3). The antibody titer decreased rapidly after termination of the rMETase challenge. The detection of antibody subtypes indicated that IgG was the dominant anti-rMETase antibody, although a low titer of IgM antibody was also observed.

The anti-rMETase antibodies appeared to be neutralizing *in vitro*. However, we found that both plasma rMETase enzyme activity and methionine depletion in the rMETase-treated monkeys were not influenced when the anti-rMETase antibody was induced (Fig. 2; Table 3). Although plasma rMETase activity on day 66 seemed to be lower after the challenge dose of 4000 units/kg was given than that on days 1, 5, 9, and 14, similar plasma methionine depletion profiles on days 1 and 66 indicated that anti-rMETase antibodies produced in the monkey plasma were not sufficient to reduce the methionine depletion efficacy of rMETase. These data indicated that the anti-rMETase antibodies produced in the plasma of the monkeys were not sufficient to reduce the methionine depletion efficacy of rMETase; rMETase antibodies, therefore, did not have a significant neutralizing effect.

The present results show that consistently low plasma methionine levels of less than 2 μM can be achieved in monkeys at the doses used in the present study. Although the monkeys can tolerate the 2-week rMETase treatment, there are some systemic toxicities and anaphylactic reactions that were not found previously in mice. Continuous infusion as a clinical option may achieve even lower methionine levels (26, 27).

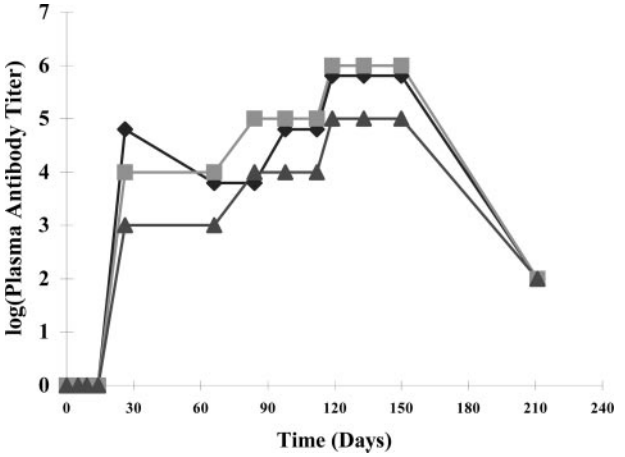

Fig. 3 Plasma anti-rMETase antibody titers in monkeys receiving repeated injections of rMETase and four-interval challenges of rMETase. Monkeys were treated with 4000 units/kg rMETase for 14 days three times per day as described in "Materials and Methods." Monkeys were then challenged with 4000 units/kg rMETase on days 28, 66, 86, and 116. Blood was collected at the indicated time points. Anti-rMETase antibodies were measured as described in "Materials and Methods." ◆, Monkey 1; ■, Monkey 2; ▲, Monkey 3.

These studies suggest that the current toxicity is limited and that methods such as polyethylene glycol conjugation (PEGylation) of the enzyme might additionally reduce the toxicity observed, provide a longer half-life of methioninase depletion, and have less associated immunological reactivity. PEG conjugation masks the surface of the protein and increases its molecular size, thereby increasing the circulating time of the enzyme by reducing its renal clearance and degradation by proteolytic enzymes. As a result, the effective dose could be reduced due to prolonged half-life, which may lead to reduction of some dose-related toxicity. In addition, PEG conjugation could prevent antibody binding or antigen processing and thereby reduce rMETase antigenicity. Future studies will evaluate PEG-rMETase in primates.

The work of Kokkinakis *et al.* (10) suggests that the combination of an O^6 methylguanine methyltransferase inhibitor along with rMETase may achieve synergistic antitumor efficacy. DNA methyltransferase is a promising target to investigate with compounds such as azacytosine in combination with rMETase in future studies.

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