Recombinant Methioninase Infusion Reduces the Biochemical Endpoint of Serum Methionine with Minimal Toxicity in High-Stage Cancer Patients

YUYING TAN[1], JORGE ZAVALA, SR.[2], QINGHONG HAN[1], MINGXU XU[1], XINGHUA SUN[1], XUEZHONG TAN[1], XIUYING TAN[1], REY MAGANA[1], JACK GELLER[1] and ROBERT M. HOFFMAN[1]

[1]*AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111, U.S.A.;*
[2]*Comprehensive Cancer Center of Northwest Mexico, Tijuana, Mexico*

Abstract. *The tumor-specific increased minimal requirement for methionine has been shown to be a highly promising therapeutic target. To attack this target we have previously cloned the methioninase gene from Pseudomonas putida and produced recombinant methioninase (rMETase). A pilot Phase I clinical trial has been carried out to determine rMETase toxicity, rMETase pharmacokinetics, and serum MET-depletion in cancer patients. Patients with advanced breast cancer, lung cancer, renal cancer and lymphoma were given a single rMETase treatment at doses ranging from 5,000 to 20,000 units by i.v. infusion over 6-24 hours. No clinical toxicity was observed in any patient after rMETase treatment. rMETase levels reached 0.1 to 0.4 units per ml of serum in the patients which correspond to therapeutic levels in vitro. The lowest serum methionine levels in rMETase-treated patients were 0.1% of the pre-treatment levels corresponding to approximately 0.1 μM, which also correlates to therapeutic levels in vitro. The results of the rMETase pilot Phase I clinical trial therefore indicate that i.v. infusion of rMETase is safe and effectively depletes its biochemical target of serum methionine suggesting potential efficacy in future clinical trials.*

The goal of the next generation of cancer chemotherapy is tumor-selective effective therapy. A broad tumor-selective target with high therapeutic potential is the increased minimal methionine requirement of tumor cells relative to normal cells which we have termed methionine dependence [1,2]. The *in vitro* studies in our laboratory have shown that methionine dependence occurs frequently, if not universally, in many types of human cancer as well as in fresh human tumor specimens but not in normal cells [3-6]. Methionine dependence was found in 20 of 20 tested human tumor cell lines from the NCI Human Tumor Cell Line Screen including kidney, colon, lung, prostate, and melanoma tumor cell lines (Xu, M. *et al* unpublished data).

Recent results reported by Goseki *et al* have demonstrated the clinical efficacy of methionine depletion in Phase II clinical trials with a methionine-depleted total parenteral solution (TPN) given to high-stage gastric cancer patients. The methionine-depleted TPN solutions doubled the response and survival rate in combination with 5-FU and mitomycin C as compared to the latter two drugs given with methionine-replete TPN [7-8].

We initially purified endotoxin-free METase from *Pseudomonas putida* in order to develop anti-methionine chemotherapy targeting the methionine dependence of tumors *in vivo* [9,11]. The *in vivo* pre-clinical studies carried out in our laboratory have shown that methioninase effectively inhibited the growth of the Yoshida sarcoma and the human lung tumor H460 in nude mouse-xenograft models [10]. Clinical pharmacokinetics studies showed that native methioninase depleted serum methionine levels more than 100-fold without toxicity [11].

Recently, we have cloned the *Pseudomonas putida* METase gene into a high expression vector which was transformed *into E.coli*. With this high expression clone, we have established scale-up protocols which produce high-purity, low-endotoxin recombinant methioninase (rMETase) [12]. Preclinical studies have shown that rMETase has high potential as an effective broad-spectrum tumor-selective agent without acute toxicity in nude mice with human tumor xenografts (Tan, Y. *et al* unpublished data). *In vitro* studies with rMETase demonstrated universally lower IC_{50} values by approximately an order-of-magnitude, in a broad range of human tumor cells compared to five types of normal cells [Xu, M. *et al*, unpublished data]. We report here that pilot clinical phase I trials of rMETase infusion in 9 high-stage cancer patients have shown that serum levels of rMETase reach potential therapeutic levels. rMETase depleted serum methionine levels to 0.1% of untreated levels without any clinical toxicity. This depleted level of serum methionine also corresponds to a potential therapeutic level.

Correspondence to: Robert M. Hoffman, Ph.D., AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111, USA. TEL: 619-654-2555, FAX: 619-268-4175, E-mail: antica@ix.netcom.com

Key Words: Methioninase, cancer, methionine dependence, methionine depletion, clinical trial.

Table I. *Patient characteristics and rMETase toxicity.*

Name	Diagnosis		Methionine (μM)		METase		Toxicity	
	Cancer	Stage	Base	Lowest	Units	Hours	Symptom	Laboratory
A.M.E.	Breast cancer	IV	28.6	0.5	10,000	8	–	–
M.R.	Kidney cancer	III	N/A	N/A	20,000	24	–	–
O.P.	Breast cancer	III	N/A	N/A	5,000	24	–	–
P.J.	Lung cancer	IV	N/A	N/A	10,000	6	–	–
C.M.	Lymphoma	III	N/A	N/A	10,000	8	–	–
B.O.	Breast cancer	IV	35	0.28	10,000	8	–	–
R.M.	Lymphoma	III	N/A	N/A	10,000	8	–	–
L.S.	Gastric cancer	IV	34	1.27	10,000	8	–	–
S.N.	Breast cancer	IV	34.7	0.1	20,000	8	–	–

Materials and Methods

Production of rMETase.

High expression clone of rMETase and fermentation conditions (12). The pAC-1 rMETase high expression clone established by AntiCancer was used for the production of rMETase. The fermentation procedure for host *E.coli* cells and the purification protocol for rMETase were as previously described [12]. pAC-1 was constructed with the pT7-7 vector containing the T7 RNA polymerase promotor for high expression of the rMETase gene which was cloned from *Pseudomonas putida* [12]. The host *E.coli* were grown in Terrific Broth and harvested when the OD_{600} reached 20. The expression level of rMETase reached approximately lg/liter of fermentation broth under these conditions.

Purification of rMETase (12). Pre-column treatment. The bacterial pellet was disrupted with a cavitator-type homogenizer (Microfluidics Corp., Newton, MA, model # HC 8000). Heat treatment of the homogenate was carried out at 50°C for one minute.

First column. DEAE Sepharose FF (pH 7.2) (Pharmacia, Uppsala, Sweden) (12). rMETase was eluted with a linear gradient of 40 to 200 mM potassium chloride in 10 mM potassium phosphate buffer pH 7.2. The fractions containing rMETase were identified by yellow color and activity assay.

Second column. DEAE Sepharose FF (pH 8.3) (12). rMETase was eluted with a linear gradient of 80 to 200 mM potassium chloride in 10 mM potassium phosphate buffer (pH 8.3). The fractions containing rMETase were identified by yellow color and activity assay.

Third column. Acticlean Etox (Sterogen,Arcadia, CA) (12). To eliminate endotoxin, purified rMETase (10-20 mg protein/ml) was applied on an Acticlean Etox column. The protein was eluted with elution buffer (0.12 M sodium chloride in 10 mM sodium phosphate pH 7.2). The fractions containing rMETase were identified by yellow color and activity assay.

Analysis of rMETase (12).

HPLC: An Hitachi L-6200A Intelligent pump (Hitachi, Ltd, Tokyo, Japan) with a Supelco Progel™ TSK column (G3000 SW_{XL}, 30 cm x 7.8 mm) column (Supelco, Bellefonte, PA) was used for all HPLC analysis of rMETase purity. A sample was loaded and eluted with elution solution (0.12 M sodium chloride in 10 mM sodium phosphate buffer, pH 7.2). The protein-containing fractions were identified with a spectrophotometer (Hitachi U2000) at a wavelength of 280 nm.

Electrophoresis (12). Electrophoresis is carried out in 7.5% polyacrylamide-precasted plates in 0.2 M Tris-glycine buffer, pH 8.3, both with and without 0.1% SDS.

Potency activity assay (12). The assay was carried out in a one ml volume of 50 mM phosphate buffer pH 8.0, containing 10 μM pyridoxal phosphate and 10 mM methionine for 10 minutes at 37°C with varying amounts of enzyme. and detected with 3-methyl-2-benzothiazolinone hydrazone at OD_{335}. The specific activity was calculated as units/mg protein, with one unit of enzyme defined as the amount that catalyzes the formation of 1 mmol of α-ketobutyrate per minute [12].

Endotoxin assay (12). The endotoxin level was measured with the Limulus Amebocyte Lysate (LAL) test (Bio Whittaker, Walkerville, MD). The concentration of endotoxin was measured at a wavelength of 410 nm.

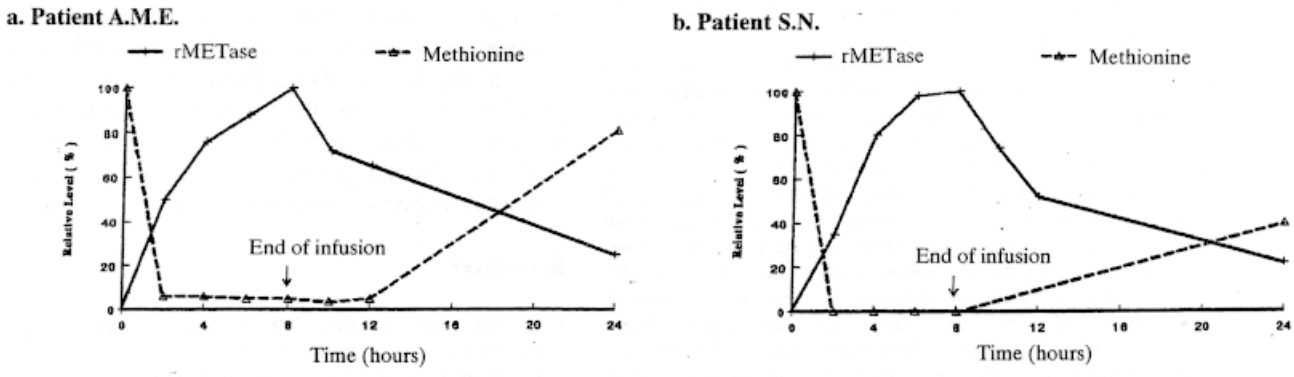
Serum methionine measurement. Serum methionine measurement was performed by HPLC (Hitachi L-6200A Intelligent pump, Hitachi, Ltd, Tokyo, Japan) after derivatization of serum amino acids with a fluoraldehyde reagent, o-phthaldialdehyde (OPA) [13]. A methionine standard (Sigma) was derivatized by OPA. Serum samples (25 μl) were precipitated by acetonitrile (75 μl). Ten μl of supernatent was mixed with 5 μl of OPA. After 1 minute, 50 μl of 0.1 M sodium acetate (pH7.0) was added, and a 20 μl sample was loaded on a reversed-phase Supelcosil LC-18DB (particle size 5 μm, 25 cm x 4.8 mm) column at room temperature. Resolution of the amino acid denvatives was accomplished with solution A (tetrahydrofuran/methanol/0.1M sodium acetate pH 7.2; 5/95/900), solution B (methanol). A gradient from 20%-60% solution B was run at a flow rate of 1.5 ml/minute. The eluate was read by a fluorescence spectrophotometer (Hitachi, F1000) at wavelength of 350-450 nm. The limit of detection was approximately 0.1 μM methionine.

Patients and treatment. Nine patients with advanced stage breast cancer, lung cancer, kidney cancer and lymphoma participated in the ongoing Phase I trial of rMETase (Table I). Patient selection requirements included no chemotherapy or radiotherapy in the four previous months before this study. The diagnosis of the original cancer and the metastases was confirmed by pathologic analysis. There were no other coexistent medical problems of sufficient magnitude to jeopardize full compliance with this study. Informed consent forms followed IRB approval guidelines.

Formulation for rMETase. rMETase was formulated at a concentration of 10-20 mg/ml in 0.12 M sodium chloride, 10 mM sodium phosphate buffer (pH 7).

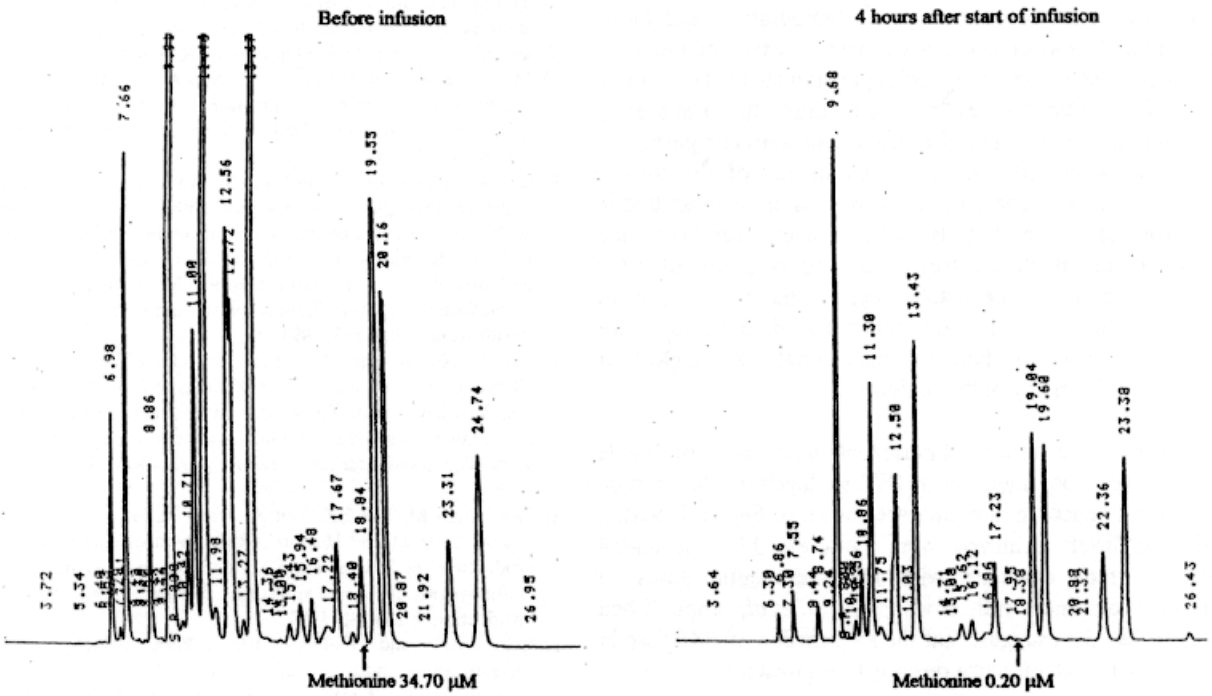
The rMETase administered to the patients was more than 99% pure with an endotoxin level of less than 2 EU/mg protein.

Infusion. rMETase was diluted with sterilized 0.9% sodium chloride solution in a total volume 800-1,200 ml just before infusion. Patients



Patients (A.M.E. and S.N.) with breast cancer received an 8- hour iv infusion of 10,000 units and 20,000 units of rMETase, respectively. Blood samples were collected before treatment, every four hours during treatment and 24 hours after the start of the infusion. Serum rMETase activities were measured as described in the Materials and Methods. Serum methionine measurement was performed by HPLC after derivatization of serum amino acids with o-phthaldialdehyde (OPA) as described in the Materials and Methods.

Figure 1. *rMETase pharmacokinetics and serum MET levels in patients. rMETase was measured by activity assay (see text). MET levels were determined by HPLC (see text and Figure 2).*



Patient (S.N.) with stage IV breast cancer had an 8-hour iv infusion of 20,000 units of rMETase. Blood samples were collected before treatment, every four hours during treatment and16 hours after the end of infusion. Serum methionine measurement was performed by HPLC after derivatization of serum amino acids with o-phthaldialdehyde (OPA) as described in the Materials and Methods.

Figure 2. *Serum methionine measurement was performed by HPLC (Hitachi L-6200A Intelligent pump, Hitachi, Ltd, Tokyo, Japan) after derivatization of serum amino acids with a fluoraldehyde reagent, o-phthaldialdehyde (OPA) [13]. A methionine standard (Sigma) was derivatized by OPA. The eluate was read by a fluorescence spectrophotometer (Hitachi, F1000) at wavelength of 350-450 nm. The limit of detection was approximately 0.1 µM methionine.*

received 5,000 units (0.25g) to 20,000 units (1.0g) of rMETase by continuous i.v. infusion for 6-24 hours.

Patient observation and sample collection. All the patients were observed carefully for vital signs. Pulse, respiration rate, blood pressure, and temperature were measured every two hours. The blood samples and urine samples were collected before treatment, during treatment every four hours, and at 24 hours. The blood samples were used for rMETase pharmacokinetics (50 µl), serum methionine measurement (25 µl) and laboratory analysis. Laboratory tests and measurements included: a complete blood count with differential, prothrombin time, partial thromboplastin time, electrolytes, protein, albumin, blood urea nitrogen, creatinine, lactic dehydrogenase, alkaline phosphatase, calcium, uric acid, total and direct bilirubin, serum alanine aminotransferase, and urinalysis. Chest X-ray, electrocardiogram, and pertinent radiographic studies for evaluable/measurable disease were also done. Toxicity was determined according to WHO criteria.

Results and Discussion

Patient characteristics. A total of nine patients were evaluated with rMETase infusion. Of these nine patients, four had stage III or IV breast cancer. One had stage III renal cancer, one had stage IV lung cancer, two had stage III lymphoma, and one had stage IV gastric carcinoma. Their characteristics are listed in Table I.

Toxicity evaluation. None of the nine cases had any observable clinical toxicity during or after a single i.v. infusion of rMETase. Pulse, respiration rates, temperature and blood pressure had almost no change during and after treatment.

Toxicity was evaluated according to standard WHO criteria [14]. The physical examination and the laboratory determination before and after treatment were compared. No acute clinical toxicity was observed in any of the toxicity criteria measured. The patients were treated as described in the protocol of Table I. Blood and urine were collected before treatment, during treatment, and 24 hours after the start of treatment. The results suggest that i.v. infusion of rMETase did not cause any functional damage to major organs. There was no hematological, renal, neurological, or cardiac toxicity in any of the patients.

Pharmacokinetics results. Pharmacokinetic data of single infusion were obtained for rMETase levels in the serum. Typical pharmacokinetics data are shown in Figure 1. Serum rMETase levels achieved were between 0.1 units/ml-0.4 units/ml which correlate with the therapeutic doses of rMETase for cancer cells *in vitro* (Xu M. *et al*, unpublished data). These data suggest that the i.v. infusion of rMETase in patients can reach effective doses without toxicity.

Depletion of methionine in the serum. The depletion of serum methionine started within 60 minutes of the infusion, and reached maximum depletion after two hours. Methionine depletion was maintained during the infusion and for at least two hours after the infusion was completed. Methionine was depleted as low as approximately 0.1 µM within two hours (Table I, Figure 2) in patients corresponding to more than a 300-fold depletion. These levels of depleted methionine correspond to therapeutic levels for cancer cells *in vitro* (Xu, M *et al*, unpublished data). These results demonstrated that rMETase could essentially completely deplete methionine without any observed clinical toxicity, which is the basis for inhibition of tumor growth. The results justify further clinical trials of this novel and promising agent.

References

1 Hoffman RM and Erbe RW: High *in vivo* rates of methionine biosynthesis in transformed human and malignant rat cells auxotrophic for methionine. Proc Natl Acad Sci USA *73:* 1523-1527, 1976.

2 Hoffman RM: Altered methionine metabolism, DNA methylation, and oncogene expression in carcinogenesis: A review and synthesis. Biochem Biophys Acta Reviews on Cancer *738:* 49-87, 1984.

3 Mecham JO, Rowitch D, Wallace CD, Stern PH and Hoffman RM: The metabolic defect of methionine dependence occurs frequently in human tumor cell lines. Biochem Biophys. Res Commun *117:* 429-434, 1983.

4 Guo H, Herrera H, Groce A and Hoffman RM: Expression of the biochemical defect of methionine dependence in fresh patient tumors in primary histoculture. Cancer Res *53:* 2479-2483, 1993.

5 Kreis W and Goodenow M: Methionine requirement and replacement by homocysteine in tissue cultures of selected rodent and human malignant and normal cells. Cancer Res *38:* 2259-2262, 1978.

6 Guo H, Lishko V, Herrera H, Groce A, Kubota T and Hoffman RM: Therapeutic tumor specific cell-cycle block induced by methionine starvation *in vivo*. Cancer Res *53:* 5676-5679, 1993.

7 Goseki N, Yamazaki S, Shimojyu K, Kando F, Maruyama M, Endo M, Koike M and Takahashi H. Synergistic effect of methionine-depleting total parenteral nutrition with 5-fluorouracil on human gastric cancer. A randomized, prospective clinical trial. Jpn J Cancer Res *86:* 484-489, 1995.

8 Goseki N, Yamazaki S and Endo M: Antitumor effect of methionine-depleting total parenteral nutrition with doxorubicin administration on Yoshida sarcoma-bearing rats. Cancer *69:* 1865-1872, 1992.

9 Lishko VK, Lishko OV and Hoffman RM: The preparation of endotoxin-free L-methionine-α-deamino-γ-mercaptomethane-lyase (L-methioninase) from *Pseudomonas putida*. Protein Expression and Purification *4:* 529-533, 1993.

10 Tan Y, Xu M, Sun X, Kubota T and Hoffman RM: Anticancer efficacy of methioninase *in vivo*. Anticancer Res *16:* 3931-3936, 1996.

11 Tan Y, Zavala Sr J, Xu M, Zavala Jr J and Hoffman RM: Serum methionine depletion without side effects by methioninase in metastatic breast cancer patients. Anticancer Res *16:* 3937-3942, 1996.

12 Tan Y, Xu M, Tan XZ, Tan X, Wang X, Saikawa S, Nagahama T, Sun X, Lenz M and Hoffman RM: Overexpression and large-scale production of recombinant L-methionine-α-deamino-γ-mercapto-methane-lyase for novel anticancer therapy. Protein Expression and Purification *9:* 233-245, 1997.

13 Jones BN and Gilligan JP: o-Phthaldialdehyde precolumn derivatization and reversed-phase high-performance liquid chromatography of polypeptide hydrolysates and physiological fluids. J Chromatog *266:* 471-482, 1983.

14 Guidelines for reporting of adverse drug reactions. Bethesda, MD: Division of Cancer Treatment, National Cancer Institute, 1985.

Received May 5, 1997
Accepted June 9, 1997