Serum Methionine Depletion without Side Effects by Methioninase in Metastatic Breast Cancer Patients

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Abstract. *The growth dependence of human tumors on elevated levels of methionine has been shown in preclinical in vitro and in vivo studies to be a frequently occurring, highly effective, tumor - selective therapeutic target. High purity endotoxin - free methioninase was produced from* Pseudomonas putida *in order to develop anti - methionine chemotherapy targeting of human tumors. A pilot Phase I clinical trial has been initiated in order to determine methioninase toxicity, the pharmacokinetics of methioninase and methionine - depletion and maximum tolerated dose. A two hour i.v. infusion of 5,000 units (0.4 g) and 10,000 units (0.8 g) and a ten hour i.v. infusion of 20,000 units (1.6 g) of methioninase was administered to patient - 1, patient - 2, and patient - 3, respectively. All patients had advanced breast cancer. Blood and urine samples were obtained at frequent intervals between 0 and 24 hours. The toxicity evaluations were carried out according to FDA criteria. Pharmacokinetics data were obtained for both methioninase and methionine levels in the serum. No acute clinical toxicity was observed for all the toxlcity criteria measured in patient - 1, patient - 2 and patient - 3. The depletion of serum methionine started within 30 minutes of the infusion, and was maintained for 4 hours after the infusion was completed in patient - 1 and patient - 2. The lowest serum methionine levels were 35% and 19% of the pretreatment level, respectively, in patient - 1 and patient - 2. Patient - 3 received a ten hour i.v. infusion of 20,000 units of methioninase without any signs of side effects. Patient - 3 maintained serum levels of methioninase as high as 50% of the maximum level for a subsequent 6 hours after infusion. Methionine was depleted over 200 - fold from 23.1 μM to 0.1 μM by the 10-hour infusion of patient - 3. No clinical toxicity was observed whatsoever in all the toxicity criteria measured in patient - 3. The results of the methioninase pilot Phase I clinical trial suggested that i.v. infusion of the methioninase is safe and effectively depletes serum methionine without any signs of side effects. Clinical studies are continuing to determine the maximum length of time complete serum methionine depletion can be tolerated.*

A major limitation of current chemotherapy is dose - limitating toxicity due to lack of antitumor - selectivity.

Over the past 23 years our laboratories and others have identified the elevated methionine requirement of tumors as a potential target of high antitumor selectivity (1 - 3). This tumor - specific metabolic defect has been termed methionine dependence. Initial *in vitro* studies indicated two key aspects of the elevated methionine requeirement of tumors: a) Unlike normal cells, many tumor cells will not grow when methionine is replaced by its immediate precusor homocysteine in the growth medium. Recently obtained unpublished results [Guo, H., Tan, Y. and Hoffman, R.] indicated that removal of both methionine and homocysteine from the growth medium completely blocked growth of all 20 human tumor cell lines tested while allowing normal cells to survive for long periods. b) When tumor cells stop growing due to methionine depletion, they specfically arrest in the late - S/G_2 phase of the cell cycle, allowing for the first time, selective synchrony of tumor cells for cell cycle - specific chemotherapy (4,5). Such anti - methionine therapy has been shown to effect the selective elimination of tumor cells from mixed cultures of tumor and normal cells (6).

To attack the methionine target of tumors *in vivo*, we have recently purified endotoxin - free methioninase from *Pseudomonas putida* that is suitable for therapeutic use (7). Purified methioninase has been shown to be effective against rodent and human tumors growing in nude mice without noticeable

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side effects (8). In clinical studies, methionine - free total parenteral nutrition (TPN), which only partially lowers the serum methionine levels, demonstrated efficacy against advanced gastric carcinoma in combination with 5 - fluorouracil and mitomycin C (9). These data gave the background for a pilot evaluation of highly purified endotoxin - free methioninase in a limited clinical trial of patients with metastatic breast cancer. The endpoints of this dose escalating study were toxicity and serum methioninase and methionine levels, the results of which are described in this report.

Materials and Methods

a) *Source of methioninase.* Methioninase was isolated from *Pseudomonas putida* as described below.

b) *Conditions of fermentation and harvest.* The AC - 1 strain of *Pseudomonas putlda* from the AntiCancer Master Cell Bank was incubated in 5 ml LB containing 50 μg/ml kanamycin at 26°C and agitated at 250 rpm/minute for 18 hours. Two ml of bacteria were added to 2L LB containing 50 μg/ml kanamycin at 26°C with agitation at 200 rmp/minute overnight (18 hours). Two liters of bacteria were then added to a 40 L carboy containing special medium (10% LB, 0.1% potassium phosphate buffer pH 7.2, 0.1% glycerol, 0.1 % urea, 0.025% yeast extract, 0.01% magnesium sulfate and 0.25% methionine) with optimal aeration conditions at 26°C for 20 hours. The final cell density in the carboy reached approximately OD_{600}1.8.

The cells were harvested with an AGT column (Model UFP - 500 - E - 55 cartridge) at 4°C, then centrifuged with an automatic refrigerated centrifuge (SORVALL superspeed RC2 - B) at 4°C, 7000 rpm for 10 minutes. The cells were suspended in extraction solution (20 mM potassium phosphate pH 9.0, 10 μM pyridoxal phosphate and 0.01% ß - mercaptoethanol) at a density of 500 g wet cell/L, and disrupted with a cavitator - type homogenizer (Microfluidics Corp. model # HC 8000). The homogenate was then stored at -80°C immediately. The homogenate protein concentration was 10 - 20 mg/ml, and the methioninase specific activity was 0.05 - 0.1 units/mg in the supernatant.

c) *Purification and formulation of methioninase.* Pre - column purification of methioninase: The homogenate was heated in a water bath to 50°C and put on ice immediately. The suspension was centrifuged with an automatic refrigerated centrifuge (SORVALL superspeed RC2 - B) at 4°C at 13 k rpm for 30 minutes. The supernatant was collected and was filtered by a Millipore Prep/Scale - TFF PLHK 100 k 2.5 ft^2 cartridge.

Chromatographic purification of methioninase. First column: Toyopearl DEAE - 650M column (10/50). A sample of about 20 - 40 g of total protein (2 - 4 mg/ml) in extraction buffer (10 mM potassium phosphate buffer pH 7.2, 10 μM pyridoxal phosphate and 0.01% ß - mercaptoethanol) was applied to the first column, Toyopearl DEAE - 650 M (total volume of the column was 4 L). The column was equilibrated with equilibration buffer A (40 mM potassium chloride in extraction buffer). The concentration of protein applied to the column was 5 - 10 mg / ml. The column was pre - washed with 20 - 30 L of the equilibration buffer until the reading at OD_{280} dropped below 0.1. The column was then eluted with a linear gradient of potassium chloride at concentrations starting at 40 mM increasing to 300 mM in extraction buffer. Elution fractions of 400 ml were collected. The fractions containing methioninase were determined by activity assay and were then pooled.

Second column: DEAE Sephadex A 50 (5 / 20). The pooled fractions from the first column were dialyzed with equilibration buffer B (150 mM potassium chloride in 10 mM potassium phosphate buffer pH 8.3,10 μM pyridoxal phosphate and 0.01% ß - mercaptoethanol) for 24 hours. A sample of about 1 - 2 g of total protein (1 - 2 mg / ml) was applied to the second column (the volume of the column gel was 700 ml). The concentration of protein applied on the column was about 2 - 3 mg / ml gel. The column was then eluted with a linear gradient of potassium chloride at a concentration beginning at 150 mM increasing to 500 mM in 10 mM potassium phosphate buffer pH 8.3, 10 μM pyridoxal phosphate and 0.01% ß - mercaptoethanol. Elution fractions of 150 ml were collected. The fractions containing methioninase were pooled.

Third column: Acticlean Etox (5 / 30). Purified methioninase (3 - 5 mg protein / ml) in a volume of 100 - 200 ml was applied on 500 ml of Acticlean Etox (Sterogene), and eluted with elution buffer (0.12 M sodium chloride in 10 mM sodium phosphate pH 7.2). The methioninase was essentialy endotoxin free after this column.

d) *Formulation of methioninase:* The final eluant was concentrated with 30 K Amicon Centriprep Concentrators. The final fomulation of methioninase was 120- 160 units / 10 mg / ml in the formulation buffer (0.12 M sodium chloride in 10 mM sodium phosphate pH 7.2). The enzyme was stored at -80°C. Before treatment, methioninase was sterilized by filtration with 0.22 μM filters, and then diluted with normal saline solution to a final concentration of 20 - 25 units / ml.

Patients. Three patients with breast cancer and measurable axillary lymph nodes metastases were studied (Table I). Patient selection requirements included no chemotherapy or radiotherapy in the four months previous to this study. The diagnosis of the original cancer and the metastases were confirmed' by pathologic analysis. There were no other coexistent medical problems of sufficient magnitude to jeopardize full compliance with this study. The informed consent form followed institutional guidelines consistent with those of the Food and Drug Administration (10). Patients were informed of potential toxicities.

Table I. *Patient characteristics.*

	Patient #1 O.R.L.	Patient #2 S.N.K.	Patient #3 S.N.O.
Primary cancer	Breast cancer	Breast cancer	Breast cancer
Metastasis	lung and axillary lymph nodes	Axillary lymph nodes	Axillary lymph nodes
Sex	Female	Female	Female
Age	46	54	45
Dose of methioninase	5,000 units	10,000 units	20,000 units
Hours of i.v. infusion	2 hours	2 hours	10 hours
Sample collection	Before treatment, during treatment every hour, 10 hours after treatment	Before treatment, during treatment every hour, 10 hours after treatment	Before treatment, during treatment every hour, 10 hours after treatment

Table II. *Vital parameters of patients treated with methioninase.*

Patient	Treatment	Temperature	Pulse	Respiration rate	Blood pressure
	Before	36.7	134	21	110/70
# 1	During (Max)	36.8	138	22	120/80
O.R.L.	(Min)	36.4	125	24	110/70
	After	36.6	127	24	120/80
	Before	36.7	74	14	118/78
# 2	During (Max)	36.6	74	12	120/80
S.N.K.	(Min)	36.6	70	14	110/70
	After	36.7	74	14	110/74
	Before	36.4	67	14	124/82
# 3	During (Max)	37.0	76	14	128/74
S.N.O.	(Min)	36.0	66	12	90/70
	After	36.3	65	12	114/88

Table III. *Toxicity of methionase in pilot phase I clinical trial.*

Physical and laboratory examination	Toxicity grade of FDA standard Patient #1	Patient #2	Patient #3
Hematological	0	0	0
Gastrointestinal	0	0	0
Renal	0	0	0
Pulmonary	0	0	0
Fever	0	0	0
Allergic	0	0	0
Phlebitis	0	0	0
Cutaneous	0	0	0
Cardiac	0	0	0
Neurological	0	0	0

Chest X-ray, electrocardiogram, and pertinent radiographic studies for evaluable /measurable disease were also done.

Treatment. Patient-1 received 5,000 units (0.4 g) of methioninase by i.v. infusion over 2 hours, patient - 2 received 10,000 units (0.8 g) of methioninase by i.v. infusion over 2 hours, and patient - 3 received 20,000 units (1.6 g) of methioninase by i.v. infusion over 10 hours.

Observation and sample collection. All patients were observed carefully for vital signs. Pulse, respiration rate, blood pressure and temperature were measured every hour. The blood samples and urine samples were collected before treatment, during treatment every two hours and after treatment for laboratory examination. Pretreatment 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 FDA criteria. Blood samples were also collected before treatment, every hour during treatment and every two hours after treatment. The levels of methionine and methioninase were measured and the relative levels were calculated as described below (Figures 1 - 3).

Pharmacokinetic study. a) Methioninase levels in the serum: The levels of methioninase were determined by activity assay. The assay was carried out in a 1 ml volume of 50 mM phosphate buffer pH 8.0, containing 10 μM pyridoxal phosphate and 10 mM methionine for 10 min at 37°C with varying amounts of serum (20-50 μl). The reaction was stopped by adding 0.5 ml of 4.5% TCA. The suspension was centrifuged at 15 K rpm for 2 minutes with an Eppendorf centrifuge (Brinkmann). 0.5 ml of supernatant along with 0.5 ml of 0.05% 3-methyl-2-benzothiazolinone hydrazone in 1 ml of 1 M sodium acetate pH 5.2 was incubated at 50°C for 30 minutes. α-ketobutyrate was then determined by spectrophotometry at OD_{335}. The amount of protein was determined using the Lowry Reagent Kit (Sigma). The specific activity was calculated as units/mg protein.

Methionine levels in the serum. The levels of methionine were determinated by HPLC after methionine derivitization with IPTG and measured by a protein and peptide C18 column or with an amino acid analyzer.

Toxicity criteria of methioninase. The toxicity of methioninase was evaluated using standard FDA criteria.

Results and Discussion

Patient characteristics: Patient characteristics are shown in Table I. Vital signs are shown in Table II. None of the three patients had any side effects during or after the i.v. infusion of methioninase. Pulse, respiration rates, temperature and blood pressure had almost no change during and after the treatment.

Pharmacokinetics results. Pharmacokinetic data of single infusion were obtained for methioninase levels in the serum. The half life of methioninase in patient - 1 and patient - 2 who received i.v. infusions for 2 hours was 2 hours (Figure 1 and Figure 2) and in patient - 3 who received i.v. infusion for ten hours it was ten hours (Figure 3). The results suggested that the half life of methioninase was related to the infus on time rather than the dose. The infusion time should be further studied in future trials.

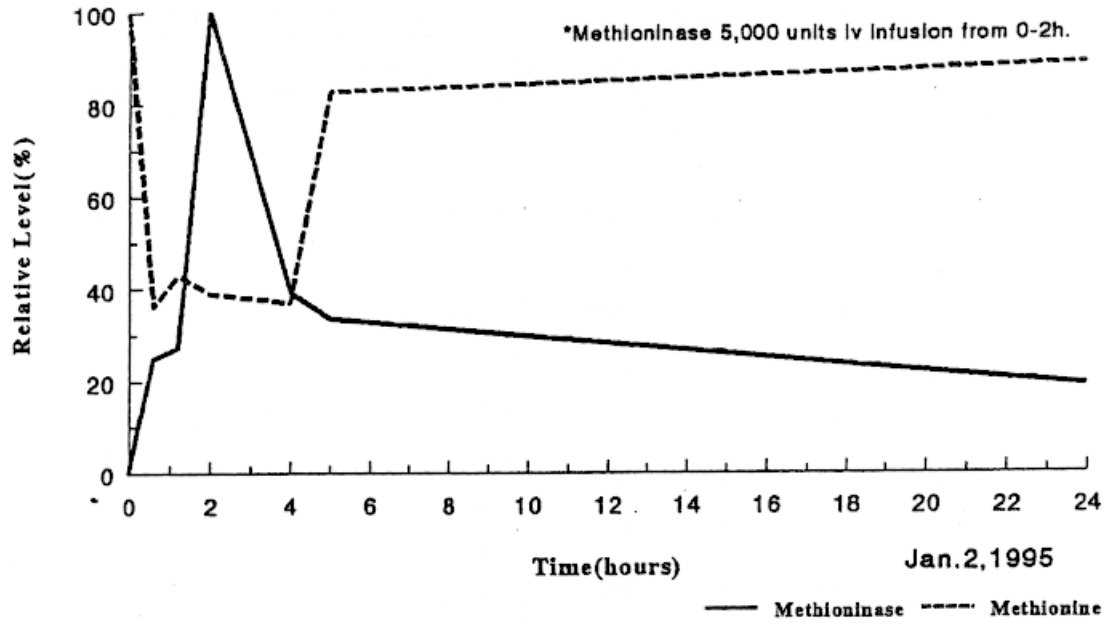

Figure 1. *Patient - 1 received an i.v. infusion of methioninase for 2 hours with 5,000 units of methioninase. The blood samples were collected before treatment, every hour during treatment and every two hours up to 20 hours after treatment. The levels of methionine and methioninase were measured, and the relative levels were calculated.*

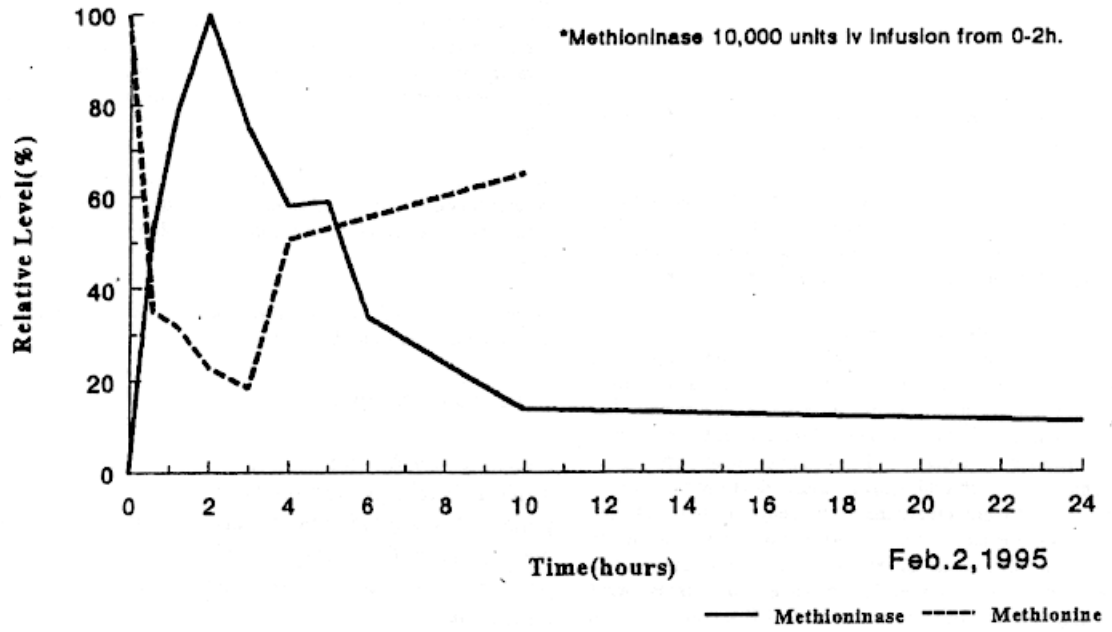

Figure 2. *Patient - 2 received an i.v. infusion of methioninase for 2 hours with 10,000 units of methioninase. The blood samples were collected before treatment, every hour during treatment and every two hours up to 20 hours after treatment. The levels of methionine and methioninase were measured, and the relative levels were calculated.*

Depletion of methionine in the serum. The depletion of serum methionine started within 30 minutes of infusion, and was maintained for 4 hours after the infusion was completed in patient - 1 and patient - 2. The lowest serum methionine levels were 35% and 19% of the pretreatment level, respectively, in patient - 1 and patient - 2 (Figure 1 and Figure 2). Patient - 3 received a ten hour i.v. infusion of 20,000 units of methioninase without any signs of side effects and

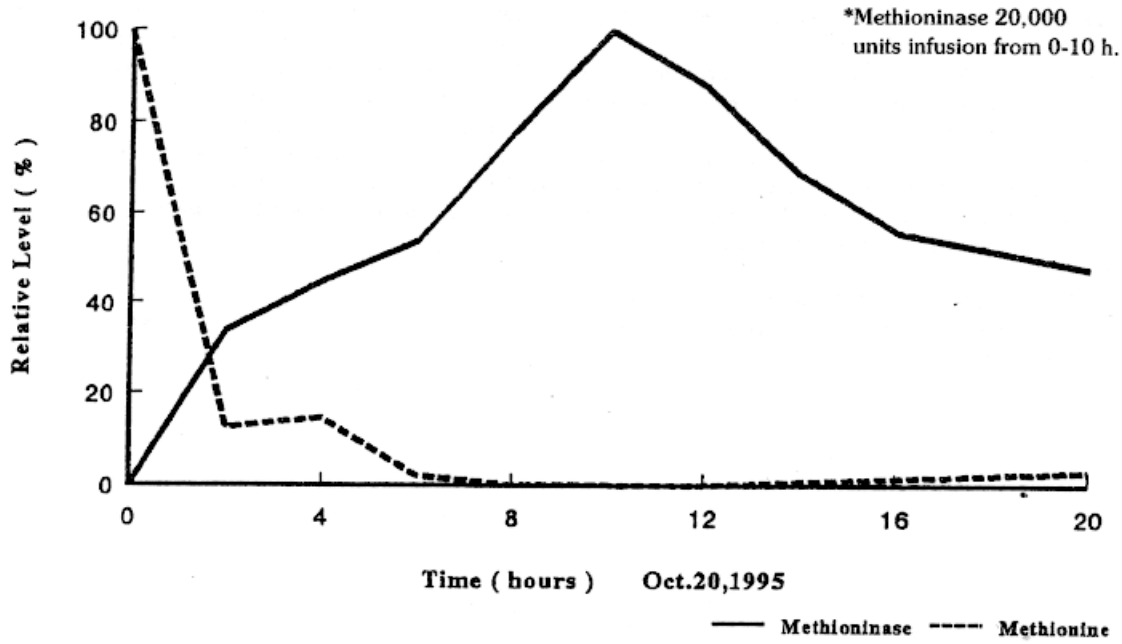

Figure 3. *Patient-3 received an i.v. infusion of methioninase for 10 hours with 20,000 units of methioninase. The blood samples were collected before treatment every hour during treatment and every two hours up to 20 hours after treatment. The levels of methionine and methioninase were measured, and the relative levels were calculated.*

maintained serum levels of methioninase as high as 50% of the maximum level for another 10 hours after infusion. Methionine was depleted over 200 - fold from 23.1 μM to 0.1 μM within 10 hours of infusion (Figure 3). These results demonstrated that methioninase could deplete methionine to a very low level within one hour of i.v. infusion. Methionine could be maintained at low levels after i.v. infusion for a long period without any observed side effects. This could be the basis for methioninase inhibition of tumor growth (8).

Toxicity results. Toxicity was evaluated according to the standard toxicity criteria (11). The physical examination and the laboratory determination before and after treatment were compared. No acute clinical toxicity was observed for all the toxicity criteria measured in patient - 1, patient - 2 and patient - 3 (Table III). The patients were treated as in the protocol of Table I, blood and urine were collected before treatment and 12 hours after treatment. The measurements were carried out according to WHO toxicity criteria and results are shown in Table III. The results suggest that i.v. infusion of methioninase did not cause any functional damage to the major organs. There was no hematological toxicity, renal, neurological, or cardiac toxicity (Tables IV-VI). There was a slight, transient blood pressure drop in patient - 3 during the treatment for about 20 minutes and after the infusion rate slowed, it disappeared. There was no allergic toxicity, no gastrointestinal toxicity and only a slight nausea was observed in patient - 2 during the treatment, which disappeared after the i.v. infusion slowed.

The results suggested that doses up to 20,000 units (1.6 g) as i.v. infusion over ten hours were safe. The i.v. infusion of methioninase effectively depleted serum methionine without any signs of side effects. The depletion of methionine levels could be maintained after the 10-hour i.v. infilsion for an additional 10 hours. Clinical studies are continuing to determine the maximum length of time complete methionine depletion can be tolerated, for subsequent efficacy studies.

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