Broad Selective Efficacy of rMETase and PEG-rMETase on Cancer Cells *In Vitro*

YUYING TAN, MINGXU XU and ROBERT M. HOFFMAN

AntiCancer, Inc., San Diego, CA 92111, U.S.A.

Abstract. *The elevated dependence on methionine of tumor cells is a cancer-specific metabolic defect. In current studies, the recombinant L-methionine α,γ-lyase (rMETase), an L-methionine depleting enzyme cloned from Pseudomonas putida, was shown to have efficacy in a broad series of cancer cell lines. Twenty-one different human tumor cell lines (4 lung, 4 colon, 4 kidney, 4 melanoma, 3 CNS, and 2 prostate) from the NCI Human Tumor Cell Line Screen and 7 human normal cell strains were treated with rMETase in vitro. We showed that rMETase had mean IC_{50} (units rMETase/ml) for the following cancer cell types: renal cancer, 0.07; colon cancer, 0.04; lung cancer, 0.12; prostate cancer, 0.01; melanoma, 0.19; and CNS cancer, 0.195, which was approximately one order of magnitude lower than that for normal cell strains: skin fibroblasts, 4; aortic smooth muscle cells, 0.88; aortic endothelial cells, 0.8; keratinocytes, 0.75, and bronchial epithelial cells, 0.75. rMETase was also conjugated with polyethylene lycol (PEG). PEG-rMETase also had high cell-kill activity. In vitro studies, animal studies and clinical trials have now shown that methionine restriction is an effective anticancer strategy. Cells from many different types of cancers are methionine dependent. The most effective strategy to deplete methionine is with the use of rMETase. PEG-rMETase offeres additional advantages of increasing the circulating half-life and reducing the immunogenicity of rMETase which is a bacterial protein. The results of the current study demonstrate the broad clinical potential for rMETase and rMETase for cancer treatment.*

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 (1-3). Methionine dependence is a metabolic defect seen only in cancer cells and precludes the cells from growing in medium in which methionine is depleted (4, 5). Normal mammalian cells proliferate normally in the absence of methionine as long as homocysteine is present in the growth medium (1). Animals fed diets in which methionine has been replaced by homocysteine also grow normally (2, 3). However, most cancer cells are dependent on exogenous, preformed methionine and grow, even in the presence of homocysteine (5-8).

Dietary methioninase restriction causes tumor regression of animal tumors, including cancer xenografts in nude mice (9, 10) and inhibits metastasis (3, 11). One clinical trial of chemotherapy combined with methionine restriction by total parenteral nutrition showed preliminary evidence of activity against gastric cancer (12). Tumors are more sensitive than normal tissues to methionine restriction. In contrast, restriction of other essential amino acids is either very toxic or ineffective (13).

A phase I clinical trial of dietary methionine restriction for adults with advanced solid tumors was carried out (14). All patients on the trial were maintained on an external diet. Plasma methionine declined 58%. The only side effect was weight loss of approximately 0.5% of body mass index (0.5 kg) per week. Thus, an enteral dietary methionine restriction is safe and tolerable in adults with metastatic solid tumors and results in significant reduction in plasma methionine levels.

Preclinical studies showed a better antitumour activity using MET restriction plus 5-FU than either treatment administered separately (15-18). A clinical trial in preoperative advanced gastric cancer patients also demonstrated that MET-deprived total parenteral nutrition with 5-FU gave a better histological response than conventional total parenteral nutrition with 5-FU (19). Moreover, Machover *et al*. (18) have demonstrated in CCRF-CEM, human leukemia cell line, a cytotoxic synergism of MET deprivation in combination with the association of 5-FU and folinic acid. MET restriction also enhances cyotoxic activity of platinum compound, cisplatin (20-22).

Current standard first-line regimens for metastatic colorectal cancer are FOLFOX (infusional 5-FU/LV with oxaliplatin) and FOLFIRI (infusional 5-FU/LV with irinotecan) (23). Since MET deprivation can potentiate the different chemotherapeutic

Correspondence to: Robert M. Hoffman, AntiCancer, Inc., 7917 Ostrow St., San Diego, CA 92111, U.S.A. Tel +18586542555, Fax +8582684175, e-mail: all@anticancer.com

Key Words: Methioninase, PEG-methioninase, cancer, methionine dependence, efficacy, selectivity.

agents used in the FOLFOX regimen, MET restriction was used in combinations with these regimens. A clinical trial combining MET restriction with the FOLFOX regimen in colorectal cancer patients was carried out. Plasma MET concentration was reduced by dietary MET restriction, with a depletion of 58% at the 1st day of the MET-free diet. MET-free diet and FOLFOX regimen produced minimal toxicity. Among the 4 evaluable patients for response, 3 experienced a partial response and 1 patient a disease stabilization.

L-methionine α-deamino-γ-mercaptomethane lyase (methioninase, METase), is a pyridoxal-L-phosphate (PLP)-dependent enzyme that cleaves methionine. METase has been demonstrated to be a powerful approach to methionine depletion *in vivo* (24, 25). The enzyme has been cloned from Pseudomona putida and produced in *Escherichia coli* (26, 27) (recombinant methioninase, METase) for extensive preclinical testing.

rMETase alone or in combination with chemotherapeutic agents such as cisplatin, 5-fluorouracil (5-FU), and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) have shown efficacy and synergy, respectively, in mouse models of colon cancer, lung cancer, and brain cancer (21, 28-30). The findings from a pilot Phase I clinical trial showed that METase depleted plasma methionine levels without observed clinical toxicity over a period up to ~24 hours in patients with advanced cancer (31, 32).

Conjugation of protein therapeutics with polyethylene glycol (PEG) has been shown to confer important therapeutic benefits, most importantly reduced antigenicity (33). The Food and Drug Administration has approved the PEGylated forms of several protein therapeutics for clinical use including adenosine deaminase, asparaginase, α-interferon, and a growth hormone antagonist (34-38).

To improve the rMETase therapeutic potential, rMETase was coupled to methoxypolyethylene glycol succinimidyl glutarate-5000 (MEGC-PEG-5000) (39). Pharmacokinetic evaluation in mice showed that MEGC-PEG-rMETase increased the serum half-life of the enzyme up to 20-fold and increased methionine depletion time up to 12-fold compared with unmodified rMETase. In addition, a further prolongation of *in vivo* activity and effective methionine depletion by MEGC-PEG-rMETase was achieved by the simultaneous administration of pyridoxal-5'-phosphate (PLP) (40).

The current study investigated the efficacy of rMETase and PEG-rMETase in a broad range of cancer cell lines *in vitro* compared to normal cell strains.

Materials and Methods

Fermentation of E. coli expressing rMETase. Every production fermentation was started with one vial from the Cell Bank. Ten microliters of bacteria from the Cell Bank were seeded into 5 ml LB medium with 100 μg ampicillin and grown at 37˚C at 400 rpm overnight. This culture was transferred to 800 ml Terrific Broth (TB) (38) in 6 L flasks and grown overnight at 37˚C at 400 rpm at which time the OD_{600} was approximately 10. The 800-ml cultures were then transferred into 10 800-ml TB-medium cultures in 6 L flasks and grown at 37˚C at 400 rpm for 16 h at which time the OD_{600} was approximately 10. The medium was changed with fresh TB and the incubation was continued for another 6 h. When the OD_{600} reached 20, the bacteria were harvested by centrifugation at 4000g at 4˚C for 10 min (41).

Purification of rMETase.

(1) Precolumn treatment of the sample. The bacterial pellet was suspended in extraction solution (20 mM potassium phosphate, pH 9.0, 10 μM pyridoxal phosphate and 0.01% β-mercaptoethanol) and disrupted with a cavitator-type homogenizer (Microfluidics Corp., Newton, MA, USA; model HC 8000). Heat treatment of the homogenate was then carried out up to 50˚C for 1 min. The suspension was centrifuged with an automatic refrigerated centrifuge (SORVALL, superspeed RC 2-B) at 4˚C at 8000g for 30 min. The supernatant was then collected. This step was followed by ultrafiltration by a Millipore (Bedford, MA, USA) Prep/Scale-TFF PLHK 100k, 2.5 ft^2 cartridge with buffer (10 Mm potassium phosphate, pH 8.3). The pH was adjusted to 7.2 by ultrafiltration (41).

Chromatographic conditions.

First column: DEAE Sepharose FF (pH 7.2). The first column was 100 mm diameter and 30 cm height, with a volume of 2400 ml of DEAE Sepharose FF (Pharmacia, Uppsala, Sweden). The elution flow rate was 15-50 ml/min. Forty to eighty grams of total protein (10-20 mg/ml) was applied on the column. After loading, the column was prewashed with 40 mM potassium chloride in PPM buffer (10 mM potassium phosphate, pH 7.2, containing 10 mM pyridoxal phosphate and 0.01% b-mercaptoethanol) for approximately 10 vol, until the OD_{280} dropped below 0.1. The protein was then eluted with a linear gradient of 40 to 300 mM potassium chloride in PPM buffer. Elution fractions of 500 ml were collected. The fractions containing rMETase were identified by yellow color and activity assay (41).

Second column: DEAE Sepharose FF (pH 8.3). The height of the second column (XK 50/30) was 25 cm, with a volume of 500 ml. The elution flow rate was 6-8 ml/min. After 24 h dialysis in 80 mM potassium chloride and 10 mM potassium phosphate (pH 8.3), 5-10 g of total protein (2-5 mg/ml) were applied on the second column. After loading, the column was prewashed with 80 mM potassium chloride and 10 mM potassium phosphate (pH 8.3) for approximately 4 vol, until the OD280 dropped below 0.1. rMETase was eluted with a linear gradient of 80 to 300 mM potassium chloride in 10 mM potassium phosphate buffer (pH 8.3). Elution fractions of 300 ml were collected. The fractions containing rMETase were identified by yellow color and activity assay (41).

Third column: ActiClean Etox. To eliminate endotoxin, purified rMETase (10-20 mg protein/ml) in a volume of 200-300 ml was applied on an 800-ml Acticlean Etox (Sterogen, Arcadia, CA) column (25×60) with a bed height of 40 cm. The protein was eluted with elution buffer (0.12 M sodium chloride in 10 mM sodium phosphate, pH 7.2), at a flow rate of 1 ml/min. The enzyme fractions, identified by yellow color and activity assay, were collected (41).

The final eluant was concentrated with 30K Amicon (Lexington, MA, USA) Centriprep concentrators by centrifugation at 4000g for 30 min at 4˚C. Sterilization was performed with 0.2 μm Nylon filter (Nalgene) (41).

Formulation of rMETase. rMETase in solution consisted of 0.12 M sodium chloride, 10 mM sodium phosphate buffer (pH 7.2), at a concentration of 10-20 mg/ml. This formulation was used *in vivo*. rMETase lyophilization rMETase, in solution, was frozen on dry ice and acetone and then lyophilized (Freeze mobil 24, Vertis) at –80˚C, under a vaccum of 100 millibar for 72 h (41).

(3) Analysis of rMETase.

HPLC. An Hitachi L-6200A Intelligent pump (Hitachi, Ltd, Tokyo, Japan) with a Supelco Progel-TSK column (G3000 SWXL, 30 cm × 7.8 mm) (Supelco, Bellefonte, PA, USA) was used for all HPLC experiments. A sample of 20 μl (0.1-0.5 mg/ml) was loaded and eluted with elution solution (0.12 M sodium chloride in 10 mM sodium phosphate buffer, pH 7.2) at a flow rate of 1.0 ml/min. The protein-containing fractions were identified with a spectrophotometer (Hitachi U2000) at a wavelength of 280 nm. Bovine serum albumin (MW 66,000) and sweet potato b-amylase (MW 200,000) (Sigma, Louis, MO, USA) were used as MW standards (41).

Electrophoresis. Electrophoresis was 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. Molecular weight standards used were Kaleidoscope Prestained Standards (Bio-Rad, Hercules, CA) (41).

Activity assay. The assay was carried out in a 1-ml vol 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 enzyme. The reaction was stopped by adding 0.5 ml of 4.5% TCA. The suspension was centrifuged by eppendorf centrifuge at 13 krpm for 2 min. One-half milliliter of supernatant was added to 0.5 ml of 0.05% 3-methyl-2-benzothiazolinone hydrazone in 1 ml of 1 M sodium acetate, pH 5.2, and incubated at 50˚C for 30 min. The amount of reaction product was determined by spectrophotometry at OD_{335}. The amount of protein was determined with the Lowry Reagent kit (Sigma) with bovine serum albumin as a standard. The specific activity was calculated as units/mg protein, with one unit of enzyme defined as the amount that catalyzes the formation of 1 μmol of a-ketobutyrate (41).

Endotoxin assay. The endotoxin level was measured by the Limulus Amebocyte Lysate (LAL) test (BioWhittaker, Walkersville, MD, USA). A sample was mixed with the LAL and incubated at 37˚C for 10 min. A substrate solution supplied with the kit was then mixed with the sample and incubated at 37˚C for an additional 6 min. The reaction was stopped with stop reagent supplied with the kit. The absorbance of the sample was determined with a spectrophotometer (Hitachi, U 2000) at 410 nm. The concentration of endotoxin was calculated from a standard curve which was constructed from the endotoxin supplied in the kit at concentrations from 0.1 EU/ml to 1 EU/ml (41).

Preparation and purification of PEGylated rMETase. Methoxy-polyethylene glycol succinimidyl glutarate-5000 (MEGC-50HS-PEG or MEGC-PEG) (NOF Corporation, Kawasaki-shi, Kanagawa, Japan, Lot No. M21514) had a polydispersity of 1.02, substitution 94.2%, dimer content 0.84% and purity by ^{1}H-NMR of 98.4%. The average molecular weight was 5461 Da. (42).

The activated PEG derivative was used at a molar excess (1-4 fold) of PEG to free lysines in rMETase (32 per rMETase molecule), which corresponds to molar ratios of PEG to rMETase of 30-120/1. For each reaction, 120 mg/ml rMETase in 100 mM borate buffer (pH 8.8) was used. Based on 30-120/1 molar ratios of activated PEG *versus* rMETase (equal to 0.87-3.5/1 weight ratio of activated PEG *versus* rMETase), a given amount of the activated PEG was added to the rMETase solution with three stepwise additions at 30 min intervals. The PEGylation reactions were carried out at 20-25˚C under gentle stirring for 90 min (42).

To eliminate an excess of unreacted activated PEG, the resulting PEGrMETase conjugate was applied on a Sephacryl S-300 HR gel filtration column (HiPrep 26/60, Amersham Pharmacia Biotech, Piscataway, NJ, USA) immediately after the PEGylation reaction. PEG-rMETase was eluted with 80 mM sodium chloride in 10 mM sodium phosphate, pH 7.4, containing 10 μM PLP at a flow rate of 120 ml/h (42).

The fractions containing the PEG-rMETase conjugate were further purified by DEAE Sepharose FF column (XK 16/15, Amersham Pharmacia Biotech) to remove trace amounts of un-PEGylated rMETase. The column was equilibrated and eluted with 80 mM sodium chloride in 10 mM sodium phosphate pH 7.2, containing 10 μM PLP at a flow rate of 180 ml/h. The fractions containing the PEG-rMETase conjugate were collected. PEG-rMETase-containing fractions were concentrated with an Amicon centriprep YM-30 (Millipore Corp, Bedford, MA, USA) and sterilized by filtration with a 0.22 μM membrane filter (Fisher Scientific, Tustin, CA, USA). Storage was at –80˚C (42).

Determination of protein content. Protein was measured with the Wako Protein Assay Kit (Wako Pure Chemical, Osaka, Japan) according to the instruction manual with slight modification (43). 50 μl of each sample or standard protein (BSA) was added to 3 ml of chromophore solution (pyrogallol red-molybdate complex) and vortexed well. The mixture was incubated at room temperature for 20 min without shaking and then measured for absorbance at 600 nm. The protein content of the sample was determined from the BSA standard calibration curve.

Determination of PEGylation degree of rMETase. The degree of modification of PEGylated rMETase was estimated both by the fluorescamine assay (44) and by MALDI. For the fluorescamine assay, various amounts of rMETase and PEGylated rMETase in 2 ml of 0.1 M sodium phosphate buffer, pH 8.0 were mixed with 1 ml fluorescamine solution (0.3 mg/ml in acetone) and incubated for 5 min at room temperature. Samples were then assayed with a fluorescence spectrometer at 390 nm excitation and 475 nm emission. Results were plotted as fluorescence units *versus* concentration, with the slope of the line being determined by linear regression. The percent of PEGylated primary amines was determined according to the following formula: 1- (slope PEGylated rMETase/slope naked rMETase) ×100. MALDI analysis of naked and PEG-rMETase was performed at the Scripps Research Institute using a PerSeptive Biosystems Voyager-Elite mass spectrometer.

Inhibition of human cancer cells by rMETase and PEG-rMETase in vitro. Human lung, colon, kidney, brain, prostate, and melanoma cancer cells and various types of normal cells were incubated in methionine-containing RPMI 1640 medium supplemented with 10% FBS. Various concentrations (0.1-4 units/ml) of rMETase or PEGrMetase were added to the medium and incubated with the cells for 4 days at 37˚C, 5% CO_2. The relative growth inhibition was calculated from cell counts.

Table I. *rMETase IC_{50} for tumor cell lines of major organs.*

Tumor type	Renal		Colon		Lung		Prostate		Melanoma		CNS	
		IC_{50}*		IC_{50}		IC_{50}		IC_{50}		IC_{50}		IC_{50}
Cell lines	A498	0.06	HCT15	0.04	H460	0.02	DU145	0.14	UACC257	0.2	SNB-75	0.19
	CAKI-1	0.13	Colo205	0.12	H522	0.12	PC-3	0.125	UACC62	0.18	SF-295	0.2
	SN12C	0.05	SW620	0.1	H23	0.1			SK-MEL-5	0.17		
	RXF393	0.05	HT29	0.05	SW620	0.1			Lox	0.2		
Mean IC_{50}	0.07±0.04		0.08±0.04		0.12±0.1		0.13±0.01		0.19±0.15		0.195±0.01	
IC_{50} Range	0.05-0.13		0.04-0.12		0.02-0.12		0.125-0.14		0.17-0.2		0.19-0.2	

*Inhibition concentration (units/ml).

Table II. *rMETase IC_{50} for human normal cell strains.*

Cell type fibroblasts	Foreskin muscle cells	Aortic smooth endothelial cells	Aortic epithelial cells	Keratinocytes	Bronchial
Cell strains	Hs-68 Hs-27	AOSMC (2.5 Mo.) AOSMC (22 YOD) AOSMC (54 YOD)	HAEC	NHEK	NHBE
IC_{50} *(units/ml)	>4	0.88±0.04	0.8	0.75	0.75

*Inhibition concentration (units/ml).

Results

Tumor-selective growth inhibitory effect of rMETase on human cancer cells in vitro: IC_{50} studies. The elevated dependence on methionine of tumor cells is a cancer-specific metabolic defect. In current studies, the recombinant L-methionine α,γ-lyase (rMETase), an L-methionine depleting enzyme cloned from *Pseudomonas putida*, was shown to have efficacy in a broad series of cancer cell lines. Twenty-one different human tumor cell lines (4 lung, 4 colon, 4 kidney, 4 melanoma, 3 CNS, and 2 prostate) from the NCI Human Tumor Cell Line Screen and 7 human normal cell strains were treated with rMETase *in vitro*. We showed that rMETase had mean IC_{50} (units rMETase/ml) for the following cancer cell types: renal cancer, 0.07; colon cancer, 0.04; lung cancer, 0.12; prostate cancer, 0.01; melanoma, 0.19; and CNS cancer, 0.195 (Table I). In contrast, normal cell strains had a much higher IC_{50}: skin fibroblasts, 4; aortic smooth muscle cells, 0.88; aortic endothelial cells, 0.8; keratinocytes, 0.75, and bronchial epithelial cells, 0.75 (Table II).

rMETase was also conjugated with polyethylene lycol (PEG). PEG-rMETase also had high cell-kill activity. The PEG-rMETase IC_{50} (units/ml) for human colon cancer was 0.28; for brain cancer, 0.25; for liver cancer, 0.17; for prostate cancer, 0.17; for lung cancer, 0.09; for pancreatic cancer, 0.17; for melanoma, 0.19; for kidney cancer, 0.22; for ovarian cancer, 0.25; for breast cancer, 0.14; for submaxillary cancer, 0.35, for mouse breast cancer, 0.08; for melanoma, 0.11. In contrast, the IC_{50} for human normal fibroblasts was 72 (Table III).

Discussion

In vitro studies, animal studies and clinical trials have now shown that methionine restriction is an effective anticancer strategy. Cells from many different types of cancers are methionine dependent. The most effective strategy to deplete methionine is with the use of rMETase. PEG-rMETase offers additional advantages of increasing the circulating half-life and reducing the immunogenicity of rMETase which is a bacterial protein. The results of the current study demonstrate the broad clinical potential for rMETase and rMETase for cancer treatment.

References

1 Stern PH and Hoffman RM: Enhanced *in vitro* selective toxicity of chemotherapeutic agents for human cancer cells based on a metabolic defect. J Natl Cancer Inst *76*: 629-639, 1986.

2 Du Vigneaud V, Chandler JP, Moyer AW and Keppel DM: The effect of choline on the ability of homocysteine to replace methionine in the diet. J Biol Chem *131*: 57-76, 1939.

Table III. *rMETase IC_{50} for human normal cell strains.*

	Human colon							Human brain					
	Colo205	HCT116	RKO	Colo320	HT29	Cal27	Average	SF295	Daoy	324	C6	U87	Average
IC_{50} (U/ml)	0.12	0.22	0.24	0.32	0.33	0.47	0.28	0.12	0.22	0.24	0.32	0.33	0.25
IC_{70} (U/ml)	0.91	0.32	0.36	1.7	1.45	0.95	0.95	0.91	0.32	0.36	1.7	1.45	0.95
IC_{90} (U/ml)	4.68	0.56	0.7	7.54	15.6	3	5.35	4.68	0.56	0.7	7.54	15.6	5.82

	Human liver			Human prostate		
	Hep2	SK-Hep-1	Average	LnCap	PC-3	Average
IC_{50} (U/ml)	0.12	0.22	0.17	0.12	0.22	0.17
IC_{70} (U/ml)	0.91	0.32	0.62	0.91	0.32	0.62
IC_{90} (U/ml)	4.68	0.56	2.62	4.68	0.56	2.62

	Human lung	Human pancreas	Human melanoma	Human kidney	Human ovary	Human breast	Human submaxillary	Mouse breast cancer	Mouse melanoma	Human normal fibroblasts
	H460	MIA-PaCa	Lox	SN12C	Ovcar-3	MA11	A253	MMT060562	B16F0	Fs6
IC_{50} (U/ml)	0.09	0.17	0.19	0.22	0.25	0.4	0.35	0.08	0.11	>2
IC_{70} (U/ml)	0.24	1	0.62	0.97	1.01	1	0.92	0.23	0.37	
IC_{90} (U/ml)	1.22	18.39	1.7	5.96	14.2	46.6	2.93	0.95	1.2	

3 Breillout F, Hadida F, Echinard-Garin P, Lascaux V and Poupon MF: Decreased rat rhabdomyosarcoma pulmonary metastases in response to a low methionine diet. Anticancer Res *7*: 861-867, 1987.

4 Tisdale MJ: Utilization of preformed and endogenously synthesized methionine by cells in tissue culture. Br J Cancer *49*: 315-320, 1984.

5 Halpern BC, Clark BR, Hardy DN, Halpern RM and Smith RA: The effect of replacement of methionine by homocystine on survival of malignant and normal adult mammalian cells in culture. Proc Natl Acad Sci USA *71*: 1133-1136, 1974.

6 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.

7 Tisdale M and Eridani S: Methionine requirement of normal and leukaemic haemopoietic cells in short term cultures. Leuk Res *5*: 385-394, 1981.

8 Guo HY, 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.

9 Poirson-Bichat F, Gonfalone G, Bras-Gonçalves RA, Dutrillaux B and Poupon MF: Growth of methionine-dependent human prostate cancer (PC-3) is inhibited by ethionine combined with methionine starvation. Br J Cancer *75*: 1605-1612, 1997.

10 Guo H, Lishko VK, 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.

11 Breillout F, Antoine E and Poupon MF: Methionine dependency of malignant tumors: a possible approach for therapy. J Natl Cancer Inst *82*: 1628-1632, 1990.

12 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.

13 Sugimura T, Birnbaum SM, Winitz M and Greenstein JP: Quantitative nutritional studies with water-soluble, chemically defined diets. VIII. The forced feeding of diets each lacking in one essential amino acid. Arch Biochem Biophys *81*: 448-455, 1959.

14 Epner DE, Morrow S, Wilcox M and Houghton JL: Nutrient intake and nutritional indexes in adults with metastatic cancer on a Phase I clinical trial of dietary methionine restriction. Nutrition and Cancer *42*: 158-166, 2002.

15 Hoshiya T, Kubota T, Inada T, Kitajima M and Hoffman RM: Methionine-depletion modulates the efficacy of 5-fluorouracil on human gastric cancer in nude mice. Anticancer Res *17*: 4371-4376, 1997.

16 Goseki N and Endo M: Thiol depletion and chemosensitization on nimustine hydrochloride by methionine-depleting total parenteral nutrition. Tohoku J Exp Med *161*: 227-239, 1990.

17 Yoshioka T, Wada T, Uchida N, Maki H, Yoshida H, Ide N, Kasai H, Hojo K, Shono K, Maekawa R, Yagi S, Hoffman RM and Sugita K: Anticancer efficacy *in vivo* and *in vitro*, synergy with 5-fluorouracil, and safety of recombinant methioninase. Cancer Res *58*: 2583-2587, 1998.

18 Machover D, Zittoun J, Broet P, Metzger G, Orrico M, Goldschmidt E, Schilf A, Tonetti C, Tan Y, Delmas-Marsalet B, Luccioni C, Falissard B and Hoffman RM: Cytotoxic synergism of methioninase in combination with 5-fluorouracil and folinic acid. Biochem Pharmacology *61*: 867-876, 2001.

19 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.

20 Poirson-Bichat F, Gonçalves RA, Miccoli L, Dutrillaux B and Poupon MF: Methionine depletion enhances the antitumoral efficacy of cytotoxic agents in drug-resistant human tumor xenografts. Clin Cancer Res *6*: 643-653, 2000.

21 Tan Y, Sun X, Xu M, Tan X, Sasson A, Rashidi B, Han Q, Tan X, Wang X, An Z, Sun FX and Hoffman RM: Efficacy of recombinant methioninase in combination with cisplatin on human colon tumors in nude mice. Clin Cancer Res *5*: 2157-2163, 1999.

22 Hoshiya Y, Kubota T, Matsuzaki SW, Kitajima M and Hoffman RM: Methionine starvation modulates the efficacy of cisplatin on human breast cancer in nude mice. Anticancer Res *16*: 3515-3517, 1996.

23 Goldberg RM: Advances in the treatment of metastatic colorectal cancer. Oncologist *10*: 40-48, 2005.

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

25 Lishko VK, Lishko OV and Hoffman RM: Depletion of serum methionine by methioninase in mice. Anticancer Res *13*: 1465-1468, 1993.

26 Hori H, Takabayashi K, Orvis L, Carson DA and Nobori T: Gene cloning and characterization of *Pseudomonas putida* L-methionine -deamino- -mercaptomethane-lyase. Cancer Res *56*: 2116-2122, 1996.

27 Tan Y, Xu M, Tan XZ, Tan XY, Wang X, Saikawa Y, Nagahama T, Sun X, Lenz M and Hoffman RM: Overexpression and large-scale production of recombinant L-methionine- -deamino- -mercaptomethane-lyase for novel anticancer therapy. Protein Expr Purif *9*: 233-245, 1997.

28 Yoshioka T, Wada T, Uchida N, Maki H, Yoshida H, Ide N, Kasai H, Hojo K, Shono K, Maekawa R, Yagi S, Hoffman RM and Sugita K: Anticancer efficacy *in vivo* and *in vitro*, synergy with 5-fluorouracil and safety of recombinant methioninase. Cancer Res *58*: 2583-2587, 1998.

29 Kokkinakis DM, Schold SC Jr, Hori H and Nobori T: Effect of long-term depletion of plasma methionine on the growth and survival of human brain tumor xenografts in athymic mice. Nutr Cancer *29*: 195-204, 1997.

30 Kokkinakis DM, Wick JB and Zhou Q-X: Metabolic response of normal and malignant tissue to acute and chronic methionine stress in athymic mice bearing human glial tumor xenografts. Chem Res Toxicol *15*: 1472-1479, 2002.

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

32 Tan Y, Zavala J, Sr, Han Q, Han Q, Xu M, Sun X, Tan X-Z, Tan X-Y, Magana R, Geller J and Hoffman RM: Recombinant methioninase infusion reduces the biochemical endpoint of serum methionine with minimal toxicity in high-stage cancer patients. Anticancer Res *17*: 3857-3860, 1997.

33 Yang Z, Wang J, Yoshioka T, Li B, Lu Q, Li S, Sun X, Tan Y, Yagi S, Frenkel EP and Hoffman RM: Pharmacokinetics, methionine depletion, and antigenicity of recombinant methioninase in primates. Clin Cancer Res *10*: 2131-2138, 2004.

34 Kozlowski A and Harris JM: Improvements in protein PEGylation: pegylated interferons for treatment of hepatitis C. J Controlled Release *72*: 217-224, 2001.

35 Maeda H, Kabanov A, Kataska K, Okano T (eds.). Advances in Experimental Medicine and Biology: Polymer Drugs in the Clinical Stage. Vol. 519, Kluwer Academic/Plenum Publishers Dordrecht, The Netherlands, 2003.

36 Park CWG and Chuo M: Interferon polymer conjugates. United States Patent 5,951,974, September 14, 1999.

37 Aguayo A, Cortes J, Thomas D, Pierce S, Keating M and Kantarjian H: Combination therapy with methotrexate, vincristine, polyethylene-glycol conjugated-asparaginase, and prednisone in the treatment of patients with refractory or recurrent acute lymphoblastic leukemia. Cancer *86*: 1203-1209, 1999.

38 Pool R: "Hairy enzymes" stay in the blood. Science *248*: 305, 1990.

39 Hershfield MS: PEG-ADA replacement therapy for adenosine deaminase deficiency: an update after 8.5 years. Clin Immunol Immunopathol *76*: S228-S232, 1995.

40 Sun X, Yang Z, Li S, Tan Y, Zhang N, Wang X, Yagi S, Yoshioka T, Takimoto A, Mitsushima K, Suginaka A, Frenkel EP and Hoffman RM: *In vivo* efficacy of recombinant methioninase is enhanced by the combination of polyethylene glycol conjugation and pyridoxal 5'-phosphate supplementation. Cancer Res *63*: 8377-8383, 2003.

41 Tan Y, Xu M, Tan X-Z, Tan X-Y, Wang, X, Saikawa Y, Nagahama T, Sun X, Lenz M and Hoffman RM: Overexpression and large-scale production of recombinant L-methionine-α-deamino-γ-mercaptomethane-lyase for novel anticancer therapy. Protein Expr Purif *9*: 233-245, 1997.

42 Sun X, Yang Z, Li S, Tan Y, Zhang N, Wang X, Yagi S, Yoshioka T, Takimoto A, Mitsushima K, Suginaka A, Frenkel EP and Hoffman RM: *In vivo* efficacy of recombinant methioninase is enhanced by the combination of polyethylene glycol conjugation and pyridoxal 5' phosphate supplementation. Cancer Res *63*: 8377-8383, 2003.

43 Watanabe N, Kamei S, Ohkubo A, Yamanaka M, Ohsawa S, Makino K and Tokuda K: Urinary protein as measured with a pyrogallol red-molybdate complex, manually and in a Hitachi 726 automated analyzer. Clin Chem *32*: 1551-1554, 1986.

44 Li S, Yang Z, Sun X, Tan Y, Yagi S and Hoffman RM: A simultaneous colorimetric assay of free and protein-coupled polyethylene glycol. Anal Biochem *313*: 335-337, 2003.

Received January 26, 2010
Accepted February 3, 2010