

Broad Selective Efficacy of Recombinant Methioninase and Polyethylene Glycol-modified Recombinant Methioninase on Cancer Cells *In Vitro*

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Abstract. *Elevated dependence on methionine of cancer cells is a cancer-specific metabolic defect. Recombinant L-methionine α,γ -lyase (rMETase), an L-methionine depleting enzyme cloned from *Pseudomonas putida*, was shown to have efficacy on 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 a mean IC_{50} (units rMETase/ml) for the following cancer cell types: renal, 0.07; colon, 0.04; lung, 0.12; prostate, 0.01; melanoma, 0.19; and CNS, 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 glycol (PEG). PEG-rMETase also had high cancer cell-killing 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 cancer 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 PEG-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 (1-12). Methionine dependence

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is a metabolic defect seen only in cancer cells and precludes the cells from growing in medium in which methionine is depleted. Non-malignant mammalian cells proliferate normally in the absence of methionine as long as homocysteine is present in the growth medium (4). Animals fed diets in which methionine has been replaced by homocysteine also grow normally (4). However, most cancer cells are dependent on exogenous, preformed methionine and do not grow, even in the presence of homocysteine (1-8).

Dietary methioninase restriction causes tumor regression of animal tumors, including cancer xenografts in nude mice (9-13) and inhibits metastasis (9). One clinical trial of chemotherapy combined with methionine restriction by total parenteral nutrition showed preliminary evidence of activity against gastric cancer (14). Tumors are more sensitive than normal tissues to methionine restriction. In contrast, restriction of other essential amino acids is either very toxic or ineffective (15).

A phase I clinical trial of dietary methionine restriction for adults with advanced solid tumors was carried out (16). 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, enteral dietary methionine restriction is safe and tolerable in adults with metastatic solid tumors and results in significant reduction in plasma methionine levels.

Preclinical and clinical studies showed a better antitumor activity using MET restriction plus 5-fluorouracil (5-FU) than either treatment administered separately (17-21). A clinical trial in preoperative advanced gastric cancer patients demonstrated that MET-deprived total parenteral nutrition with 5-FU gave a better histological response than conventional total parenteral nutrition with 5-FU (21). Moreover, Machover *et al.* (20) have demonstrated in the CCRF-CEM human leukemia cell line a cytotoxic synergism of MET deprivation in combination with 5-FU and folinic acid. MET restriction also enhances the cytotoxic activity of cisplatin (22-24).

Current standard first-line regimens for metastatic colorectal cancer are FOLFOX (infusional 5-FU/leucovorin [LV] with oxaliplatin) and FOLFIRI (infusional 5-FU/LV with irinotecan)

(25). Since MET deprivation can potentiate the different chemotherapeutic 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. The 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 (26).

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* (27, 28). The enzyme has been cloned from *Pseudomonas putida* and produced in *Escherichia coli* (29, 30) (recombinant methioninase, rMETase) for extensive preclinical testing.

rMETase alone or in combination with chemotherapeutic agents such as cisplatin, 5-FU, and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) have shown efficacy and synergy, respectively, in mouse models of colon, lung and brain cancer (19, 23, 31, 32). The findings from a pilot Phase I clinical trial showed that rMETase depleted plasma methionine levels without observed clinical toxicity over a period up to ~24 hours in patients with advanced cancer (33, 34).

Conjugation of protein therapeutics with polyethylene glycol (PEG) has been shown to confer important therapeutic benefits, most importantly reduced antigenicity (35). 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 (36-41).

To improve the therapeutic potential rMETase, rMETase was coupled to methoxypolyethylene glycol succinimidyl glutarate-5000 (MEGC-PEG-5000) (42). 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 (43). 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) (44).

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 μ g ampicillin and grown 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 OD₆₀₀ 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 OD₆₀₀ was approximately 10. The medium was changed with fresh TB and the incubation was continued for another 6 h. When the OD₆₀₀ reached 20, the bacteria were harvested by centrifugation at 4000 xg at 4°C for 10 min (30).

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 μ M 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 8000 xg for 30 min. The supernatant was then collected. This step was followed by ultrafiltration with a Millipore Corp. (Bedford, MA, USA) Prep/Scale-TFF PLHK 100k, 2.5 ft² cartridge with buffer (10 mM potassium phosphate, pH 8.3). The pH was adjusted to 7.2 by ultrafiltration (30).

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) were 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 PLP and 0.01% β -mercaptoethanol) for approximately 10 vol, until the OD₂₈₀ 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 (30).

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 (30).

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, USA) column (25x60) 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 (30).

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

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.

rMETase lyophilization. rMETase, in solution, was frozen on dry ice and acetone and then lyophilized (Freeze mobil 24, Vertis) at -80°C , under a vacuum of 100 millibar for 72 h (30).

Analysis of rMETase.

HPLC. An Hitachi L-6200A Intelligent pump (Hitachi, Ltd, Tokyo, Japan) with a Supelco Progel-TSK column (G3000 SWXL, 30 cm \times 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 β -amylase (MW 200,000) (Sigma, Louis, MO, USA) were used as MW standards (30).

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, USA) (30).

Activity assay. The assay was carried out in a 1-ml vol of 50 mM phosphate buffer, pH 8.0, containing 10 μM PLP and 10 mM methionine for 10 min at 37°C , with different amounts of enzyme. The reaction was stopped by adding 0.5 ml of 4.5% TCA. The suspension was centrifuged in an Eppendorf centrifuge at 13 k rpm 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 OD₃₃₅. 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 α -ketobutyrate (30).

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 (30).

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 $^1\text{H-NMR}$ 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 $20-25^{\circ}\text{C}$ 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 centrprep YM-30 (Millipore Corp) 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 (45). Fifty 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 fluorescamine assay (46) and by MALDI. For the fluorescamine assay, different 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: $1 - (\text{slope PEGylated rMETase} / \text{slope naked rMETase}) \times 100$. MALDI analysis of naked and PEG-rMETase was performed at the Scripps Research Institute using a PerSeptive Biosystems Voyager-Elite mass spectrometer. Takakura *et al.* developed advanced techniques for the purification, assay, and PEGylation of rMETase (47-49).

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.

Results

Tumor-selective growth-inhibitory effect of rMETase on human cancer cells in vitro: IC₅₀ studies. The elevated dependence on methionine of tumor cells is a cancer-specific metabolic defect. In the present study, rMETase showed efficacy in a broad series of cancer cell lines. Twenty-one different human tumor cell lines

Table I. *rMETase* IC₅₀* 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	

*Inhibitory concentration (units/ml); see Materials and Methods for details.

Table II. *rMETase* IC₅₀* for human normal cell strains.

Cell type	Foreskin fibroblasts	Aortic smooth muscle cells	Aortic endothelial cells	Keratinocytes	Bronchial cells
Cell strains	Hs-68 Hs-27	AOSMC (2.5 Mo.) AOSMC (22 YOD) AOSMC (54 YOD)	HAEC	NHEK	NHBE
Mean IC ₅₀	>4	0.88±0.04	0.8	0.75	0.75

*Inhibitory concentration (units/ml); see Materials and Methods for details.

(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*. *rMETase* had a mean IC₅₀ (units *rMETase*/ml) for the following cancer cell types: renal, 0.07; colon, 0.04; lung, 0.12; prostate, 0.01; melanoma, 0.19; and CNS, 0.195 (Table I). In contrast, normal cell strains had a much higher IC₅₀: 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 conjugated with PEG also had high cell-killing activity towards cancer cells. The PEG-*rMETase* IC₅₀ (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; and for melanoma, 0.11. In contrast, the IC₅₀ for human normal fibroblasts was greater than 2 (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 cancer are methionine dependent which may also affect their DNA methylation (4, 50-52). The most effective strategy to deplete methionine is with the use of *rMETase*. The structure of *rMETase* has been determined at 1.8 Å resolution (53).

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 PEG-*rMETase* for cancer treatment, especially for those involving combinations with cancer drugs whose clinical efficacy has been shown to be enhanced by serum methionine depletion (26, 54-56).

References

- 1 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.
- 2 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.
- 3 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.
- 4 Hoffman RM: Altered methionine metabolism, DNA methylation and oncogene expression in carcinogenesis: a review and synthesis. *Biochim Biophys Acta Reviews on Cancer* 738: 49-87, 1984.
- 5 Tisdale MJ: Utilization of preformed and endogenously synthesized methionine by cells in tissue culture. *Br J Cancer* 49: 315-320, 1984.
- 6 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.
- 7 Tisdale M and Eridani S: Methionine requirement of normal and leukaemic haemopoietic cells in short term cultures. *Leuk Res* 5: 385-394, 1981.

Table III. Efficacy of PEG-rMETase on various (human and mouse) cancer cell lines.

	Human colon cancer							Human brain cancer					
	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 cancer			Human prostate cancer		
	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 cancer	Human pancreatic cancer	Human melanoma	Human kidney cancer	Human ovarian cancer	Human breast cancer	Human submaxillary cancer	Mouse breast cancer	Mouse melanoma	Human normal foreskin fibroblasts
	H460	MIA-PaCa	LOX	SN12C	Ovarc-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	

*Inhibition concentration (units/ml); see Materials and Methods for details.

- 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 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.
- 10 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.
- 11 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.
- 12 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.
- 13 Poirson-Bichat F, Lopez R, Bras Concalves RA, Miccoli L, Bourgeois Y, Demerseman P, Poisson M, Dutrillaux B and Poupon MF: Methionine deprivation and methionine analogs inhibit cell proliferation and growth of human xenografted gliomas. *Life Sci* 60: 919-931, 1997.
- 14 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.
- 15 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.
- 16 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.
- 17 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.
- 18 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.
- 19 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.
- 20 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.
- 21 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.
- 22 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.
- 23 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.
- 24 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.
 - 25 Goldberg RM: Advances in the treatment of metastatic colorectal cancer. *Oncologist* 10: 40-48, 2005.
 - 26 Durando X, Farges MC, Buc E, Abrial C, Petorin-Lesens C, Gillet B, Vasson MP, Pezet D, Chollet P and Thivat E: Dietary methionine restriction with FOLFOX regimen as first line therapy of metastatic colorectal cancer: a feasibility study. *Oncology*, 78: 205-209, 2010.
 - 27 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.
 - 28 Lishko VK, Lishko OV and Hoffman RM: Depletion of serum methionine by methioninase in mice. *Anticancer Res* 13: 1465-1468, 1993.
 - 29 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.
 - 30 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-mercapto-methane-lyase for novel anticancer therapy. *Protein Expr Purif* 9: 233-245, 1997.
 - 31 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.
 - 32 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.
 - 33 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.
 - 34 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.
 - 35 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.
 - 36 Kozlowski A and Harris JM: Improvements in protein PEGylation: pegylated interferons for treatment of hepatitis C. *J Controlled Release* 72: 217-224, 2001.
 - 37 Maeda H, Kabanov A, Kataska K and Okano T (eds.). *Advances in Experimental Medicine and Biology: Polymer Drugs in the Clinical Stage*. Vol. 519, Dordrecht, The Netherlands, Kluwer Academic/Plenum Publishers, 2003.
 - 38 Park CWG and Chuo M: Interferon polymer conjugates. United States Patent 5,951,974, September 14, 1999.
 - 39 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.
 - 40 Pool R: "Hairy enzymes" stay in the blood. *Science* 248: 305, 1990.
 - 41 Hershfield MS: PEG-ADA replacement therapy for adenosine deaminase deficiency: an update after 8.5 years. *Clin Immunol Immunopathol* 76: S228-S232, 1995.
 - 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 Yang Z, Wang J, Lu Q, Xu J, Kobayashi Y, Takahura T, Takimoto A, Yoshioka T, Lian C, Chen C, Zhang D, Zhang Y, Li S, Sun X, Tan Y, Yagi S, Frenkel EP and Hoffman RM: PEGylation confers greatly extended half-life and attenuated immunogenicity to recombinant methioninase in primates. *Cancer Res* 64: 6673-6678, 2004.
 - 44 Yang Z, Sun X, Li S, Tan Y, Wang X, Zhang N, Yagi S, Takahura T, Kobayashi Y, Takimoto A, Yoshioka T, Suginaka A, Frenkel EP and Hoffman RM: Circulating half-life of PEGylated recombinant methioninase holoenzyme is highly dose dependent on cofactor pyridoxal-5'-phosphate. *Cancer Res* 64: 5775-5778, 2004.
 - 45 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.
 - 46 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.
 - 47 Takakura T, Takimoto A, Notsu Y, Yoshida H, Ito T, Nagatome H, Ohno M, Kobayashi Y, Yoshioka T, Inagaki K, Yagi S, Hoffman RM and Esaki N: Physicochemical and pharmacokinetic characterization of highly potent recombinant L-methionine γ -lyase conjugated with polyethylene glycol as an antitumor agent. *Cancer Res* 66: 2807-2814, 2006.
 - 48 Takakura T, Ito T, Yagi S, Notsu Y, Itakura T, Nakamura T, Inagaki K, Esaki N, Hoffman RM and Takimoto A: High-level expression and bulk crystallization of recombinant L-methionine γ -lyase, an anticancer agent. *Appl Microbiol Biotechnol* 70: 183-192, 2006.
 - 49 Takakura T, Mitsushima K, Yagi S, Inagaki K, Tanaka H, Esaki N, Soda K and Takimoto A: Assay method for antitumor L-methionine γ -lyase: comprehensive kinetic analysis of the complex reaction with L-methionine. *Anal Biochem* 327: 233-240, 2004.
 - 50 Diala ES, Cheah MSC, Rowitch D and Hoffman RM: Extent of DNA methylation in human tumor cells. *J Natl Cancer Inst* 71: 755-764, 1983.
 - 51 Cheah MSC, Wallace CD and Hoffman RM: Hypomethylation of DNA in human cancer cells: a site-specific change in the *c-myc* oncogene. *J Natl Cancer Inst* 73: 1057-1065, 1984.
 - 52 Machover D, Zittoun J, Saffroy R, Broet P, Giraudier S, Magnaldo T, Goldschmidt E, Debuire B, Orrico M, Tan Y, Mishal Z, Chevallier O, Tonetti C, Jouault H, Ulusakarya A, Tanguy M-L, Metzger G and Hoffman RM: Treatment of cancer cells with methioninase produces DNA hypomethylation and increases DNA synthesis. *Cancer Res* 62: 4685-4689, 2002.
 - 53 Kudou D, Misaki S, Yamashita M, Tamura T, Takakura T, Yoshioka T, Yagi S, Hoffman RM, Takimoto A, Esaki N and Inagaki K: Structure of the antitumor enzyme L-methionine γ -lyase from *Pseudomonas putida* at 1.8Å resolution. *J Biochem* 141: 535-544, 2007.
 - 54 Thivat E, Farges MC, Bacin F, D'Incan M, Mouret-Reynier MA, Cellarier E, Madelmont JC, Vasson MP, Chollet P and Durando X: Phase II trial of the association of a methionine-free diet with cystemustine therapy in melanoma and glioma. *Anticancer Res* 29(12): 5235-5240, 2009.
 - 55 Durando X, Thivat E, Farges MC, Cellarier E, D'Incan M, Demidem A, Vasson MP, Barthomeuf C and Chollet P: Optimal methionine-free diet duration for nitrourea treatment: a Phase I clinical trial. *Nutr Cancer* 60: 23-30, 2008.
 - 56 Thivat E, Durando X, Demidem A, Farges MC, Rapp M, Cellarier E, Guenin S, D'Incan M, Vasson MP and Chollet P: A methionine-free diet associated with nitrosourea treatment down-regulates methylguanine-DNA methyl transferase activity in patients with metastatic cancer. *Anticancer Res* 27(4C): 2779-2784, 2007.

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