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 IC50 (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 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.

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 Animals fed diets in which methionine has been replaced by homocysteine also grow normally (2, 3).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 cytotoxic 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
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-deaminogamma-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 Pseudomonas 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 1 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 to normal cell strains.

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 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 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 cavitation 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 ft² 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% 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 (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 PPM 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 (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 (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 Nylion 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 millilbar 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 and 10 mM methionine for 10 min at 37˚C, with varying amounts of phosphate buffer, pH 8.0, containing 10 μM pyridoxal phosphate (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 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 PEG-rMETase were added to the medium and incubated with the cells for 4 days at 37°C, 5% CO2. 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 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 IC50 (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 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).

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

Table III. \( r\text{METase IC}_{50} \) for human normal cell strains.

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Human</th>
<th>Human</th>
<th>Human</th>
<th>Human</th>
<th>Human</th>
<th>Human</th>
<th>Human</th>
<th>Human</th>
<th>Human</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>colon</td>
<td>liver</td>
<td>melanoma</td>
<td>kidney</td>
<td>ovary</td>
<td>submaxillary</td>
<td>breast</td>
<td>cancer</td>
<td>melanoma</td>
<td>fibroblasts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colo205</td>
<td>HCT116</td>
<td>RKO</td>
<td>Colo320</td>
<td>HT29</td>
<td>Cal27</td>
<td>Average</td>
<td>SF295</td>
<td>Daoy</td>
<td>324</td>
<td>C6</td>
</tr>
<tr>
<td>IC(_{50}) (U/ml)</td>
<td>0.12</td>
<td>0.22</td>
<td>0.24</td>
<td>0.32</td>
<td>0.33</td>
<td>0.47</td>
<td>0.28</td>
<td>0.12</td>
<td>0.22</td>
<td>0.32</td>
<td>0.33</td>
</tr>
<tr>
<td>IC(_{70}) (U/ml)</td>
<td>0.91</td>
<td>0.32</td>
<td>0.36</td>
<td>1.7</td>
<td>1.45</td>
<td>0.95</td>
<td>0.95</td>
<td>0.91</td>
<td>0.32</td>
<td>0.36</td>
<td>1.7</td>
</tr>
<tr>
<td>IC(_{90}) (U/ml)</td>
<td>4.68</td>
<td>0.56</td>
<td>0.7</td>
<td>7.54</td>
<td>15.6</td>
<td>3</td>
<td>5.35</td>
<td>4.68</td>
<td>0.56</td>
<td>0.7</td>
<td>7.54</td>
</tr>
<tr>
<td></td>
<td>Hep2</td>
<td>SK-Hep-1</td>
<td>Average</td>
<td>LnCap</td>
<td>PC-3</td>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC(_{50}) (U/ml)</td>
<td>0.12</td>
<td>0.22</td>
<td>0.17</td>
<td>0.12</td>
<td>0.22</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC(_{70}) (U/ml)</td>
<td>0.91</td>
<td>0.32</td>
<td>0.62</td>
<td>0.91</td>
<td>0.32</td>
<td>0.62</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC(_{90}) (U/ml)</td>
<td>4.68</td>
<td>0.56</td>
<td>2.62</td>
<td>4.68</td>
<td>0.56</td>
<td>2.62</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H460</td>
<td>MIA-PaCa</td>
<td>Lox</td>
<td>SN12C</td>
<td>Ovcar-3</td>
<td>MA11</td>
<td>A253</td>
<td>MMT060562</td>
<td>B16F0</td>
<td>Fs6</td>
<td></td>
</tr>
<tr>
<td>IC(_{50}) (U/ml)</td>
<td>0.09</td>
<td>0.17</td>
<td>0.19</td>
<td>0.22</td>
<td>0.25</td>
<td>0.4</td>
<td>0.35</td>
<td>0.08</td>
<td>0.11</td>
<td>&gt;2</td>
<td></td>
</tr>
<tr>
<td>IC(_{70}) (U/ml)</td>
<td>0.24</td>
<td>1</td>
<td>0.62</td>
<td>0.97</td>
<td>1.01</td>
<td>1</td>
<td>0.92</td>
<td>0.23</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC(_{90}) (U/ml)</td>
<td>1.22</td>
<td>18.39</td>
<td>1.7</td>
<td>5.96</td>
<td>14.2</td>
<td>46.6</td>
<td>2.93</td>
<td>0.95</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Received January 26, 2010
Accepted February 3, 2010