EXPERT OPINION

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Development of recombinant methioninase to target the general cancer-specific metabolic defect of methionine dependence: a 40-year odyssey

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Introduction: All tested cancer cell types are methionine dependent in that the cells arrest and eventually die when deprived of methionine, a condition that is generally nontoxic to normal cells. Methionine dependence is the only known general metabolic defect in cancer. Methionine-deprived cancer cells arrest at the S/G_2 phase, an unusual position for cell cycle arrest. In order to exploit the cancer-specific metabolic defect of methionine dependence, methioninases were developed.
Areas covered: The present Expert Opinion describes the phenomena of methionine dependence and a methioninase cloned from *Pseudomonas putida* (chemical name: L-methionine α-deamino-γ-mercaptomethane lyase [EC 4.4.1.11]). The cloned methioninase, termed recombinant methioninase, or rMETase, has been tested in mouse models of human cancer as well as in macaque monkeys and a pilot Phase I trial of human cancer patients. Efficacy of rMETase has been demonstrated against various cancer types in mouse models.
Expert opinion: The most promising application of rMETase therapy is in sequential combination therapy, whereby the cancer cells within a tumor are trapped in S/G_2 by methioninase treatment and then treated with chemotherapeutic agents active against cells in S/G_2.

Keywords: cancer, combination chemotherapy, *Escherichia coli*, gene cloning, methioninase, recombinant rMETase, methionine dependence, PEGylation, purification, S/G_2, selective cancer-cell-cycle arrest, trap

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The crucial quality of science is to encourage, not discourage, the testing of assumptions. (Halton Arp [1987] in Quasars, Redshifts and Controversies, Interstellar Media, Berkeley, CA)

1. The discovery of methionine dependence of cancer cells

L5 178Y mouse leukemia cells in culture required a minimum of 10.0 mg L-methionine per liter in order to proliferate. When the cells were deprived of L-methionine, there was no growth. In order to proliferate, L-methionine supplementation was required for optimum cell growth, even in the presence of N^5-methyltetrahydrofolate, hydroxycobalamin, transcobalamin II, and DL-homocysteine, a condition that supported the biosynthesis of L-methionine and growth of normal cells. This was the first report that cancer cells in culture are abnormally methionine dependent [1].

Subsequently, other malignant mammalian cell lines, for example, Walker-256 rat breast carcinoma (W-256) and the L1210 murine lymphocytic leukemia were found to be methionine dependent [2]. In contrast, normal rat, human, and hamster

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Article highlights.

- Methionine dependence is the only known general metabolic defect in cancer.
- Cancer cells require high levels of methionine compared to normal cells.
- Methioninase can be used to treat any cancer and selectively trap the cells in S/G_2-phase of the cell cycle, where they are susceptible to most cytotoxic chemotherapy and can be successfully eradicated.

This box summarizes key points contained in the article.

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fibroblasts proliferated well under methionine depletion [3-6]. Thus, the malignant cells are methionine auxotrophs, that is, cell lines that manifest a nutritional requirement not observed in the genetically-related normal cells.

2. Alteration of methionine metabolism in cancer cells

Work on this topic was begun by the author in 1974.

Three lines of evidence indicated that the malignant and transformed cells synthesize large amounts of methionine endogenously through the reaction catalyzed by N^5-methyltetrahydropteroyl-L-glutamate: L-homocysteine S-methyltransferase (EC 2.1.1.13).

- The activity of the methyltransferase involved in methionine biosynthesis was comparable in extracts of malignant and normal cells.
- The uptake of radioactive label from [5-^{14}C]methyltetrahydropteroyl-L-glutamic acid (N^5-methyl-H_4PteGlu) was at least as great in the malignant cells as in the normals and was nearly totally dependent on the addition of homocysteine, the methyl acceptor.
- The majority of the labeled methyl groups incorporated by cancer cells was recovered as methionine. The malignant and transformed cells were unable to grow in homocysteine alone, but in the presence of otherwise limiting amounts of exogenous methionine, homocysteine greatly stimulated the growth of these cells. These results indicated that the methionine auxotrophy (dependence) of the malignant cells does not result simply from the inability to synthesize and incorporate methionine from homocysteine and N^5-methyl-H_4PteGlu [6].

Out of 23 cell lines derived from diverse types of human tumors, 11 did not grow at all in methionine-depleted, homocysteine-containing (MET$^-$HCY$^+$) medium and were absolutely methionine-dependent, whereas 3 grew only slightly in this medium. Many of the cancer cell lines tested have little else in common other than the fact that they are methionine-dependent. The high frequency of occurrence of methionine dependence in diverse types of human cancer cells indicated that methionine dependence could be an important aspect of oncogenic transformation. Normal unestablished cell strains thus far characterized grow well in MET$^-$HCY$^+$ medium [7,8].

Bone marrow cells from non-leukemic patients required a lower concentration of L-methionine in the culture medium for optimal incorporation of [methyl ^{3}H]thymidine (DNA synthesis) than did bone marrow cells from leukemic patients. The size of the cytoplasmic pool for L-methionine was larger (approximately twofold) in marrow cells from normal subjects than leukemia patients [9].

During methionine deprivation, the *S*-adenosylmethionine/*S*-adenosylhomocysteine (AdoMET/AdoHCY) ratio, an indicator of the methylation potential of the cell, became abnormally low in cancer cells, with very low levels of AdoMET and very high levels of AdoHCY. The amount of free methionine was low in malignant methionine-dependent SV40-transformed human fibroblasts in MET$^-$HCY$^+$ medium compared to normal human diploid fibroblasts. In contrast, in MET$^+$HCY$^-$ medium, the amount of free methionine was comparable in cancer and normal types [10].

In a diverse set of human tumor cell lines, all were found to be defective in at least one aspect of methionine metabolism, giving rise to the possibility of a general metabolic defect in cancer [11].

When the W-256 cancer cell line was cultured for 24 h in MET$^-$HCY$^+$ medium, the extent of methylation of nucleic acids and the acid-soluble pool of methionine were decreased. However, there was increased methylation activity of both endogenous substrate and *Escherichia coli* tRNA. Methionine deprivation of the W-256 cells resulted in a large increase in the Vmax value for methylation of tRNA, without any change in the Km value for AdoMET [12].

We observed that cancer cells have enhanced overall rates of transmethylation compared to normal human fibroblasts. Transmethylation rates were measured by blocking AdoHCY hydrolase and measuring AdoHCY, which accumulates as a result of transmethylation. The enhanced transmethylation rates may be the basis of the methionine dependence of cancer cells which explains the low levels of free methionine and the low AdoMET/AdoHCY ratio in cancer cells under methionine deprivation. The alteration of such a fundamental process as transmethylation in cancer may be indicative of its importance in the oncogenic process [13].

Rare cells from methionine-dependent cancer cell lines regained the normal ability to grow in MET$^-$HCY$^+$ medium. These lines were termed methionine-independent revertants. Two revertants isolated from SV40-transformed cells had regained the ability to grow similar to normal cells in MET$^-$HCY$^+$ medium without substantial changes in methioninase biosynthesis activity. Increased methionine biosynthesis thus is not a prerequisite to reversion from methionine dependence to independence [14].

Methionine-independent revertants had much lower basal transmethylation rates than parental methionine-dependent cell lines. For example, when comparing the parent SP1 cell line and its methionine-independent revertant, SP1-R, the

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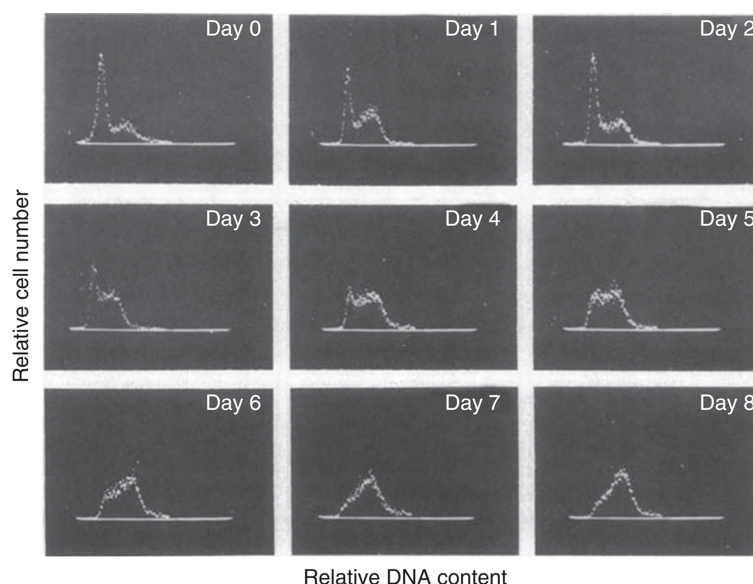

Figure 1. Fluorescence-activated cell sorting to determine cell-cycle position in a malignant SV40-transformed human line. Days indicate time of incubation in MET^-HCY^+ containing medium. Note how the cancer cells become trapped in late S/G_2 phase of the cell cycle [17].

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revertant reduced its transmethylation activity and rates. These results further suggested that methionine dependence is due to an increase in the rate of transmethylation reactions [15].

3. Relationship of methionine-dependence and other features of malignancy

We then demonstrated that methionine-independent revertants of both malignant human fibroblasts transformed by simian virus 40 and malignant rat fibroblasts concomitantly revert for characteristics associated with cancer. Of the 13 cancer-cell methionine-independent revertants characterized, 5 showed increased anchorage dependence as reflected by reduced cloning efficiencies in methylcellulose, 8 showed an increased serum requirement for optimal growth, 8 showed decreased cell density in medium containing high serum, and 3 altered their cell morphology significantly. Thus, the methionine-independent revertants become more normal-like. Eight of the 13 had increased chromosome numbers, which probably played a role in reversion. All lines tested contained simian virus 40 T-antigen. Thus by selecting for methionine independence, it is possible to select for heterogeneous malignant transformation revertants, which became less malignant, indicating further a relationship between altered methionine metabolism and oncogenic transformation [16].

4. S/G_2 cell cycle arrest of cancer cells deprived of methionine

Growth arrest of methionine-dependent cancer cells in MET^-HCY^+ medium resulted in a reduction in the percentage of mitotic cells in the cell population. Fluorescence-activated cell cycle analysis demonstrated that the cells are arrested in the S/G_2 phases of the cell cycle in MET^-HCY^+ medium. This is in contrast to a G_1-phase accumulation of cells, which occurs only in methionine-supplemented medium at very high cell densities and is similar to the G_1 block seen in cultures of normal fibroblasts at high density (**Figure 1**) [17].

When the human PC-3 prostate cancer cell line was cultured in MET^-HCY^+ medium, there was an extreme increment in DNA content without cell division, suggesting a cell cycle block in late-S/G_2 [18].

PC-3 and human prostate cancer DU 145 cells stopped proliferating within 6 days in MET^-HCY^+ medium. In contrast, proliferation of human prostate cancer LNCaP cells was only partially inhibited by the absence of methionine. Flow cytometry demonstrated that methionine restriction caused PC-3 cells to arrest in all phases of the cell cycle, but predominantly in the G_2/M phase. Methionine deprivation led to accumulation of the cyclin-dependent kinase inhibitors p21 and p27 and inhibited the enzymatic activities of the cyclin-dependent kinases CDK2 and cdc2. PC-3 cells underwent apoptosis, as indicated by DNA degradation after methionine deprivation [19].

In order to determine whether methionine dependence occurs in fresh patient tumors, they were histocultured on Gelfoam® [20]. Nuclear DNA content was measured by image analysis to determine the cell-cycle position in MET^-HCY^+ compared to MET^+HCY^- medium in 21 human patient tumors. Cell cycle arrest in late-S/G_2 was used as a marker of methionine dependence for histocultured patient tumors. We found that 5 of 21 human tumors, including tumors of

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the colon, breast, ovary, prostate, and a melanoma, were methionine dependent, based on cell cycle analysis [21].

5. Methionine-deprivation based chemotherapy

In cultures that were initiated with equal amounts of cancer cells and human diploid fibroblasts, substitution of homocysteine and doxorubicin for methionine in the culture medium, followed by methionine repletion with vincristine, was totally effective at selectively eliminating a methionine-dependent human sarcoma and 3 methionine-dependent human carcinomas. The above protocol was nearly totally effective against a partially methionine-independent revertant of the sarcoma. The chemotherapeutic procedure used was not lethal to normal cells growing alongside the cancer cells and was ineffective when conducted totally in MET^+ HCY^- medium. The optimal procedure was doxorubicin in MET^- HCY^+ medium for 10 days followed by vincristine in MET^+ HCY^- medium for 1 day, in turn followed by drug-free MET^+ HCY^- medium. These results demonstrate the potential for treatment of solid tumors with chemotherapy based on the methionine dependence of cancer cells compared to normal cells and the S/G_2 cell cycle block selectively induced in cancer cells under methionine deprivation [22].

6. Efficacy of methionine-depleted diets on cancers *in vivo*

The first suggestion that tumors may require elevated amounts of methionine was obtained in rat models in which specific amino acids could be added or withheld [23]. Yoshida sarcoma-bearing nude mice were deprived of dietary methionine, which caused the tumor to eventually regress. The antitumor effect of methionine depletion resulted in the extended survival of the tumor-bearing mice. The mice on the methionine-deprived diets maintained their body weight for the time period studied, indicating that tumor regression was not a function of body weight loss [24].

Replacement of methionine with homocysteine in the diet fed to rats with a rhabdomyosarcoma resulted in a significant decrease of pulmonary metastases [25].

Methionine depletion modulated the efficacy of cisplatinum against the MX-1 human breast carcinoma cell line when grown in nude mice. The tumor-bearing nude mice were fed a methionine-free diet and subsequently treated with cisplatinum. The MX-1 tumor was relatively resistant to both methionine starvation and cisplatinum alone but was very sensitive to the combination of methionine starvation and cisplatinum [26].

The combination of ethionine, a methionine analog, and HCY slowed the proliferation of prostate cancer cells *in vitro* and *in vivo*, decreased ATP synthesis and caused cell cycle arrest in S/G_2 and apoptosis [27].

In gastric-cancer-bearing rats, the average survival time was 18.6 days in methionine-containing total parenteral nutrition (TPN)-treated mice, 31 days in methionine-deprived TPN-treated mice, 27.5 days in methionine-containing TPN+5-FU-treated mice, and 43 days in methionine-deprived TPN+5-FU-treated mice ($p < 0.05$) [28].

7. Development of methioninase for cancer treatment

L-methioninase, isolated from *Clostridium sporogenes*, inhibited the Walker 256 carcinosarcoma of the rat. Compared with a methionine-free diet, this enzyme had greater growth-inhibiting activity and did not cause weight loss of the host. A single intravenous (i.v.) injection of L-methioninase depleted the plasma levels of methionine to below 8% of the control [29].

Plasma methionine was lowered to a steady state of <5 µM in mice with a combination of dietary restriction of methionine, homocysteine, and choline and synchronous treatments with L-methioninase and homocystine, each administered at 12-h intervals. No weight loss or toxicity in the liver or pancreas occurred. Human medulloblastoma tumor growth was inhibited. This regimen of methionine deprivation blocked the cell cycle in G_2 and induced apoptosis. Tumor growth arrest was achieved in 100% of treated animals within 4 days of treatment, and regression was seen in one-third of animals after a 10-day period [30].

Bone marrow cells from patients with leukemia were found more sensitive to methioninase than bone marrow from patients with nonmalignant diseases. Depletion of L-methionine by methioninase was similar to MET^-HCY^+ medium. After 24 h in MET^-HCY^+ medium, leukemic cells showed a lower DNA synthesis. There was also an elevation of methionine adenosyltransferase, tRNA methyltransferase and *S*-adenosylmethionine decarboxylase [31].

The methioninase gene from *Pseudomonas putida* was cloned in *E. coli* using the polymerase chain reaction. The methioninase (METase) gene was then ligated into the pT7-7-overexpression plasmid containing the T7 RNA polymerase promoter and re-cloned in *E. coli* strain BL21(DE3). A scaled-up production protocol of recombinant methioninase (rMETase), which contained a heat step, two DEAE Sepharose fast flow (FF) ion-exchange columns, and one ActiClean Etox endotoxin-affinity chromatography column was established. The resulting pAC-1 clone produced rMETase at ~ 10% of the total soluble protein and up to 1 g/liter in shake-flask culture. The half-life of rMETase was 2 h, when rMETase was administered by i.v. injection in mice [32].

In order to prevent immunological reactions that might be produced by multiple dosing of rMETase and to prolong the serum half-life of rMETase, the *N*-hydroxysuccinimidyl ester of methoxypolyethylene glycol propionic acid (M-SPA-PEG 5000) was coupled to rMETase. The PEGylated molecules (PEG-rMETase) were purified from unreacted PEG with

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Amicon 30 K centriprep concentrators or by Sephacryl S-300 HR gel-filtration chromatography. Unreacted rMETase was removed by DEAE Sepharose FF anion-exchange chromatography. The resulting PEG-rMETase subunit, from a PEG/rMETase ratio of 30/1 in the synthetic reaction, had a molecular mass of ~ 53 kda determined by matrix-assisted laser desorption/ionization mass spectrometry. These results indicated the conjugation of two PEG molecules per subunit of rMETase and eight per tetramer. PEG-rMETase molecules obtained from reacting ratios of PEG/rMETase of 30/1 had enzyme activities of 70% of unmodified rMETase. PEGylation of rMETase increased the serum half-life of the enzyme in rats to ~ 160 min compared to 80 min for unmodified rMETase. PEG-rMETase could deplete serum methionine levels to < 0.1 µM for ~ 8 h compared to 2 h for rMETase in rats [33].

Mini-osmotic pumps containing various concentrations of pyridoxal-5'-phospate (PLP) were implanted in BALB-C mice. PLP-infused mice were then injected with a single dose of 4000 or 8000 units/kg PEG-rMETase. Pumps containing 500 mg/ml PLP increased the half-life of PEG-rMETase holoenzyme 4.5-fold from 1.5 to 7 h. Thus, PLP infusion could extend the period of MET depletion by PEG-rMETase by ~ 10-fold in a dose-dependent manner. The extended time of MET depletion by PEG-rMETase was due to the maintenance of active PEG-rMETase holoenzyme by infused PLP. The infused PLP either bound to apo-PEG-rMETase and/or inhibited dissociation of PLP from holo-PEG-rMETase, thereby maintaining the holoenzyme form of PEG-rMETase *in vivo* [34].

8. Recombinant methioninase (rMETase) combination chemotherapy *in vivo*

rMETase alone arrested growth of HCT15 and HT29 colon tumors in nude mice for 1 week after treatment termination. Colo 205 and SW 620 colon tumors were partially arrested by rMETase. Cisplatinum (CDDP) in combination with rMETase resulted in tumor regression of Colo 205 and growth arrest of SW 620 in nude mice. The combination treatment resulted in two of the six animals having no detectable tumor. SW620 was resistant to CDDP alone and only partially sensitive to rMETase alone. However, when SW 620 was treated with rMETase from days -5 to -9 and CDDP on day -5, tumor growth was arrested [35].

The combination treatment of the Lewis lung carcinoma with rMETase and 5-FU resulted in a dose-dependent enhanced antitumor efficacy for survival and tumor growth inhibition. Thus, methionine depletion by rMETase potentiates the antitumor efficacy of 5-FU [36].

The growth of Daoy, SWB77, and D-54 brain tumors in athymic mice was arrested after the depletion of mouse plasma methionine with a combination of a methionine-and choline-free diet and rMETase. Toxic effects of methionine depletion were prevented with daily intraperitoneal homocysteine. This regimen depleted plasma methionine to below 5 µM. MET depletion for 10 – 12 days induced cell-cycle arrest, apoptosis and necrosis in tumors but did not prevent tumor regrowth after cessation of the treatment. *N*,*N'*-bis (2-chloroethyl)-*N*-nitrosourea (BCNU), which was not effective as a single therapy in any of the tumors, was given at the end of the methionine depletion regimen and up to a > 80-day tumor growth delay was observed (**Figure 2**). Methionine-depleting regimens also tripled the efficacy of temozolomide (TMZ) when TMZ was administered after a 10-12 day period of MET depletion [37].

rMETase treatment may be more effective against high-grade gliomas such as U87, which are resistant to chemotherapy, than against more differentiated astrocytic tumors [38].

Microtubule depolymerization agents such as vincristine, vinorelbine, vinblastine, and mebendazole were synergistic in combination with rMETase. Cell cycle analysis showed that rMETase arrested cancer cells in G_2 and not M phase. Synergism between rMETase and DNA damaging agents was also observed [39,40].

Reengineering the pyridoxal phosphate-dependent human enzyme cystathionine-γ-lyase (hCGL) resulted in a human methionine-degrading enzyme. Three amino acid substitutions of hCGL resulted in an engineered enzyme that degraded methionine with a long serum half-life (hCGL-NLU). Treatment of neuroblastoma mouse xenografts with PEGylated hCGL-NLV resulted in tumor growth delay similar to *P. putida* rMETase (**Figure 3**) [41].

9. Safety and toxicity of rMETase and polyethylene glycol (PEG) rMETase in monkeys

A 4000 units/kg dose of rMETase reduced plasma methionine to an undetectable level (< 0.5 µM) by 30 min that was stable for 8 h in macaque monkeys. rMETase was eliminated with a $T_{½}$ of 2.49 h. A 2-week i.v. administration of 4000 units/kg every 8 h/day for 2 weeks resulted in plasma methionine of < 2 µM. There was decreased food intake and slight weight loss. Re-challenge on day 28 resulted in anaphylactic shock and death in one animal. Subsequent pretreatment with hydrocortisone prevented the anaphylactic reaction, although vomiting was frequently observed. Re-challenge was carried out at days 66, 86, and 116. Anti-rMETase antibodies at 10^{-3} were found after the first challenge, and these increased to 10^{-6} after the fourth challenge and decreased to 10^{-2} by 2 months post therapy. The main rMETase antibody was IgG, and although it had some *in vitro* features of being a neutralizing antibody, each challenge dose was effective in depleting plasma methionine levels. Thus, rMETase was able to effectively deplete plasma methionine levels with minimal toxicity in a primate model [42].

A single dose of 4000 units/kg of PEG-rMETase to macaque monkeys reduced plasma methionine to < 5 µM

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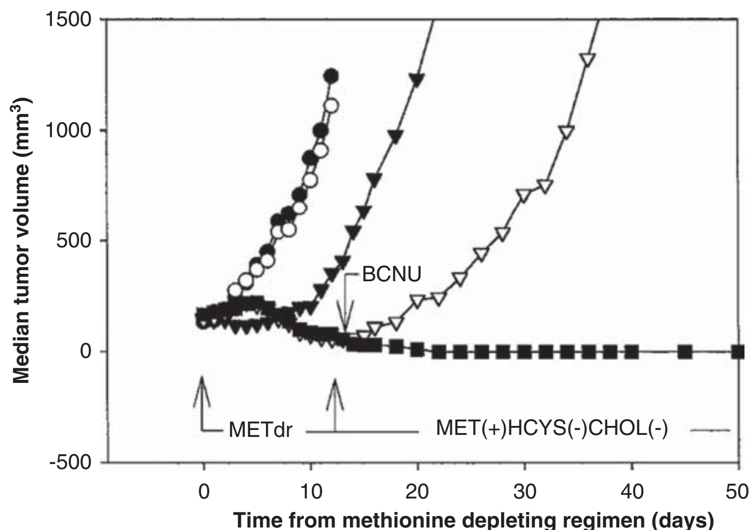

Figure 2. Efficacy of 12-day methionine depletion regimen (METdr) and subsequent BCNU treatment on the growth of the human (Daoy) medulloblastoma xenograft implanted subcutaneously in athymic mice. The animals were fed a MET(-)HCY(-)CHOL(-) diet and treated with 1500 units/kg rMETase i.p. every 8 h and 20 mg/kg HCY i.p. every 24 h. This treatment was initiated when tumors reached a volume of 120 – 160 mm^3. ○, untreated controls fed a MET(+)HCY(-)CHOL(-) diet; ●, mice given BCNU (35 mg/m^2) with a MET(+)HCY(-)CHOL(-) diet throughout; ▼, mice given BCNU (65 mg/m^2) with a MET(+)HCY(-)CHOL(-) diet throughout; ▽, mice on a 12-day MET depletion regimen followed by a MET(+)HCY(-)CHOL(-) diet; ■, animals on a MET depletion regimen followed by a MET(+)HCY(-)CHOL(-) diet + a single treatment with BCNU (35 mg/m^2) administered 8 h after termination of MET depletion [37].

BCNU: N,N'-bis(2-chloroethyl)-N-nitrosourea; CHOL: Choline.

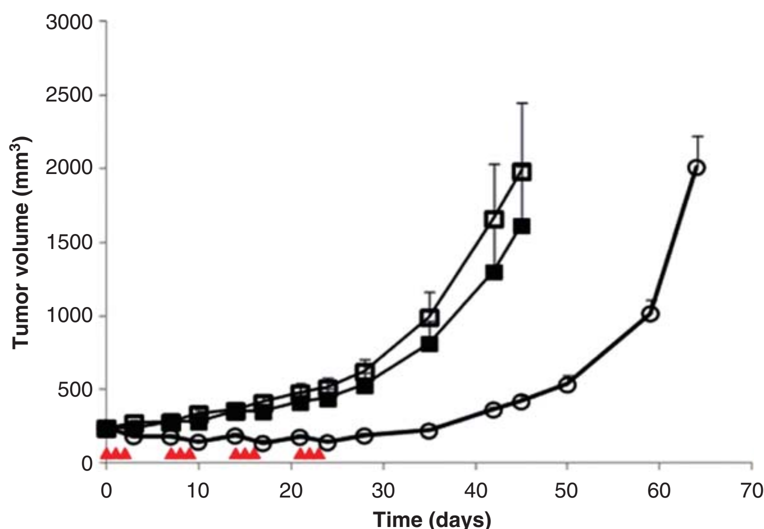

Figure 3. Efficacy of PEGylated-hCGL-NLV (methioninase) in athymic mice with neuroblastoma xenografts. (□) Control mice on a normal diet (n = 10); (■) mice on a MET(-)HCY(-)CHOL(-) diet (n = 10); (○) mice treated with 100 U PEG-hCGL-NLV in combination with a MET(-)HCY(-)CHOL(-) diet (n = 10). Treatment days are designated by (▲). *$p < 0.01$ for the PEG-hCGL-NLV treated mice relative to untreated controls [41].

hCGL: Human enzyme cystathionine-γ-lyase.

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for 12 h. PEG-rMETase holoenzyme activity was eliminated with a half-life of 1.3 h. The PEG-rMETase apoenzyme was eliminated with a half-life of 90 h, a ~ 36-fold increase compared with non-PEGylated rMETase. A single dose at 2000 units/kg of PEG-rMETase resulted in an apoenzyme half-life of 143 h. A 7-day i.v. administration of 4000 units/kg every 12 h resulted in a steady-state depletion of plasma methionine to < 5 µM. The only toxicity was decreased food intake and slight weight loss. Red-cell values and hemoglobin declined transiently during treatment but recovered after cessation of treatment. Subsequent challenges on days 29, 50 and, 71 did not result in any immunologic reactions. Although anti-PEG-rMETase antibodies were produced, no neutralizing antibody was identified, and each challenge dose was effective in depleting plasma methionine levels. The results demonstrate that PEGylation greatly prolongs serum half-life of the rMETase apoenzyme and eliminated anaphylactic reactions [43].

10. Clinical studies of methionine-depleted diets

Advanced gastric cancer patients were given a methionine-depleting TPN for 7 days before surgery with continuous intravenous infusion of 5-FU. The other patients received methionine-containing TPN with 5-FU. All patients underwent subsequent gastrectomy. Resected tumors in the methionine-depleted patients were decreased in size, whereas almost no effect was seen in the control group with methionine-containing TPN [44].

A Phase I clinical trial showed that dietary methionine restriction was nontoxic for the treatment of patients with advanced cancer. One patient with hormone-independent prostate cancer had a 25% reduction in prostate-specific antigen after 12 weeks on the methionine-restricted diet, and a second patient with renal cell cancer had an objective radiographic response [45].

A Phase I clinical trial of dietary MET restriction in combination with chloroethyl-nitrosourea (cystemustine) for patients with recurrent glioma or metastatic melanoma was carried out. A total of 10 patients received a methionine-free diet and cystemustine. There was good tolerance of the methionine-free diet and cystemustine treatment [46].

5-FU, leucovorin, and oxaliplatin (FOLFOX) in combination with dietary methionine restriction was evaluated with patients with metastatic colorectal cancer. Of the 4 evaluated patients, 2 experienced a partial response and 1 had disease stabilization [47,48].

11. Pilot Phase I trial of METase and rMETase

In a pilot Phase I clinical trial of methioninase from *P. putida*, no acute clinical toxicity was observed for all criteria measured in three breast cancer patients. The depletion of serum methionine started within 30 min of the infusion and was maintained for 4 h 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 10 h i.v. infusion of 20,000 units of methioninase without any signs of side effects. Patient-3 maintained serum levels of methioninase at 50% of the maximum level for a subsequent 6 h after infusion. Methionine was depleted from 23.1 to 0.1 µM by the 10-h infusion of patient-3 [49].

A pilot Phase I clinical trial was carried out to determine rMETase toxicity. Patients with advanced breast cancer, lung cancer, renal cancer and lymphoma were given a single rMETase treatment at doses ranging from 5000 to 20,000 units by i. v. infusion over 6 – 24 h. No clinical toxicity was observed in any patient after rMETase treatment. rMETase levels reached 0.1 – 0.4 units per ml of serum in the patients which correspond to therapeutic levels *in vitro*. Serum methionine was depleted to as low as 0.1% of the pretreatment levels to ~ 0.1 µM [50].

12. Conclusion

Methionine dependence may be the only known general and very widespread metabolic defect in cancer. Twenty-one different human tumor cell lines (4 lung, 4 colon, 4 kidney, 4 melanoma, 3 CNS, and 2 prostate) and normal cell strains were treated with rMETase *in vitro*. rMETase had a mean IC_{50} for the cancer cells, which was one order of magnitude lower than that for normal cell strains. PEG-rMETase also had high cancer cell-killing activity [51].

Methionine-depletion is a powerful tool for broad-based cancer treatment that is best carried out in a combination of rMETase and cytotoxic chemotherapy.

A major impediment to the response of tumors to chemotherapy is that the large majority of cancer cells in a tumor are quiescent in G_0/G_1, where cancer cells are resistant to chemotherapy [52]. To attempt to solve this problem of quiescent cells in a tumor, cancer cells were treated with rMETase, which selectively blocks cancer cells in S/G_2. The cell cycle phase of the cancer cells was visualized with the fluorescence ubiquitination-based cell cycle indicator (FUCCI). The extent and time course of becoming trapped in S/G_2 is a facile and quantitative means to determine the degree of methionine dependence of any cancer cell type cells [53].

At the time of rMETase-induced S/G_2-phase blockage, identified by FUCCI imaging, the cancer cells were administered S/G_2-dependent chemotherapy drugs, which block DNA synthesis including doxorubicin, cisplatin, or 5-fluorouracil. Treatment of cancer cells with drugs only led to the majority of the cancer-cell population being blocked in G_0/G_1 phase. The G_0/G_1 blocked cells were resistant to the chemotherapy. In contrast, blockage of cancer cells in S/G_2 phase by rMETase treatment followed by chemotherapy was highly effective in killing the cancer cells [53].

The basis of synergy between methionine depletion and chemotherapy is the S/G_2 trap. A limitation of this approach

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would be dormant cells in a tumor which do not cycle and therefore would not be trapped in S/G_2.

S/G_2 phase blockage of cancer cells by rMETase appears to be a more effective methionine depletion strategy. The S/G_2-phase blocked cancer cells become highly sensitive to cytotoxic chemotherapy. A PEGylated rMETase appears most feasible to use, since long-term treatment could be required to induce the S/G_2 block, which is then followed by chemotherapy.

Methionine metabolism is also related to polyamine and glutathione metabolism. For a recent review, please see [54].

13. Expert opinion

13.1 Key findings and weaknesses

The most important finding over the past 40+ years is that perhaps all cancer cell types are methionine dependent, that is, they exhibit an elevated requirement for methionine compared to any normal cells. Even more exciting is the possibility that every cancer is methionine dependent. Methionine dependence is the only known metabolic defect that may be general across all cancer types.

Another important finding is that different cancer cell types arrest in the S/G_2 phase of the cell cycle when deprived of methionine. This is the most sensitive phase of the cell cycle for most currently-used chemotherapy drugs. The S/G_2 phase trap of cancer cells induced by methionine depletion has been shown to sensitize the cancer cells to chemotherapy [17,22,53].

Another important development is recombinant methioninase (rMETase) for the treatment of cancer. *In vivo* studies with rMETase have shown efficacy and higher efficacy in combination with chemotherapy drugs.

Proof-of-principle of the efficacy of methionine depletion has been demonstrated in cancer patients on a methionine-depleted diet, which the patients tolerated and which, in some cases, arrested the progression of their cancer. It is expected that clinical treatment with rMETase will result in S/G_2 trapping of the cancer and subsequent highly-effective chemotherapy.

A weak point is the paucity of clinical data on rMETase. It is imperative that rMETase be rapidly-developed clinically through the approval process, as it has great potential as a widely-used chemotherapy agent. Another weak point is that many, if not most, tumors contain dormant cancer cells. If these cancer cells do not cycle during the treatment period with rMETase, they cannot be trapped in late S/G_2 for subsequent eradication with chemotherapy. Dormant cells within tumors could remain and possibly regrow later, resulting in possible metastasis. It is, therefore, imperative that a means be developed to decoy quiescent and/or dormant cancer cells in a tumor to cycle and, thereby, be potentially-trapped by rMETase-mediated methionine depletion. Initial studies have demonstrated that quiescent/dormant cancer cells can be decoyed to cycle by treatment with an adenovirus or tumor-targeting *Salmonella typhimurium* A1-R [52,55].

13.2 Potential of the research and ultimate goal

The major goal is to develop S/G_2-trap cancer chemotherapy using rMETase as the trapping agent. In order to achieve this goal, rMETase must be developed clinically. rMETase is potentially antigenic and, therefore, PEG conjugation of the protein would decrease antigenicity and increase circulating half-life [34,42,43,56]. The clinical development of rMETase would need to go through Phase I, Phase II, and possibly Phase III trials, which will still take some years to finalize. In addition, the optimization of combination chemotherapy with rMETase to take advantage of the S/G_2 trap would also require clinical trials.

13.3 What is the biggest challenge to achieve this goal

The biggest challenge is the clinical development of rMETase. The next challenge is optimizing S/G_2-trap chemotherapy, especially the ability to decoy quiescent cancer cells to cycle.

13.4 Future prospects

A new paradigm of chemotherapy using the combination of rMETase and chemotherapy for broad-based treatment for most types of cancer is possible. After 40 years, it is hoped that this “promised land” can now be entered.

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Declaration of interest

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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