ISSN 0006-2979, Biochemistry (Moscow), 2023, Vol. 88, No. 7, pp. 944-952 © The Author(s) 2023. This article is an open access publication.
Published in Russian in Biokhimiya, 2023, Vol. 88, No. 7, pp. 1162-1172.

REVIEW

Targeting Methionine Addiction of Cancer Cells with Methioninase

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Received February 11, 2023
Revised March 12, 2023
Accepted March 13, 2023

Abstract—All types of cancer cells are addicted to methionine, which is known as the Hoffman effect. Restricting methionine inhibits the growth and proliferation of all tested types of cancer cells, leaving normal cells unaffected. Targeting methionine addiction with methioninase (METase), either alone or in combination with common cancer chemotherapy drugs, has been shown as an effective and safe therapy in various types of cancer cells and animal cancer models. About six years ago, recombinant METase (rMETase) was found to be able to be taken orally as a supplement, resulting in anecdotal positive results in patients with advanced cancer. Currently, there are 8 published clinical studies on METase, including two from the 1990s and six more recent ones. This review focuses on the results of clinical studies on METase-mediated methionine restriction, in particular, on the dosage of oral rMETase taken alone as a supplement or in combination with common chemotherapeutic agents in patients with advanced cancer.

DOI: 10.1134/S0006297923070076

Keywords: methionine addiction, Hoffman effect, methionine γ-lyase, methionine-degrading enzyme, oral methioninase, clinical study, methionine restriction

INTRODUCTION

Based on more than 60 years of research on the metabolism of cancer cells, it has been observed that a lack of certain amino acids can significantly inhibit the growth and proliferation of cancer cells vs. normal cells [1]. Sugimura et al. were the first to observe that removal of methionine (L-Met) from the diet of tumor-bearing rats reduced tumor growth to a greater extent than removal of other amino acids [1]. Fifty years ago, it was discovered that cancers are addicted to L-Met [2] – the Hoffman effect [3], which is due to a high demand of cancer cells for transmethylation reactions [2, 4-8].

L-Met restriction can be an important strategy in the treatment of cancer cells that need L-Met for their growth and survival, while in normal cells, the deficit of L-Met produces no effect as long as L-homocysteine (L-Hcy) is present [2, 4, 9, 10]. Several *in vivo* studies using L-Met-restricted diets have reported inhibition of tumor growth in animals and humans [11, 12].

Vegan diets, some of which are low in L-Met, can be an effective diet strategy to prevent cancer growth in humans [11]; however, low L-Met might be insufficient

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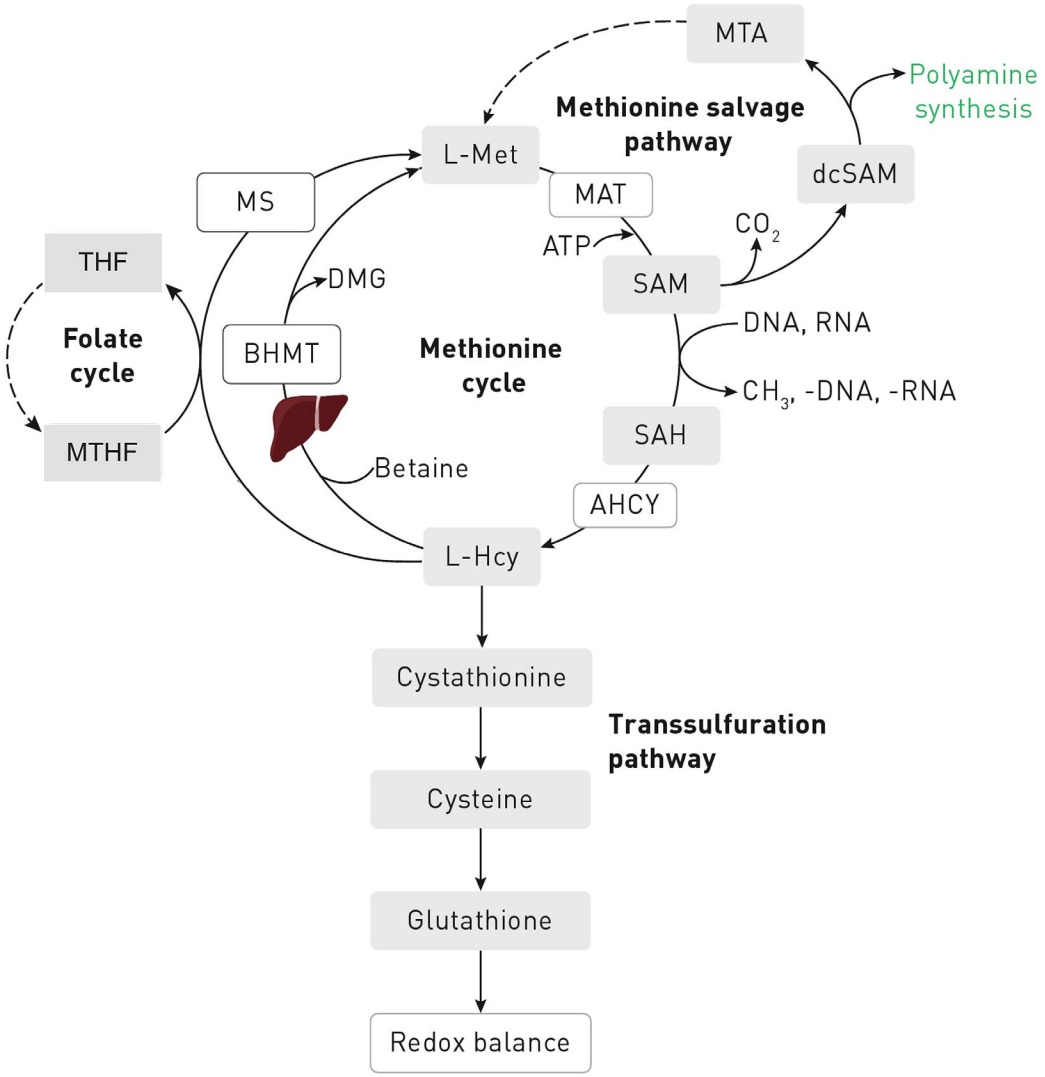
to effectively lower the content of L-Met in the body to regress or eradicate cancer. Another effective strategy for preventing cancer growth may be the use of methioninase (METase; methionine γ-lyase, EC 4.4.1.11), an enzyme that reduces the amount of L-Met in the bloodstream [13]. L-Met restriction in combination with chemotherapy to selective eradicate cancer cells in the presence of normal cells was first demonstrated almost 40 years ago [14]. The use of METase in combination with chemotherapy agents is currently tested in several clinical studies [15-20].

The present review focuses on the efficacy of METase, in particular, oral recombinant METase (o-METase) (alone as a supplement or in combination with chemotherapeutic agents) in the treatment of patients with late-stage cancer, including prostate cancer, ovarian cancer, pancreatic cancer, rectal cancer, and invasive lobular breast cancer.

METHIONINE METABOLISM

L-Met is one of the common sulfur-containing amino acids essential for cell viability and growth. The metabolism of L-Met involves three distinct pathways: (i) the L-Met cycle, which produces S-adenosylmethionine (SAM) [21]; (ii) the L-Met salvage pathway, which recycles L-Met from the byproducts of polyamine synthesis (S-methyl-5'-thioadenosine) [3]; and (iii) the transsulfuration pathway, which produces the antioxidant glutathione (figure) [3]. Additionally, since folate metabolism and the L-Met cycle are closely linked, both of them can indirectly affect nucleotide biosynthesis. Folates are necessary for L-Met synthesis (figure), and not surprisingly, cancers are addicted to folate [10].

As the first step of L-Met metabolism, methionine adenosyltransferase (MAT; EC 2.5.1.6), in the



Metabolism of L-Met. Abbreviations: SAM, S-adenosylmethionine; MAT, methionine adenosyltransferase; SAH, S-adenosylhomocysteine; AHCY, adenosylhomocysteinase; MS, methionine synthase; BHMT, betaine-homocysteine S-methyltransferase; dcSAM, decarboxylated SAM; MTA, methylthioadenosine; DMG, dimethylglycine; 5-MTHF, 5-methyltetrahydrofolate; THF, tetrahydrofolate

presence of ATP, catalyzes L-Met conversion to SAM, which serves as a cofactor in the majority of methylation reactions and as a principal methyl donor for DNA, RNA, and chromatin and other methylation reactions [3, 9, 22]. SAM is converted to S-adenosylhomocysteine (SAH) through transmethylation reactions. SAH is converted by adenosylhomocysteinase (AHCY; EC 3.13.2.1) into L-Hcy, which can be remethylated back into L-Met by methionine synthase (MS; EC 2.1.1.13) in the presence of N^5-methyltetrahydrofolate (N^5-MTHF) and cobalamin to complete the methionine cycle [3].

Under normal conditions, MS remethylates approximately 50% of L-Hcy into L-Met in all tissues. The activity of MS is not only necessary to remethylate L-Hcy, but also to maintain the cellular levels of reduced tetrahydrofolate, an essential component of one-carbon metabolism [3, 23]. L-Hcy can also be remethylated using betaine, produced from choline, by betaine-homocysteine S-methyltransferase (BHMT; EC 2.1.1.5), which is expressed predominantly in the liver and kidney and does not depend on folates [3].

Alternatively, cystathionine β-synthase (EC 4.2.1.22) can convert L-Hcy to cystathionine in the transsulfuration pathway, which is then transformed to cysteine by cystathionase (EC 4.4.1.1) to be used in glutathione synthesis and redox balance maintenance [3].

Low L-Met levels cause a drop in the SAM content with the following consequences: (i) inhibition of BHMT and methylenetetrahydrofolate reductase (MTHFR) and prevention of activation of cystathionine β-synthase to maintain the flow through the L-Met cycle [3]; (ii) accumulation of 5-MTHF, which inhibits glycine N-methyltransferase and directs SAM utilization towards DNA methyltransferases [3]; and (iii) preferential upregulation of *MAT* expression, suggesting that SAM production is controlled to maintain its cellular level [3].

The methionine-salvage pathway also replenishes L-Met levels [3]. In the methionine-salvage pathway, SAM is decarboxylated and utilized in the synthesis of polyamines [3]. Methylthioadenosine (MTA) is converted to methylthioribose and ultimately to methylthiooxobutyrate (MTOB) via multiple enzymatic steps leading to L-Met synthesis.

This coordinated network supports methylation reactions, protein and polyamine synthesis, and other methylation-related processes required for cell growth and survival on a low-L-Met diet.

METHIONINE ADDICTION AS A VULNERABILITY OF CANCER CELL METABOLISM

Addiction to L-Met is a ubiquitous and fundamental hallmark of cancer cells [24, 25]. It was first described as methionine dependence of tumors in rats and later as inability of cancer cells to survive and grow in a culture medium, in which L-Met was replaced with its immediate precursor L-Hcy [2, 8, 26, 27].

Normal cells and cancer cells produce L-Met via resynthesis from L-Hcy via MS [2]; however, cancer cells have a higher demand for MS activity because they extensively use L-Met for transmethylation reactions and thus are more vulnerable to L-Met restriction [5, 27, 28].

So far, all studied normal cell lines have been shown to be L-Met-independent; they grow in $Met^{-}Hcy^{+}$ media almost as well as in $Met^{+}Hcy^{-}$ media by synthesizing L-Met from L-Hcy [24, 25]. Out of 23 cell lines derived from various types of human malignancies, 11 cannot grow in $Met^{-}Hcy^{+}$ media, being completely L-Met-dependent, and three cell lines grow minimally in this medium [24]. All tested cancer cells have been found to be addicted to L-Met based on their very high sensitivity to METase compared to normal cells [25]. According to these findings, L-Met dependency is typical for human cancer cells, suggesting that it might be a significant factor in their oncogenic transformation, as well as a possible therapeutic target.

Clinical positron emission tomography (PET) using [^{11}C] L-Met (METPET) was more sensitive and accurate than PET with [^{18}F] deoxyglucose, providing additional evidence that L-Met addiction affects all types of cancer [7, 29]. These findings suggest that the difference between cancer and normal cells in their requirements for L-Met is more pronounced than the difference in their requirements for glucose.

L-Met addiction of cancer cells is due to the excess and altered transmethylation reactions [26-28], which may cause DNA hypomethylation [22] leading to aneuploidy [30] and the overmethylation of histone H3 lysine residues [26-28].

The growth arrest of L-Met-dependent cancer cells in $Met^{-}Hcy^{+}$ medium was accompanied by the reduction in the percentage of mitotic cells in the cell population [31]. In contrast to the accumulation of cells in the G_1 phase occurring in the cultures of normal fibroblasts at a very high density in an L-Met-supplemented medium, L-Met restriction selectively arrested cancer cells in the S/G_2-phase and eliminated clonogenic cells (equivalents of tumor-initiating cells) and rendered cancer cells sensitive to cell-cycle-specific drugs [14, 28, 31].

METHIONINE RESTRICTION

Cancer cells have a high demand for L-Met because of oncogenic reprogramming [26, 27] leading to excess transmethylation in cancer [5, 26, 27, 28]. When this increased demand for L-Met is combined with a limited availability of L-Met, selective proliferation arrest of the cancer cells reveals a metabolic vulnerability

termed "methionine addiction"[2, 28], which is a general hallmark of cancer cells. Targeting L-Met addiction with a low-methionine diet has shown promise in cancer patients [11]. The use of a methionine-degrading enzyme alone or in combination with a low-methionine diet and chemotherapy agents to deplete L-Met in the body is a promising new paradigm for cancer treatment [32, 33].

METase is a bacterial pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes γ-elimination of L-Met with the generation of α-ketobutyric acid, methyl mercaptan, and ammonia [34]. METase was originally used to treat cancer in 1973 by Kreis et al., who found that METase isolated from *Clostridium sporogenes* inhibited the growth of Walker 256 carcinosarcoma in rats [35]. METase suppressed cancer growth more efficiently than a methionine-free diet and did not induce weight loss in the animals [35]. A single intravenous (i.v.) dose of METase decreased L-Met plasma level to less than 8% of the control value [35]. Since then, the METase gene from *Pseudomonas putida* was cloned and expressed in *Escherichia coli* cells [13], and the recombinant enzyme (rMETase) has been shown to be effective against all major types of cancer *in vitro* and *in vivo* [36-48]. PEGylation of rMETase allowed an increase in its half-life in the blood circulation and minimized the host immune response *in vivo* [49].

The only adverse effect of intravenous injection of PEGylated rMETase in macaque monkeys was transient anemia [49]. After i.v. injection, PLP quickly dissociated from rMETase, causing a decline in the enzyme activity [49]. The issues of anaphylaxis and PLP dissociation from rMETase were successfully resolved when it was found that rMETase could be efficiently administered orally (o-rMETase), since it can act in the gastrointestinal tract without entering the circulation. O-rMETase has been very effective against refractory sarcoma, pancreatic cancer, colon cancer, and melanoma in patient-derived orthotopic xenograft (PDOX) mouse models [39, 41, 42, 44].

CLINICAL CASE REPORTS

Early clinical trials of dietary methionine restriction, with or without chemotherapy, showed promising results in some advanced cancer patients [11, 22]. From the 1990s until the present, pilot Phase I clinical studies have been conducted to assess the efficacy of L-Met depletion, as well as toxicity, pharmacokinetics, and maximum tolerated doses of METase, rMETase [33, 50], and recently, o-rMETase [15-20].

Phase I pilot clinical trial with purified non-recombinant METase from *P. putida* showed no acute clinical toxicity or side effects of the enzyme administration at a dose of less than 20,000 units via a single i.v. infusion over 10 h [50]. In patient 1 (5000 units) and patient 2 (10,000 units) with advanced breast cancer, L-Met depletion in the serum was observed within 30 min after the start of the treatment and lasted for another 4 h after infusion had been completed. By the end of the therapy course, the content of L-Met in the serum of patients 1, 2, and 3 (20,000 units over 10 h via i.v. infusion) was reduced by 19, 35, and 50%, respectively [50]. The results of this study suggested a correlation between the decline in the L-Met content and METase dose or infusion time.

In another Phase I pilot clinical study, rMETase cloned from *P. putida* and expressed in *E. coli* cells was used to deplete serum L-Met in cancer patients. Nine patients with advanced-stage lung cancer, breast cancer, lymphoma, and kidney cancer were given a single i.v. infusion of 5000 to 20,000 units of rMETase over 6 to 24 h (table). None of the patients demonstrated signs of clinical toxicity of rMETase. Depletion of serum L-Met was observed within 1 h after the start of the treatment, reached the maximum level within 2 h, and was maintained during the course of treatment and for another 2 h after infusion had been completed. Within 2 h of the infusion, the content of L-Met in the patients was as low as 0.1 μM (300-fold depletion} without noticeable toxic effects [33]. However, the authors of the study did not assess the antitumor activity of rMETase. According to these findings, rMETase is safe for intravenous administration and efficiently depletes serum L-Met (its biochemical target), indicating its potential effectiveness in clinical studies.

The oral form of rMETase (o-rMETase) that can be taken as a supplement alone or in combination with chemotherapy agents was developed and then tested in eight patients with advanced solid cancers, including prostate cancer, ovarian cancer, pancreatic cancer, rectal cancer, and invasive lobular breast cancer [15-20].

In the early clinical studies conducted by Han et al. [18-20], the blood levels of L-Met in a 67-year-old female with late-stage ovarian cancer decreased by 50% within 4 h after taking 250 units of o-rMETase. Also, four adult patients with advanced prostate cancer were administered 500 units of o-rMETase daily as two oral doses of 250 units each for varying time periods. In patient 1, the level of prostate-specific antigen (PSA) decreased by 70% (from over 2000 ng/ml at the beginning of therapy to approximately 600 ng/ml) over three months of combined administration of o-rMETase and oral dexamethasone (table), both of which may have contributed to the reduction of PSA level. After 4 weeks of o-rMETase administration, patients 2 and 3 demonstrated 38 and 20% reduction in the PSA level, respectively, which then stabilized. Patient 2 fasted periodically and was on a vegan low-L-Met diet, which may have also affected his L-Met and PSA levels. In patient 2, the levels of L-Met dropped by 42.7% over a period of 12 days of o-rMETase administration. Six weeks

METase clinical studies

Disease (status)	METase formulation	Number of patients	Treatment dose	Combination with other chemotherapy drugs	Monitoring of disease progression	References
Advanced breast cancer	non-recombinant METase	3	from 5000 to 20,000 units METase *via* single i.v. infusion over 4 or 10 h	–	–	[50]
Late-stage lymphoma, and lung, breast, and kidney cancers	recombinant METase	9	from 5000 to 20,000 units rMETase over 6 to 24 h *via* single i.v. infusion	–	–	[33]
Advanced ovarian and prostate cancers	oral rMRTase	2	500 units of o-rMETase daily divided into two oral doses of 250 units for 1 month for a patient with ovarian cancer or for 3 months for a patient with prostate cancer	one patient was treated with oral dexamethasone and received a low-Met diet	PSA level decreased; hemoglobin increased	[20]
Advanced prostate cancer	oral rMETase	2	500 units of o-rMETase daily divided into two oral doses of 250 units for 1 month	one patient was on a low-L-Met diet	PSA level decreased	[19]
Advanced prostate cancer	oral rMETase	1	500 units of o-rMETase daily divided into two oral doses of 250 units	–	PSA level stable	[18]
Stage IV pancreatic cancer	oral rMETase	1	500 units of o-rMETase daily divided into two oral doses of 250 units for 19 months	FOLFIRINOX or FOLFIRI every two weeks and a low L-Met diet	CA19-9 level decreased; tumor volume did not change as measured by computed tomography	[16]
Rectal cancer	oral rMETase	1	500 units of o-rMETase daily divided into two oral doses of 250 units for 18 months	low-L-Met diet	CEA level decreased; tumor volume did not change as measured by computed tomography	[15]
Invasive lobular carcinoma (breast cancer)	oral rMETase	1	1000 units of o-rMETase daily divided into four oral doses every 6 h for 6 months	low-L-Met diet, doxorubicin and cyclophosphamide for the first 3 months followed by docetaxel for the next 3 months and a low L-Met diet	CEA level decreased; axillary lymph node metastases were measured by computed tomography and METPET	[17]

prior to beginning therapy, the PSA level in patient 4 increased sharply from 38 to 56 ng/ml, but then stabilized at 62 ng/ml by the fifteenth week after administration of o-rMETase. No clinical toxicity or side effects were observed in any patient with advanced prostate cancer after treatment with o-rMETase alone or in combination with a vegan low-L-Met diet. These results suggest that altering the diet to include food low in L-Met, as well as changing the dosage of o-rMETase, may help to further reduce the content of L-Met and PSA in the blood, thereby preventing the development of prostate cancer.

According to statistical data, only 5% of stage IV pancreatic cancer patients taking FOLFIRINOX demonstrate disease stabilization within 18 months after diagnosis [16]. In a clinical study, L-Met restriction with o-rMETase taken twice a day as a supplement and a low-L-Met diet showed apparent synergistic efficacy in combination with FOLFIRINOX or FOLFIRI administered every two weeks. Progression of the disease was monitored by measuring the level of cancer antigen 19-9 (CA19-9) in the bloodstream and computed tomography (table). The patient demonstrated stabilization of the late-stage pancreatic cancer and stayed alive for at least 19 months after diagnosis [16].

Another clinical study was conducted in a rectal cancer patient, who was treated with o-rMETase and a low-L-Met diet. The levels of carcinoembryonic antigen (CEA) remained stable for 18 months after the start of the therapy. The patient was regularly assessed by sigmoidoscopy and computed tomography (CT), according to which the tumor size has not changed (table). Hence, the use of o-rMETase as a supplement and a low-L-Met diet can be effective in rectal cancer and can result in long-term disease stabilization [15].

L-Met restriction with o-rMETase used as a supplement every 6 h for six months and a low-L-Met diet, showed synergistic efficacy with neoadjuvant doxorubicin and cyclophosphamide administration for the first 3 months, followed by docetaxel for the next 3 months, leading to a remarkable response that is typically expected in fewer than 10% of patients with invasive lobular carcinoma (breast cancer) treated with neoadjuvant chemotherapy alone. Disease progression was monitored by assessment of the blood CA19-9 levels and computed tomography, and a complete response was demonstrated by METPET at the completion of therapy (6 months) (table) [17].

None of the clinical studies using prolonged treatment with o-rMETase, as a supplement, at a dose of 500 units daily (two oral doses of 250 units) or 1000 units daily (four oral doses of 250 units) showed clinical toxicity or side effects of o-rMETase. These findings suggest that METase is a safe and effective agent capable of lowering circulating and intracellular L-Met levels, which should accelerate clinical application of L-Met restriction.

CONCLUSIONS

The above results indicate that o-rMETase may be used as an important supplement in the treatment of patients with various types of cancer. Due to the absence of side effects or toxicity, o-rMETase might be an ideal agent for combination chemotherapy strategies in late-stage cancer. Development of o-rMETase has addressed the problems of immune complications related to i.v. injection and the need for frequent administration. Recent technological improvements have made it possible to conduct extensive pharmacokinetic and pharmacodynamic studies of o-rMETase as a supplement, which has a substantial bearing on effectiveness and design of the ideal dose regimen for clinical applications. The best results are achieved by pharmacologically guided and individualized therapeutic strategies. There is hope that the specifics of such strategies will soon be worked out in order to achieve the full potential of rMETase in clinical practice. Finally, identification of L-Met-dependent tumor biomarkers, such as important epigenetic fingerprints, and a deeper knowledge of cellular response to L-Met restriction in cancer cells may provide more effective ways to target this unique metabolic vulnerability of cancer cells, including in combination with chemotherapeutic agents to achieve synergy [51].

Contributions. V.S.P., L.A.Q., and E.A.D. collected and analyzed information, wrote and edited the manuscript, and created the figure; Q.H. and R.M.H. reviewed the manuscript; R.M.H. revised the manuscript. All authors approved the final version of the manuscript before its submission for publication.

Funding. This study was supported by the State Program of the Ministry of Science and Higher Education of the Russian Federation (no. 075-01551-23-00; FSSF-2023-0006).

Ethics declarations. The authors declare no conflict of interest. This article does not contain description of studies involving human participants or animals performed by any of the authors.

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