Methioninase: a Therapeutic for Diseases Related to Altered Methionine Metabolism and Transmethylation: Cancer, Heart disease, Obesity, Aging, and Parkinson’s Disease

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Abstract: Methionine metabolism and transmethylation are central to the metabolism and differentiation of all known cells. In eukaryotic organisms, methionine metabolism and transmethylation are of paramount importance in modification and regulation of proteins, lipids, and nucleic acids. The differential methylation of genes regulates their expression in the myriad of cells in eukaryotic organisms. Disruption and abnormalities in methionine metabolism and transmethylation seems to be associated with the major diseases of mankind, including cancer, heart disease, aging, obesity, and Parkinson’s disease. In this review, we describe how aberrant and abnormal methionine metabolism and transmethylation are related to these major diseases. Most importantly, we review and hypothesize how the developing therapeutic recombinant methioninase (rMETase) can be utilized to cure or prevent all of these diseases.

Keywords: methioninase, cancer, cardiovascular disease, obesity, aging, neurological disease

Methioninase for Cancer

Tumor-Selective Target of Methionine Dependence

Approximately 25 years ago, our group and others identified a number of malignant cell lines that had an absolute requirement for methionine in vitro. The malignant lines would not grow on the methionine precursor homocysteine in place of methionine as opposed to normal cells. These data suggested that methionine could be a possible tumor-selective therapeutic target. We have demonstrated that actual patient tumors are also frequently methionine dependent indicating the clinical potential of the methionine-dependence target.

Studies on the mechanism of altered methionine metabolism in cancer have indicated that methionine-dependent tumor cells generally synthesize methionine at a normal rate from homocysteine although there may be some exceptions in some cancer cell types where vitamin B12 metabolism is altered. There seems to be an abnormally high rate of methionine utilization in methionine-dependent tumor cells for methylation reactions that require more methionine than the cell can synthesize from homocysteine during methionine starvation. Some tumors are also altered in the methionine salvage pathway, which may
also impact methionine dependence\textsuperscript{12}.

When methionine-dependent tumor cells in vitro are deprived of methionine in a homocysteine-containing medium they reversibly arrest in the late-S/G\textsubscript{2} phase of the cell cycle\textsuperscript{13-30}. The tumor-selective cell-cycle arrest allows methionine depletion to modulate the efficacy of many currently used chemotherapeutic agents\textsuperscript{39} (please see below).

Dietary methionine starvation extended the life span of the tumor-bearing animals and lowered the metastatic rate of the methionine-dependent tumors\textsuperscript{41-49}. Methionine-free total parenteral nutrition (TPN) doubled the response and survival rate of high-stage gastric patients treated with 5-fluorouracil and mitomycin C compared to patients treated with these drugs and fed methionine-containing TPN\textsuperscript{47}. This clinical trial demonstrated that methionine depletion has clinical activity. However, dietary methionine starvation is insufficient to completely deplete serum methionine and therefore does not completely arrest tumor growth.

Development of Methioninase

A methionine-cleaving enzyme would lower methionine levels more than methionine starvation and thereby could have more therapeutic efficacy. For this purpose Kreis and Hession\textsuperscript{48} attempted to purify a methioninase (METase) from \textit{Clostridium sporogenes}. This enzyme had a molecular weight of approximately 150 kilodaltons. The enzyme slowed the growth of the Walker 256 carcinoma in rats more than a methionine-free diet did. However, the enzyme preparation was highly unstable, its yield was only 2%, and it had a high Km of 90 mM. The \textit{C. sporogenes} enzyme therefore did not have the properties to be developed into a therapeutic.

Ito \textit{et al.} purified a METase from \textit{Pseudomonas putida}\textsuperscript{49}. This enzyme had a molecular weight of approximately 170 kilodaltons and catalyzed the \(\alpha, \gamma\)-elimination of methionine to \(\alpha\)-ketobutyrate, methanethiol, and ammonia in the presence of pyridoxal 5\textsuperscript{\textprime} phosphate. This enzyme had a Km of approximately 1 mM. Endotoxin was not removed from these METase preparations, precluding the METase from being used as a therapeutic.

A therapeutically useful METase requires not only sufficient activity and an efficient method of purification, it is also critical that the preparation be free of endotoxin for therapeutic use. We developed a simplified purification procedure that enables high-yield production of endotoxin-free METase from \textit{P. putida} suitable for therapeutic use\textsuperscript{46,47}. The METase produced by our protocols has been shown to deplete serum methionine levels without toxicity both in mice and in patients\textsuperscript{48,49}. The \textit{P. putida} endotoxin-free METase significantly retarded the growth of the Yoshida rat sarcoma and the H460 human non-small-cell-lung carcinoma in nude mice to a greater extent than standard drugs\textsuperscript{47}. The METase did not cause weight loss or other detectable toxicity for up to 10 days treatment. In order to proceed to large scale preclinical and clinical studies of METase it was necessary to clone the METase gene to produce the protein on a large scale.

The gene encoding METase in \textit{P. putida} was cloned and was expressed at relatively low levels in \textit{E. coli} by Inoue \textit{et al.}\textsuperscript{49}. Recombinant methioninase (rMETase) was shown to be composed of 398 amino acid residues with a calculated molecular weight of 42,626, corresponding to the subunit of the homotrimeric enzyme of native METase\textsuperscript{49}.

Hori, \textit{et al.} also reported the cloning of a gene from \textit{P. putida} that they termed \(\text{L-methionine-7-deaminom-c-mercaptomethane-lyase}\textsuperscript{50}. The peptide sequence deduced from the sequence of the gene has 398 amino acids with a molecular mass of 42,720 daltons. However, this gene and corresponding protein differ significantly in sequence from that reported by Inoue \textit{et al.} and Tan \textit{et al.} and from native METase\textsuperscript{49,50}.

To selectively target the methionine dependence of tumors for treatment on a large-scale preclinical and clinical basis, the rMETase gene from \textit{Pseudomonas putida} was then ligated into the pT7-7 overexpression plasmid containing the T7 RNA polymerase promoter and recloned in \textit{E. coli}. rMETase-containing clones were identified by their yellow-orange color which is due to high enrichment of the pyridoxal phosphate-containing recombinant methioninase (rMETase) and distinguished rMETase-overproducer from rMETase-
negative colonies. A scale-up production protocol which contained a heat step, two DEAE Sepharose FF ion-exchange, and one AntiClean Btox endotoxin-affinity chromatography columns was developed. Overproducer clones produce rMETase at greater than 10% of the total soluble protein and up to 1 g/liter in shake-flask culture. The protocol can produce therapeutic rMETase at the multi-gram level per batch with high yield (>60%), high purity (>98%), high stability, and low endotoxin making it suitable for therapeutic use.

**Efficacy of Methioninase**

Studies of the antitumor efficacy of rMETase in vitro and in vivo on human tumors xenografted in nude mice demonstrated that all types of human tumors tested including those from lung, colon, kidney, brain, prostate, and melanoma were sensitive to rMETase. In contrast, normal cells were insensitive to rMETase in vitro. No toxicity was detected in vivo at the effective doses. The overexpression clone and large-scale production protocols for rMETase have enabled rMETase to be used as a tumor-selective therapeutic with broad indication and high promise for effective, low-toxicity human cancer therapy.

The greatest promise for methioninase, however, is most probably in combination therapy where it has the potential to selectively sensitize tumor cells to many classes of currently-used chemotherapy. In this way, methioninase may act not only as a universal cancer drug but universal modulator of other chemotherapy drugs. This is discussed below.

**Modulation of Cisplatin by Methioninase**

Methioninase has many potential selective modulating effects on most of the major types of currently-used chemotherapy drugs. For example, methioninase seems to sufficiently lower the cellular levels of methionine that when cisplatin is also administered, the blocking of the cellular uptake of any residual serum methionine by cisplatin becomes very effective against a cancer cell selectively weakened by methionine depletion. We initially observed that a human breast cancer growing in nude mice was very sensitive to a methionine-free diet and cisplatin but not very sensitive to either treatment alone. Subsequent as-yet-unpublished experiments have shown that combining cisplatin and methioninase resulted in tumor-selective modulation and can induce tumor regression and cure. When both drugs were used alone, cisplatin was ineffective and methioninase was just tumorstatic (Tan, Y. and Hoffman, R.M., unpublished data).

**Modulation of 5-Fluorouracil by Methioninase**

Although 5-fluorouracil (5-FU) is not alone a highly effective drug, methioninase has unique potential for its modulation. When cancer-cell methionine is depleted by methioninase, folate turnover is increased by the elevated activity of methionine synthetase which tries to satisfy the cancer cells' elevated methionine needs. This results in elevated intracellular levels of 5-formyl-tetrahydrofolate allowing 5-FU to bind and inhibit its target thymidylate synthetase more effectively. In addition, the prolonged S-phase in methioninase-treated tumors may be selectively affected by 5-FU-mediated inhibition of RNA and DNA metabolism.

**Modulation of Methotrexate by Methioninase**

Methotrexate (MTX), an inhibitor of dihydrofolate reductase, reduces intracellular methionine biosynthesis. When MTX treatment is combined with methioninase, the cancer cell should be blocked from obtaining methionine from both internal and external sources and will be selectively affected due to its elevated dependence on methionine compared to normal cells.

**Modulation of Cell-Cycle Specific Drugs by Methioninase**

Other modulating potentials of methioninase include the fact that methioninase treatment selectively arrests tumor cells in the G2-phase of the cell cycle. This tumor-selective cell-cycle arrest can modulate other drugs. Cisplatin may also be modulated by the methioninase G2 cell cycle block, since cisplatin also may be a G2 cell-cycle blocking agent. This effect of cisplatin is a tumor-selective modulation in methionine-starved tumor cells which now can become doubly blocked in G2.
When tumor cells are deprived of methionine by methioninase and are in progression toward the G1 cell-cycle block, they may have a prolongation of their DNA synthesis or S-phase making them highly susceptible to S-phase drugs. Methioninase can thus modulate the S-phase drugs to have tumor selectivity. These drugs include agents such as doxorubicin, cytosine arabinoside, and possibly 5-FU.

Tumor cells, selectively arrested in G1 by methioninase, can progress synchronously into mitosis if the methioninase treatment is temporarily halted. Anti-mitotic drugs such as Vincristine and Vinblastine or other classes of anti-metastatic drugs such as the taxanes can then have a much bigger fraction of tumor cells to act upon if given after methioninase treatment ceases. The tumor-selective synchronization in mitosis is another unique modulating capability of methioninase for antimitotic drugs.

**Modulation of Apoptosis Genes and Drugs by Methioninase**

Other possibilities also arise for modulation of other drugs by methioninase modulation of suppressor oncogene effects on apoptosis (programmed cell death). Very new work has shown that a number of normally unmethylated suppressor oncogenes become abnormally hypermethylated in cancer and thereby lose function\(^a\) (please see below). The unmethylated suppressor oncogenes may have a number of very important functions such as (a) keeping normal cells from becoming cancer cells, and (b) perhaps mediating cancer drug-induced cell death by mechanisms such as apoptosis. These functions and effects cease if the suppressor oncogenes are inactivated by abnormal hypermethylation in cancer \(^6\). Indeed, methioninase has demethylating potential due to the depletion of methionine, the ultimate cellular source of methyl groups, thereby potentially allowing re-activation of suppressor oncogenes. Many of the cytotoxic drugs in current use perhaps have minimal effectiveness in killing cancer cells because the programmed cell death mechanisms are not functioning possibly due to the hypermethylation of such apoptosis-mediating genes. By activating these suppressor oncogenes or apoptosis-mediating genes, methioninase could modulate a number of the cytotoxic drugs having different mechanisms such that these drugs can now induce apoptosis and selectively kill the cancer cells.

Methioninase may also modulate agents such as 5-azacytidine (5-aza-C), which acts as a direct DNA methylation inhibitor\(^7\) but is quite toxic due to its effect on normal cells. 5-aza-C should be able to be used in much lower doses when the cancer cell is depleted of methyl groups by methioninase making the 5-aza-C less toxic to normal cells and more tumor selective.

**Modulation of Alkylating Agents by Methioninase**

Other cancer modulatory effects of methioninase include the fact that methioninase can deplete the cancer cell of a critical DNA repair enzyme such as methylguanine methyltransferase (MGMT)\(^8\). The depletion of MGMT makes the cancer cell selectively sensitive to alkylating agents such as the nitrosoureas, for example BCNU\(^9\). The BCNU-alkylated cancer cell DNA cannot be repaired after methioninase treatment due to the depletion of the above-mentioned DNA repair enzyme MGMT, causing the cancer cell to be selectively sensitive to even low doses of BCNU \(^9\). BCNU, normally used in brain cancer with minimal effectiveness can be selectively modulated by methioninase which need not cross the blood-brain barrier, but just depletes serum methionine.

**Modulation of Methionine Analogs by Methioninase**

Other mechanisms of modulation by methioninase include modulation of the efficacy of methionine analogs such as ethionine. Methionine analogs such as ethionine are toxic and non-specific at high dose. In contrast, the methionine analogs can become highly effective at lower doses when the cancer cell is in the weakened methionine-depleted state where the methionine analogs can then effectively inhibit methionine-metabolizing enzymes. The methioninase-modulated methionine analogs then become selective cancer killing agents\(^10,11\).

The antitumor and modulation efficacy of methioninase is currently being evaluated in preclinical \(^10\) and clinical trials \(^29\).
Epigenetics, Trasnmethylation, Cancer, and Methioninase

In the early 1980’s, we proposed that cancer was basically an epigenetic disease mediated by altered methionine metabolism and transmethylation including DNA methylation\(^{(2)}\).

As Jones and Gonzalgo\(^{(3)}\) point out, methylation of CpG-rich promoters, which can block transcriptional activation, is a heritable, but reversible, epigenetic change that can alter gene expression which has important developmental and genetic consequences.

In addition to methionine dependence, alterations in the DNA methylation are among the most common changes in cancer\(^{(4,5)}\). Ushijima et al. \(^{(4)}\) used representation difference analysis (RDA) to demonstrate large areas of the genome with methylation changes in murine liver cancer. Restriction landmark genomic scanning\(^{(5)}\) and methylation-sensitive arbitrarily primed PCR\(^{(5,6,7)}\) have also identified and led to the cloning of frequently-altered methylation sites in cancer cells\(^{(8)}\). Jones points out\(^{(9)}\) that not only are overall levels of DNA methylation altered in cancer, but there are major changes in the distribution of methyl groups. This is consistent with the ideas of Borek et al. formulated more than 30 years ago that methylation is unbalanced in cancer\(^{(10)}\).

Related to these studies, de novo methylation of the promoter regions of specific tumor suppressor genes has been observed. Cases including \(Rb, p16, VHL, p53\), and other genes\(^{(11-13)}\) where CpG methylation silencing has inactivated these tumor suppressors. As Jones points out\(^{(14)}\), Frommer et al.’s development of bisulfite treatment made the analysis of cytosine methylation patterns possible during DNA sequencing.

Lengauer et al.\(^{(15-17)}\) noted another aspect of altered ability to methylate DNA in cancer by introducing exogenous CpG-rich retroviruses into human colon cancer cell lines. Five out of 10 lines did not express the retrovirally encoded \(\beta\)-galactosidase gene. These lines were also deficient in DNA mismatch repair (MMR\(^{17}\)). Cells proficient for repair (MMR\(^{17}\)) efficiently expressed the retroviral gene. Lengauer et al.\(^{(18-20)}\) state that extinguished expression of the introduced retroviruses was due to de novo methylation which probably also extinguished MMR. It is thought that chromosome instability in cancer is related to aberrant DNA methylation processes\(^{(21-23)}\). Jones\(^{(24)}\) states that the mismatches formed due to the MMR deficiency may serve as loci for de novo DNA methylation which may explain some of the hypermethylation of tumor suppressor genes described above.

Laird et al.\(^{(25)}\) demonstrated that inhibiting DNA maintenance methylation by 5-azacytidine decreased the number of colonic polyps in transgenic mice with the \(min\) mutation. Related to this result, a number of studies have indicated that 5-azacytidine can reactivate tumor suppressor genes that were inactivated due to hypermethylation\(^{(26-28)}\).

In related experiments, recent publications indicate that reduction of DNA methyltransferase (MTase) activity by MTase antisense or 5-azacytidine, which leads to DNA marked hypomethylation, can inhibit tumorigenesis\(^{(29-31)}\).

Cancer cell lines\(^{(32)}\) and human tumors\(^{(33)}\) express elevated levels of DNA MTase\(^{(34)}\). 5-azacytidine (5-AZA-C) has been the drug of choice to inhibit DNA methyltransferase and DNA methylation\(^{(35)}\). However, 5-AZA-C has significant dose-limiting toxicity\(^{(36-38)}\) and carcinogenicity\(^{(39)}\). The antisense therapeutic approach mentioned above also has many technical problems. A methyl deficient diet was also shown to inhibit polyp formation in transgenic mice\(^{(40)}\) but such a diet can also be carcinogenic\(^{(41)}\). A promising approach, however, is methioninase treatment, which selectively targets the unbalanced methylation in cancer manifesting as methionine dependence. Thus, an important part of the mechanism of methioninase efficacy may be inhibition of DNA MTase due to deprivation of methyl groups. This would lead to subsequent DNA demethylation and activation of hypermethylated tumor suppressor and other genes leading to a more normal phenotype or apoptosis.

Methioninase for Heart Disease

Elevated serum total homocysteine (tHcy) levels have emerged as a major cardio-vascular risk factor. The first publication on Hcy as a determinant in arteriosclerosis 27 years ago has found ample sup-
porting evidence in the following decades. Many groups showed in cross-sectional and retrospective studies a strong and significant association between elevated levels of Hcy and myocardial infarction, stroke, and other occlusive vascular diseases. More recently, prospective studies established Hcy as an independent, strong, and significant risk factor with no, or little, correlation between elevated Hcy and other known or suspected risk factors.

Acquired, moderate hyperhomocysteinemia due to a lack of intake of the vitamins B6, B12, and folate can be treated by supplementing the diet of patients with the appropriate cofactors. However, moderate and severe hyperhomocysteinemia due to mutations in the genes coding for methylenetetrahydrofolate reductase, cystathionine-β-lyase or other lesions are non-responsive to vitamin therapy and presents a permanent and severe risk factor for those patients.

Efforts to investigate a potential treatment of hyperhomocysteinemia by administration of an enzyme specific for the degradation of HCY appears valuable and promising for the following reasons:

METases from various sources exhibit a somewhat relaxed substrate specificity and have been shown to possess a 1.8–9.0 fold higher relative activity on HCY as compared to their “natural” substrate methionine.

In pilot clinical studies, METase and rMETase (Tan, Y. and Hoffman, R. M., et al., unpublished studies) have been shown to deplete serum methionine and homocysteine with no observable toxicity.

The somewhat relaxed substrate specificities of METases in general, and the high catalytic efficiency of the Trichomonas vaginalis enzyme towards HCY in particular [9-fold with respect to its alternative substrate HCY] strongly suggest the potential of Trichomonas vaginalis METase as a homocysteinase for heart HCY-induced cardiovascular disease.

Methioninase for Obesity and Aging

Aging

Aging has also been associated with changes in DNA methylation. Issa and Baylin have recently described progressive hypermethylation of the estrogen receptor gene in aging colon, and have related it to cancer and to hematopoietic neoplasms. It is likely that other genes will be similarly affected by aging. The promoter methylation of the IGF2 gene itself is greatly increased in human colon as a function of aging.

Orentreich et al. have reported that lifelong reduction of L-methionine, from 0.86 to 0.17% of the diet results in a 30% longer life span of male Fischer 344 rats. Methionine restriction eliminated weight gain, even though food intake was larger. Increasing the energy intake of rats fed 0.17% methionine failed to increase their rate of growth, whereas restricting the food intake of 0.85% methionine-fed rats to that of 0.17% methionine-fed animals did not reduce growth, indicating that food restriction was not a factor in life span extension in these experiments. These data strongly indicate that the chronic use of rMETase could titrate serum methionine levels to potentially increase life span.

Obesity

The work of Orentreich also suggests that rMETase can perhaps regulate obesity, since, dietary methionine deprivation controlled weight gain so exquisitely in the rats.

Recent work has indicated the importance of hormone control of obesity including the hormones leptin, neuropeptide Y (NPY) and a specific melanocortin receptor as key components of the systems in the brain that regulate body weight.

Fan et al. and Huszar et al. have shown that the melanocortin-4 receptor and its peptide ligand, melanocyte-stimulating hormone (MSH) are important in the pathogenesis of obesity in mice with the mutation yellow agouti. Erickson et al. have reported NPY can reduce obesity and other abnormalities that are seen in mice mutated in the obese (ob) gene.

The agouti gene’s product is a hair-follicle secreted factor that is overexpressed in these mice. When it is overexpressed in the hair follicles, the agouti peptide inhibits the effects of melanocyte stimulating hormone (MSH) on melanocortin-1 receptors that leads to the yellow coat color. The agouti peptide also inhibits
the action of MSH on the MC-4 receptor in the brain\textsuperscript{107,108}. Mice with mutations in the MC-4 receptor are as obese as the agouti mice, but they do not have yellow coats\textsuperscript{104}. Fan et al.\textsuperscript{132} have showed that MSH agonists inhibit food intake in normal and obese animals whereas MSH antagonists have the opposite effect. In addition, both agouti and MC-4-knockout mice have very high blood levels of another fat regulator—leptin. Thus it seems that in the absence of a functional MC-4 receptor, animals no longer decrease their weight during increased concentrations of leptin in the blood\textsuperscript{93,134}. It will be very interesting to note how \textit{rMETase}-mediated block of weight gain interacts with these hormone regulators of obesity.

\section*{Methioninase for Parkinson’s Disease}

S-Adenosyl-L-methionine has been shown to cause Parkinson’s disease-like effects that include hypokinesia, tremor, rigidity, and abnormal posture in rats when injected into the lateral ventricle\textsuperscript{110}. S-adenosyl-L-methionine is the rate-limiting endogenous methyl donor for the methylation of dops-mine\textsuperscript{111}. Therefore, S - adenosyl - L - methionine and methionine, which is its precursor, may play a role in Parkinson’s disease\textsuperscript{112}. A dose of 200 mg/kg L-dopa, the main therapeutic agent for Parkinson disease, blocked the hypokinetic effects of S-adenosyl-L-methionine, but D-dopa, the inactive analog, showed no effect\textsuperscript{113}. Therefore, these findings suggest that S-adenosyl-L-methionine-induced hypokinesia, and its associated symptomatology, may serve as a model for the study of Parkinsonism and it may in fact be involved in the etiology of the disease\textsuperscript{114}.

S-adenosyl-L-methionine is the methyl donor used by catechol-O-methyltransferase (COMT) in the O-methylation of levodopa, dopamine (DA), and 3,4-dihydroxyphenylacetic acid (DOPAC)\textsuperscript{109}. Blockade of O-methylation of levodopa and DA can increase the bioavailability of levodopa and DA\textsuperscript{109}. This should improve the beneficial effect of levodopa for the therapy of Parkinson’s disease (PD)\textsuperscript{109}. Methioninase could be used to reduce circulating methionine levels such that COMT is optimally inhibited for this therapeutic effect (Magana, R., personal communication).

Allain et al.\textsuperscript{135} compared the levels of cysteine and homocysteine in the plasma of healthy subjects and of patients with Parkinson’s disease treated by L-dopa and dopa decarboxylase inhibitors. The levels of cysteine in the plasma of controls and patients with Parkinson’s disease were not statistically different but the level of homocysteine was higher in patients. Methionine treatment will also lower the homocysteine levels preventing possible vascular and other brain pathologies related to Parkinson’s disease.

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