Anticancer Efficacy of Methioninase in Vivo

YUYING TAN1, MINGXU XU1, HUIYAN GUO1, XINGHUA SUN1, TETSURO KUBOTA2 and ROBERT M. HOFFMAN1

1Anticancer Inc. 7917 Ostrow Street, San Diego, CA 92111, U.S.A.; 2Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan

Abstract. Therapeutics that are selective for cancer would have a high potential for efficacy. We have previously shown that the metabolic defect of enhanced methionine dependence is a broad cancer-selective target. Methioninase depletion can completely arrest the growth of methionine-dependent tumor cells in vitro with a reversible pre-mitosis cell-cycle block. Dietary methioninase depletion can partially arrest the growth of methionine-dependent rodent tumors in vivo. This report demonstrates that methioninase isolated from Pseudomonas putida can arrest rodent and human tumors in nude mice with no apparent toxic side effects. Methioninase injected i.p. arrested the growth of the Yoshida sarcoma in nude mice and greatly slowed the growth of the H460 human non-small-cell lung carcinoma in nude mice. The effectiveness of methioninase against H460 was in contrast to 5-fluorouracil and vincristine, which were inactive against this tumor. The activity of the administered methioninase did not cause weight loss for up to 10 days treatment at 40-120 units/day indicating the possibility of low toxicity. In contrast, vincristine was highly toxic despite its ineffectiveness. Methioninase also induced a tumor-specific late cell-cycle block. The tumor-selective late cell-cycle block induced by methioninase should be able to be exploited to enhance the tumor specificity of antimetabolic drugs and other agents in future experiments. Thus methioninase is a highly effective antitumor agent with a new tumor-selective mode of action with minimal toxicity, demonstrating potential clinical effectiveness against solid tumors.

Correspondence to: Robert M. Hoffman, Ph.D., Anticancer Inc. 7917 Ostrow Street, San Diego, CA 92111, USA. Tel: (619) 654-2555; FAX: (619) 268-4175. E-Mail: anti@ix.netcom.com

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The chemotherapy of solid tumors, with a few exceptions, has had only limited effectiveness (1). Thus, the majority of disseminated solid cancers are generally not responsive to current chemotherapeutic regimens. For the most part, existing drugs used for solid-tumor chemotherapy are cytotoxic drugs. The cytotoxic drugs are not cancer selective, and are therefore active against both tumor and normal cells, which gives the drugs limited efficacy and significant toxicity.

Thus, it is of critical importance to identify targets and agents which are tumor selective. Asparaginase dependence is possibly one such target for the enzyme drug asparaginase (1). However, the asparaginase-dependence target may be limited only to hematological tumors (1). Approximately 23 years ago reports began to suggest that the amino acid methionine may be a tumor-specific target, since a number of malignant cell lines were identified that had an absolute requirement for methionine since they would not grow on homocysteine (2-8). It was at this time, that we began studies on the altered methioninase metabolism in cancer (see reference 8 for review of early work). Recent studies have demonstrated that in situ patient tumors are also frequently methionine dependent (9). Normal cells and tissues, unlike methionine-dependent tumors, were found to be able to use homocysteine in place of methionine for proliferation, and are therefore methionine independent (8).

Studies at the biochemical level have indicated that methionine-dependent tumor cells generally synthesize methionine at the normal rate from homocysteine, although there may be exceptions in some cancer cells where vitamin B12 metabolism is altered (25). However, there seems to be an abnormally high rate of methionine utilization in methionine-dependent cells for methylation reactions that require more methionine than the cell can synthesize from homocysteine, thereby causing the cells to arrest when exogenous methionine is unavailable (11,12,13). When methionine-dependent tumor cells in vitro are deprived of
methionine in a homocysteine-containing medium they reversibly arrest in the late-S/G2 phase of the cell cycle (14).

Methionine was first investigated as a tumor-selective therapeutic target in *in vitro* experiments. In these studies cocultures of methionine-dependent tumor cells and normal methionine-independent fibroblasts were first incubated in methionine-free medium to selectively arrest the tumor cells in late-S/G2. The cultures were then supplied with methionine and given an antimitotic agent which was selectively active against the synchronous wave of tumor cells entering mitosis. This treatment selectively eliminated the tumor cells from the co-culture while the randomly-cycling normal cells were protected since only a minority were in mitosis during treatment (15).

Recently, *in vivo* experiments have been carried out, whereby animals carrying the methionine-dependent tumors were starved for methionine. Dietary methionine starvation extended the life span of the tumor-bearing animals and lowered the metastatic rate of the methionine-dependent tumor (16,17,26,27). However, dietary methionine starvation is insufficient to completely arrest tumor growth.

Recently we have isolated a methioninase, free of endotoxin, from *Pseudomonas putida* (18). This methioninase has been shown by us to deplete circulating methionine from approximately 100 µM to 1 µM in mice without severe toxicities (19). We report here that methioninase greatly slows the growth of the Yoshida sarcoma and a human lung tumor in nude mice and induced a tumor-specific late cell-cycle block.

**Materials and Methods**

*Purification of methioninase.* The procedure for methioninase production was as published previously (18) and involved a heat step and 3-column purification steps for purification to homogeneity. The methioninase had four identical subunits of approximate molecular weight 43 KD (18).

*Tumor.* The Yoshida sarcoma cells were cultured in cell suspension with Eagle’s minimum essential medium with 10% fetal calf serum. The human H460 lung cancer was passaged via s.c. transplantation in nude mice.

*Transplantation.* 4-5 week old outbred nude nu+/nu+ mice were divided randomly into 3 groups. The mice were bred and housed in a HEPA-filtered barrier room under NIH guidelines. A suspension of Yoshida sarcoma containing 7.3 x 10^5 cells was injected into each mouse at the axillary site. The first group was the control and was fed a methionine-containing diet (MET+). The second group was fed a methionine-free diet (MET-). The third group was fed a MET diet and was treated with methioninase (see below). The H460 human lung tumor was transplanted using four pieces of tumor, one mm in diameter, sutured in the subcutis. The H460-transplanted mice were divided into the same three categories.

*Diet.* Defined diets with and without methionine (#TD93030 and #TD92077, respectively) were obtained from Teklad (Madison, Wisconsin). In each group the mice were given equal measured amounts of their respective diet.

*Methioninase treatment of animals.* In a preliminary experiment, methioninase was given i.p. in phosphate-buffered saline to Yoshida

Sarcoma bearing mice in increasing amounts until the growth of the tumors was arrested at 40 units/day. The methioninase specific activity was approximately 10 units/mg. In two subsequent experiments, 4 units of methioninase were injected i.p. into each mouse, approximately every 2 hours. Treatment was started on day-2 after transplantation. The H460-bearing mice were treated with 20 units of methioninase every four hours on a methionine-containing and a methionine-free diet beginning on day-6 after transplantation.

*Standard chemotherapy.* Vincristine was administered i.p. to H460 bearing mice at 0.9 mg/kg every 4 days. 5-fluorouracil was administered i.p. to H460-bearing mice at 60 mg/kg every 4 days. Therapy was initiated six days after transplantation.

*Tumor weight and carcass weight measurements.* The length of the major and minor axis of the tumors growing in nude mice were measured with calipers. The growth of the tumors was evaluated every day for the Yoshida sarcoma and every 2 days for the H460 human lung tumor.

**DNA staining.** Slides containing histological 3-micron sections were made from the subcutaneously-growing Yoshida sarcoma tumor. The slides were incubated in preheated 1N hydrochloric acid at 60°C for 20 minutes, rinsed with distilled water 6 times to remove excess acid, stained with Schiff's reagent for 20 minutes at room temperature, treated with two successive changes of freshly-prepared sulfurous acid rinse, and washed in running tap water for 5 minutes. After dehydration, slides were coverslipped for DNA analysis. This procedure was repeated using normal colon tissue from the same mouse used above.

**DNA analysis.** A Cambridge Instruments Quantimet-520 system was used to determine the DNA content of cell nuclei by measuring integrated optical density (IOD) of each cell. 200 cells were measured from each slide. The IOD was directly proportional to DNA content. By comparing the tumor cell population to a standard normal cell population the cell-cycle position of each tumor cell was determined. This procedure was repeated using normal colon tissue from the same mouse.

**In vitro experiments.** The methionine-dependent cell-cycle block. Based on our recent *in vitro* experiments (9), the methionine-dependent cell-cycle block (MDCCB) value was calculated by the equation G1 cells/total cells (for MET) / G1 cells/total cells (for MET+). Methionine dependence was defined as a MDCCB value of < 0.65 (9).

**Statistical analysis.** Data for tumor growth were analyzed by using the Student's t-Test (20,21).

**Results and Discussion**

Escalating doses of methioninase were used in a preliminary experiment to determine the dose and schedule that would arrest the growth of the Yoshida sarcoma in nude mice. Doses of 13 and 24 units a day still allowed slight tumor growth (data not shown). Doses were then elevated up to 40 units per day, which greatly slowed tumor growth in the preliminary experiment (data not shown).

As can be seen from Figure 1, 40 units/day of methioninase were effective in preventing significant Yoshida sarcoma tumor growth in comparison with untreated animals on a normal diet or methionine-depleted diet. There was a significant difference between the rate of tumor size increase over time at p < 0.002 (Student's t-Test) for Group One.
(MET<sup>+</sup> diet, no methioninase) versus Group Three (MET<sup>-</sup> diet, plus methioninase); and for Group Two (MET<sup>-</sup> diet, no methioninase) versus Group Three (MET<sup>-</sup> diet plus methioninase).

There was no significant difference in the final estimated carcass weight of Group One versus Group Two, Group One versus Group Three and Group Two versus Group Three (p > 0.05, Student's t-Test).

Figure 2 demonstrates that the Yoshida sarcoma tumors, arrested by methioninase, have shifted their cell-cycle position, indicating an arrest at approximately the late-S/G<sub>2</sub> phase of the cell cycle. The methionine-dependent cell-cycle block (MDCCB) was calculated to be 0.52, thus indicating methionine dependence. Normal colon tissue in a methioninase-treated mouse was found to have a MDCCB value of 0.98 (Figure 2B).

Thus the data presented here indicate that methioninase is effective in preventing growth of a very malignant tumor, the Yoshida sarcoma. The Yoshida sarcoma was chosen as a model due to its rapid growth pattern so that efficacy of methioninase could be determined rapidly.

Methioninase treatment of the human H460 lung cancer in nude mice was also effective as seen in Figure 3 (p<0.01). The efficacy of methioninase against H460 is particularly noteworthy in comparison with treatment with 5-fluorouracil and vincristine which were not effective (p<0.05). The efficacy of methioninase was also noteworthy in that the animals were fed a methionine-containing diet. Methioninase treatment of the animals on the methionine-free diet did not increase the antitumor efficacy compared to methioninase treatment on the methionine-containing diet (data not shown). This result suggests that in future clinical experiments the patients can be on a normal diet. Methioninase even at 20 units/mouse every 4 hours caused no weight loss in the mice. In contrast, vincristine, which was inactive, caused a 25% weight loss in the mice.

Although methioninase has been isolated previously (22-24), the preparations were apparently not totally pure. In contrast, we developed a purification scheme to isolate endotoxin-free methioninase (18) that produces no pyrogenic reactions in the mice (data not shown).

The target of methionine dependence seems to be very broad and probably occurs frequently in all types of cancer (2,9). The H460 lung tumor shows that although standard agents are ineffective and toxic, methioninase is effective and nontoxic. The tumor specific late-cell-cycle block induced by methioninase is the first time a tumor-selective cell-cycle-specific arrest has been achieved by a therapeutic agent in vitro. The tumor-selective cell-cycle-specific arrest gives rise to the possibility of effective combination therapy with antimitotic and other agents in future experiments with human tumors.

Thus methioninase is a highly effective antitumor agent with a new tumor-specific mode of action with minimal toxicity, demonstrating potential clinical effectiveness against solid tumors.
Figure 2. Cell cycle distribution of the Yoshida sarcoma (A) and normal colon tissue (B) from nude mice in the methioninase treatment group (MET diet plus methioninase) and in the control group (MET+ diet, no treatment). The tissues were stained with Shiff’s reagent and measured for DNA content as described in the text. Note the accumulation of cells with high DNA content indicating a pre-mitotic cell-cycle block in the Yoshida sarcoma from mice in the methioninase-treatment group as opposed to the mice from the control group (2A). Normal colon tissue, however, demonstrated no methionine-dependent cell-cycle block (2B).
Figure 3. Efficacy of methioninase vs. standard agents on the growth of the human H-460 lung tumor in nude mice. The tumor was transplanted to the subcutis as described in the text. Methioninase was administered s.c. at 120 units/day with 10 units given at 4 hour intervals. Vincristine and 5-FU were both administered s.c. every four days at 0.9 mg/kg and 60 mg/kg, respectively. Day 0 on the graph, when therapy began, is equivalent to 6 days post transplantation at which time the tumors became palpable. Tumor size (3A) and body weight (3B) were measured with calipers every two days.
References


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