

## Methionine Depletion Modulates the Antitumor and Antimetastatic Efficacy of Ethionine

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**Abstract.** *The elevated methionine requirement for the growth of tumors, termed methionine dependence, is a potentially highly effective therapeutic target. To attack this target we are developing anti-methionine chemotherapy. In this study of anti-methionine chemotherapy we have observed that the methionine analog ethionine is synergistic with methionine depletion in arresting the growth of the Yoshida sarcoma both in vitro and when transplanted to nude mice. In contrast, ethionine in vitro in a methionine-containing medium is not effective against Yoshida sarcoma cells. Similarly, ethionine administered along with a methionine-containing diet is ineffective against the Yoshida sarcoma growing in nude mice. A methionine-depleted diet alone is only partially effective against tumor growth. The Yoshida sarcoma gave rise to metastases in 75 % of the organs observed in the mice on the methionine-containing diet, and 43 % of the organs in the mice on the methionine-free diet. In striking contrast, no metastases were observed in the ethionine-treated animals on the methionine-free diet. Anti-methionine chemotherapy consisting of dietary methionine depletion and ethionine administration caused an initial weight loss but the animals weight stabilized resulting in no animal deaths. The synergism of ethionine and methionine depletion is markedly similar in vitro and in vivo suggesting the observed efficacy is due to the specific anti-methionine targeting. Thus methionine depletion highly potentiates the anti-tumor and anti-metastatic*

*effectiveness of ethionine suggesting that anti-methionine chemotherapy consisting of methionine depletion as a modulator of methionine analogs holds great promise as a new, tumor-selective therapeutic approach.*

Methionine dependence, the inability of tumor cells to grow *in vitro* when methionine is removed from the culture medium and replaced by homocysteine (MET-HCY<sup>+</sup>) occurs frequently in all types of cancer (1-7). Freshly-explanted human patient tumors in primary histoculture on collagen-containing gels are methionine dependent (8). Normal cells and tissues which have been tested are methionine independent and still grow after methionine is replaced by homocysteine (7).

*In vitro* and *in vivo* studies have suggested that targeting the methionine dependence of tumors may exert tumor-selective efficacy via a tumor-specific cell-cycle block (9,16). Under conditions of a limiting methionine source, methionine dependent-tumor cell arrest in the late-S/G<sub>2</sub> phase in the cell cycle (16). Taking advantage of the tumor-specific cell cycle block, the combination of methionine starvation and cycle-specific chemotherapy used in co-cultures of tumor and normal cells eliminated the tumor cells while allowing the normal cells to flourish (10).

Recently a number of investigations have attempted to exploit the methionine dependence of tumors for therapeutic effects *in vivo*. Breillout *et al* (11,12) found for methionine-dependent tumors that a methionine-depleted diet lowered the metastatic potential of the tumor without significant effects on local tumor growth in rats. Goseki *et al* (13) observed that a methionine-free total parental nutrition (TPN) mixture for rats bearing the Yoshida sarcoma slowed tumor growth and extended the survival of the rats, especially with the use of doxorubicin. Kreis and Hession (14), with the use

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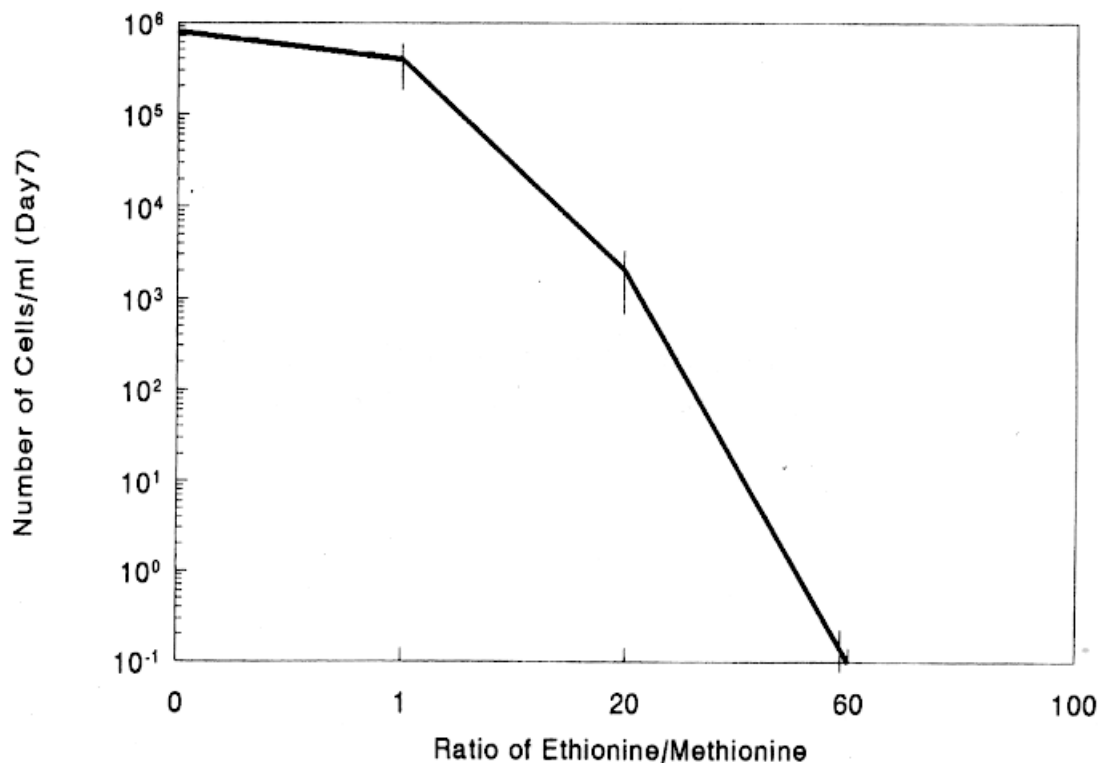


Figure 1. Effect of Ethionine and Methionine Depletion on Tumor Cell Growth in vitro. 10<sup>7</sup>/ml Yoshida Sarcoma were cultured in MEM Earle's medium containing methionine at 5 μM, 10 μM and 100 μM and ethionine at 5 μM, 50 μM, 100 μM, 300 μM, 500 μM and 1000 μM in various combinations. Cell number was counted on day 7.

of a methioninase, demonstrated an attenuation of growth of the W-256 rat carcinosarcoma growing in rats. Yoshida *et al* (15) observed that administration of methionine-depleted (TPN) slowed growth of the AH 109A ascites hepatoma in rats, perhaps by selectively enhancing tumor protein turnover.

We have demonstrated that the Yoshida tumor growing in nude mice can be induced by a methionine-free diet to have a late S/G<sub>2</sub> cell cycle block, indicating that a tumor-selective cell-cycle block can indeed be achieved *in vivo* (16). We also observed that the Yoshida tumor in nude mice can actually regress with prolonged dietary methionine starvation, resulting in an extended survival period of the mice (16). We have termed these approaches to target the methionine dependence of tumors, anti-methionine chemotherapy.

In an attempt to completely arrest the Yoshida sarcoma growing in nude mice we have administered the combination of a methionine-depleted diet and the methionine analogue ethionine which acts as a competitive inhibitor for methionine-utilizing enzymes such as methionine adenosyl transferase. Ethionine thus also targets methionine and is a component of anti-methionine chemotherapy. Our hypothesis was that ethionine should be efficacious against methionine dependent tumors under conditions of methionine depletion as opposed to normal methionine conditions. Experiments confirming this hypothesis are described in this report.

Table I. Effect of ethionine and methionine depletion on tumor growth in nude mice.

	Condition	Inhibition rate
Group I	MET-containing diet (MET <sup>+</sup> )	0
Group II	MET-depleted diet (MET <sup>-</sup> )	64.3 %* <sup>+</sup>
Group III	MET <sup>+</sup> + ETH 25 mg/kg	7.5 %
Group IV	MET <sup>-</sup> + ETH 25 mg/kg	87.1 %*# <sup>+</sup>

8 × 10<sup>5</sup> Yoshida sarcoma cells in suspension were injected in an axillary site in each nude mouse. Mice were divided into four groups. Group I mice were fed the methionine-containing diet. Group II mice were fed the methionine-free diet. Group III mice were treated with ethionine 25 mg/kg ip twice a day on the methionine-containing diet. Group IV mice were treated with ethionine 25 mg/kg ip twice a day on the methionine-free diet. Each group consisted of three animals. Tumor size was determined by caliper measurement. (see Materials and Methods). The inhibition rate was calculated on day 9 by the equation:  
 [ Tumor size (Control) - Tumor size (Treated) ] / Tumor size (Control)

\*p < 0.01 compared to group I.

# p < 0.01 compared to group II.

<sup>+</sup> p < 0.01 compared to group III.

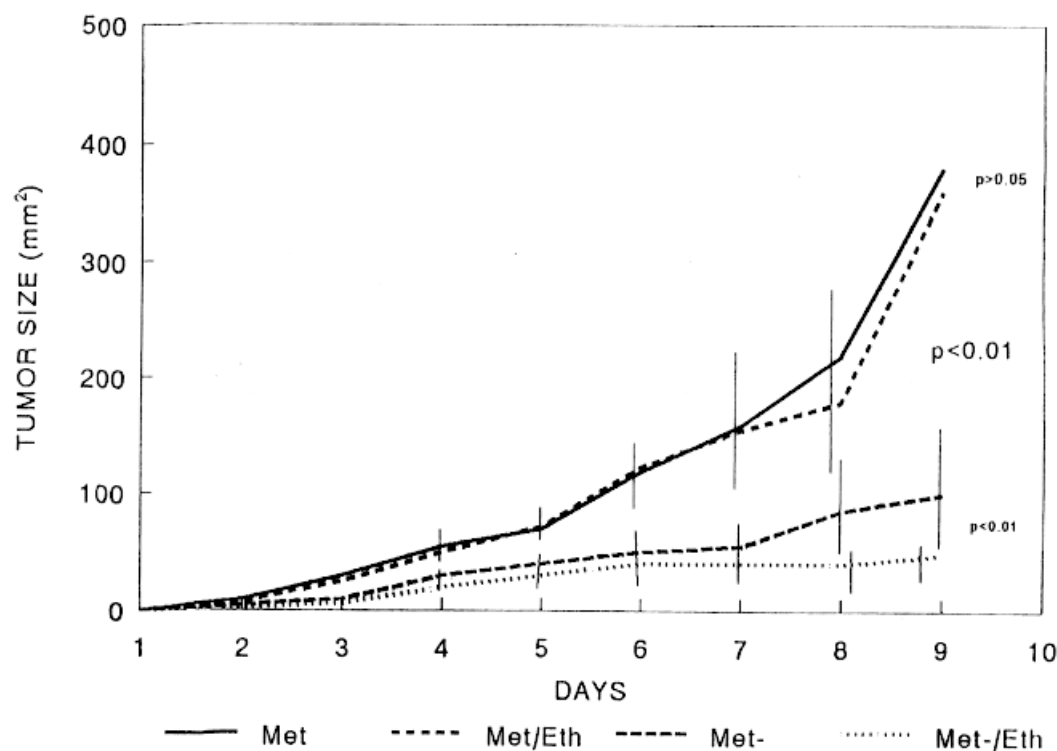


Figure 2. Effect of Ethionine and Methionine Depletion on Tumor Growth in Nude Mice.  $8 \times 10^5$  Yoshida sarcoma cells in suspension were injected in an axillary site in each nude mouse. Mice were divided to four groups. Group I mice were given a defined MET-containing diet. Group II mice were fed by a defined MET-free diet. Group III mice were treated with ethionine at 25 mg/kg ip twice a day on the MET-containing diet. Group IV mice were treated with 25 mg/kg ethionine ip twice a day on the MET-free diet. Each group consisted of three animals. The tumor size was measured by the equation: Length of longest axis  $\times$  length of shortest axis.

## Materials and Methods

**Ethionine efficacy in vitro.** The Yoshida sarcoma cell line was seeded *in vitro* at  $10^3$  cells/ml. The cells were cultured in MEM Earle's media with 10% fetal calf serum containing methionine at 5  $\mu$ M, 10  $\mu$ M and 100  $\mu$ M, and ethionine at concentrations of 5  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 500  $\mu$ M and 1000  $\mu$ M in various combinations. Cells were counted every day.

**Ethionine efficacy in vivo.** Four - five week old nude mice were randomly divided into four groups. Each group consisted of three mice.  $8 \times 10^5$  Yoshida sarcoma cells in suspension were injected in an axillary site in each nude mouse. Group I mice were fed a defined methionine-containing diet (Teklad Madison WI [16]). Group II mice were fed a defined methionine-free diet (Teklad WI [16]). Group III mice were fed the defined methionine-containing diet and given ethionine ip twice daily at 25 mg/kg. Group IV mice were treated as above with 25 mg/kg ethionine on the methionine-free diet. Tumor size was measured with a calipers each day. Tumor size was determined by the length of the longest axis times the length of the shortest axis. Body weight was measured each day.

**Histological analysis for metastases.** The liver, lung, kidney and colon of the mice were fixed in 10% formalin after the mice died. The tissues were dehydrated, embedded in paraffin and sectioned at 5  $\mu$ m. Slides were stained with hematoxylin and eosin and analyzed by light microscopy.

## Results and Discussion

**Efficacy of ethionine with and without methionine depletion on Yoshida sarcoma cells in vitro.** Ethionine at 50 - 100  $\mu$ M had no effect on the growth of the Yoshida sarcoma *in vitro* when methionine was present at 10  $\mu$ M in the medium. Even at a low methionine concentration of 5  $\mu$ M, ethionine at 50  $\mu$ M had no effect on the growth of Yoshida sarcoma *in vitro*. However, 500  $\mu$ M ethionine in medium containing 5  $\mu$ M methionine completely inhibited the growth of Yoshida sarcoma cells. Figure 1 shows that ethionine had an inhibitory effect on Yoshida sarcoma cell growth only when the ratio of methionine / ethionine in the medium was 1:20 or lower. Thus methionine depletion is essential for ethionine to have an antitumor effect *in vitro*. These data led to the hypothesis that methionine would have to be depleted *in vivo* in order that ethionine have an antitumor effect *in vivo*.

**Anti-tumor efficacy of ethionine with and without methionine depletion in vivo.** Table I and Figure 2 show that the percent inhibition of Yoshida sarcoma growth in nude mice on the methionine-free diet was 64% compared to control tumor-

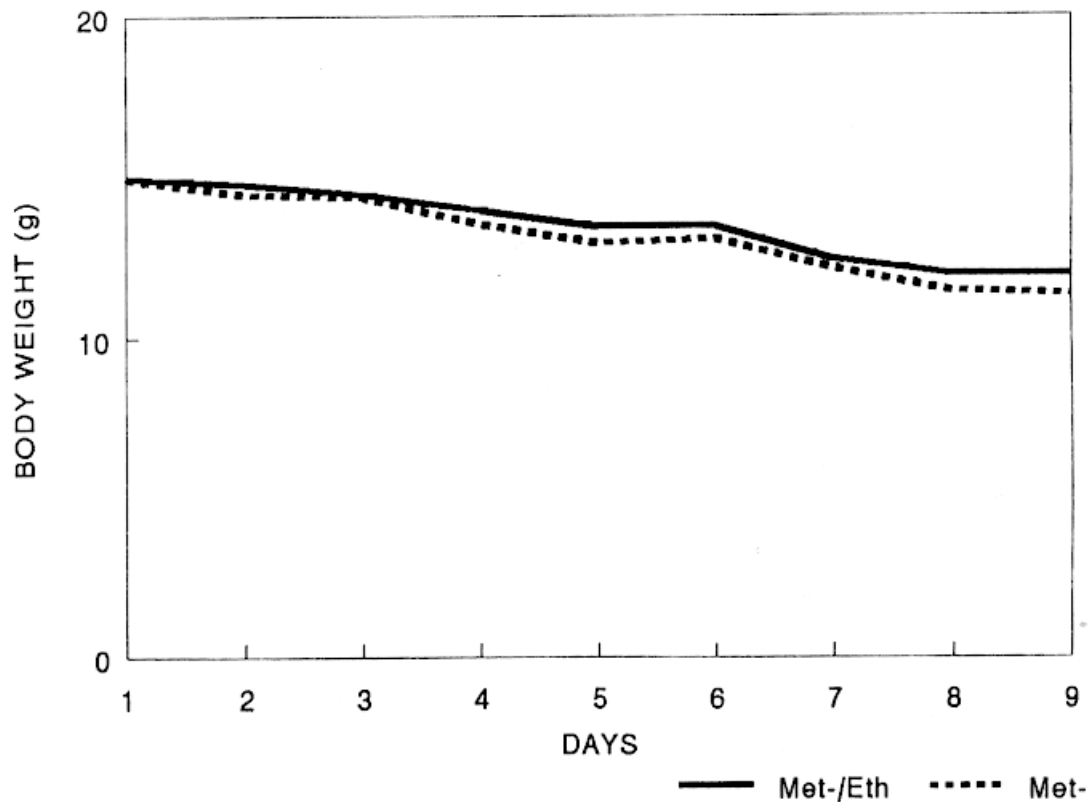


Figure 3. Effect of Ethionine and Methionine Depletion on Body Weight of Tumor-Bearing Nude Mice.  $8 \times 10^5$  Yoshida sarcoma cells in suspension were injected in an axillary site in each nude mouse. Mice were treated as in Figure 2.

bearing animals on the methionine-containing diet. 25 mg/kg ethionine given to Yoshida sarcoma - bearing nude mice on the methionine-free diet resulted in an 87.1% inhibition rate compared to control animals. However, in contrast 25 mg/kg ethionine given on the methionine-containing diet had only a 7.5% inhibition rate on the growth of the Yoshida sarcoma ( $p < 0.01$ ). Thus 25 mg/kg ethionine administration in the presence of a normal methionine diet had almost no effect on the growth of the Yoshida Sarcoma in nude mice but had an almost completely inhibitory effect on the tumor in animals on the methionine-free diet ( $p < 0.01$ ) (Table I and Figure 2). Ethionine at 7.5 mg/kg and 15 mg/kg had lesser effects on the growth of the Yoshida sarcoma than 25 mg/kg ethionine in nude mice on a methionine-free diet ( data not shown).

The body weights of the animals on the methionine-free diet with ethionine were similar to the body weights of the animals on the methionine-free diet only. The body weights of the animals under these two conditions remained essentially constant from day 4 to day 9 after an initial loss resulting in no animal deaths (Figure 3). It should be noted that as the body weights decreased in the initial period of the experiment there was slight tumor growth under these conditions. Tumor

Table II. Effect of ethionine and methionine depletion on tumor metastasis in nude mice.

Condition	Percentage of involved organs with metastases
MET-containing diet (MET <sup>+</sup> )	75 %
MET-free diet (MET <sup>-</sup> )	42.8 %
MET <sup>-</sup> + ETH 25 mg/kg	0

\* P < 0.01 compared to MET<sup>+</sup> control

Yoshida sarcoma cells were injected in nude mice and the animals were treated as described in Table I. Mouse tissues were fixed in 10% formalin, dehydrated, embedded in paraffin and sectioned at 5  $\mu$ m. Slides were stained with hematoxylin and eosin and analyzed by light microscopy. Organs observed for metastases included liver, lung, kidney and colon. Two mice were analyzed from the methionine-containing diet (MET<sup>+</sup>), two mice were analyzed from the methionine-free diet (MET<sup>-</sup>) and three mice were analyzed which were on the methionine-free diet and treated with ethionine (MET<sup>-</sup> + ETH 25 mg/kg).

growth then ceased in the animals on the methionine-free diet receiving ethionine after day 6.

The fact that the *in vivo* results matched the *in vitro* results so closely with respect to the methionine - depletion requirement for ethionine efficacy suggests that the *in vivo* anti-tumor and efficacy is due to the anti-methionine effect and not body weight loss seen at the beginning of the treatment period.

*Anti-metastatic efficacy of ethionine with and without methionine depletion.* The histology of the organs from untreated mice, organs from the mice on the methionine-free diet and organs from the mice treated with 25 mg/kg ethionine on the methionine-free diet was studied to observe the presence of metastases. 75% and 42.8% of organs from mice on methionine-containing and methionine-free diets, respectively, had tumor metastases (Table II). In striking contrast no metastases were found in Yoshida-sarcoma-bearing mice treated with 25 mg/kg ethionine on the methionine-free diet (Table II).

Our hypothesis was that a methionine-free diet, which partially lowers serum methionine levels (13), would make ethionine an effective competitor of methionine and thereby an effective antitumor agent (18). Given that methionine-dependent tumors have an elevated methionine requirement (1-7), it was thought that a reduced methionine source would make the tumor more susceptible to the methionine analogue ethionine than normal tissue. The results described in this report support this hypothesis. Both *in vitro* and *in vivo*, ethionine was only effective against the methionine-dependent Yoshida sarcoma under the conditions of a limiting methionine source. Thus although the methionine-free diet slowed the growth of Yoshida Sarcoma, ethionine could essentially totally arrest the growth of the tumor and most importantly prevent metastasis formation when administered to the mice under the methionine-limiting condition. Recently reported data indicate clinical efficacy of methionine-free TPN in combination with 5-fluorouracil in a clinical trial of gastric cancer (17). The broad spectrum of methionine dependence in cancer and the possibility to deplete methionine *in vivo* enzymatically as well as by diet or TPN, thereby modulating small - molecule methionine analogs indicates a high therapeutic potential for anti-methionine chemotherapy.

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