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Adjuvant Oral Recombinant Methioninase Inhibits Lung Metastasis in a Surgical Breast-Cancer Orthotopic Syngeneic Model

NORIHIKO SUGISAWA[1,2,3], KAZUYUKI HAMADA[1,2], QINGHONG HAN[1], JUN YAMAMOTO[1,2], YU SUN[1,2], HIROTO NISHINO[1,2], KEI KAWAGUCHI[3], MICHAEL BOUVET[2], MICHIAKI UNNO[3] and ROBERT M. HOFFMAN[1,2]

[1]*AntiCancer, Inc., San Diego, CA, U.S.A.;*
[2]*Department of Surgery, University of California, San Diego, CA, U.S.A.;*
[3]*Department of Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan*

Abstract. *Background/Aim: In the present study, we evaluated the efficacy of adjuvant administration of oral recombinant methioninase (o-rMETase) against recurrence and metastasis in a 4T1 murine breast-cancer syngeneic model. Materials and Methods: 4T1 cells were orthotopically implanted into the 2nd mammary fat pad of BALB/c mice. The 4T1 orthotopic syngeneic models were randomized into 2 groups after primary tumor resection: untreated control and o-rMETase (100 units, oral, daily, 2 weeks). Results: The frequency and extent of local recurrence were reduced by o-rMETase. The number of individual cancer cells and metastatic nodules on the lung surface was significantly lower in the o-rMETase-treated mice than the untreated control mice. Conclusion: Adjuvant o-rMETase inhibited local recurrence and lung metastasis after primary tumor resection.*

Methionine addiction is possibly the most fundamental and general hallmark of cancer (1-9) and is tightly linked to other hallmarks of cancer (2). Methionine addiction was discovered in our laboratory and is due to increased rates of transmethylation compared to normal cells (3-6). Methionine overuse by cancer cells is called the "Hoffman effect", analogous to the "Warburg effect" of glucose overuse by cancer cells (7). Methionine restriction targets the methionine addiction of cancer. For methionine restriction, we have used L-methionine-deamino-mercapto-methane-lyase (methioninase) (8, 9). Recombinant methioninase (rMETase) has been produced in *Escherichia coli* containing the methioninase gene cloned from *Pseudomonas putida* (9). We have reported the efficacy of oral administration of rMETase (o-rMETase) on various types of tumors *in vivo*, including patient-derived orthotopic xenograft (PDOX) mouse models (10-16).

Breast cancer needs follow-up for long periods after treatment. Although distant recurrence can be detected mostly in the first 5 years, there is still a significant rate of recurrence in the second and the third 5-year periods (17, 18). In order to reduce recurrence and improve survival, systemic adjuvant therapy is administered as the standard of care. Adjuvant endocrine therapy for at least 5 years is recommended for patients with hormone receptor-positive breast cancer (19, 20). Oral endocrine therapy is tolerable long term. However, patients with hormone receptor-negative breast cancer are recommended to take one regimen of adjuvant chemotherapy after surgery and be frequently followed-up for over 5-years. If a novel treatment, tolerable for 5 years, decreases the recurrence rate, it would be recommended for patients with hormone receptor-negative breast cancer.

Murine breast cancer cell lines have been used to generate orthotopic syngeneic models (21-25), especially the 4T1 cell line which has the characteristics of human triple-negative breast cancer (TNBC) and is highly metastatic (21-24). Additionally, the lung is the most frequent site for metastasis in 4T1 orthotopic models. In the present study, we evaluated the efficacy of adjuvant o-rMETase on local recurrence and metastasis after primary-tumor resection in a 4T1 murine breast-cancer orthotopic syngeneic model.

Correspondence to: Robert M. Hoffman, AntiCancer, Inc., 7917 Ostrow St., San Diego, CA 92111, U.S.A. Tel: +1 8586542555, Fax: +1 8582684175, e-mail: all@anticancer.com; Michiaki Unno, Department of Surgery, Tohoku University Graduate School of Medicine, 1-1, Seiryo-machi, Aoba-ku, Sendai, 980-8574, Japan. Tel: +81 227177201, Fax: +81 227177209, e-mail: m_unno@surg.med.tohoku.ac.jp

Key Words: Breast cancer, surgical resection, recurrence, methionine restriction, recombinant methioninase, lung metastasis, inhibition, adjuvant therapy.

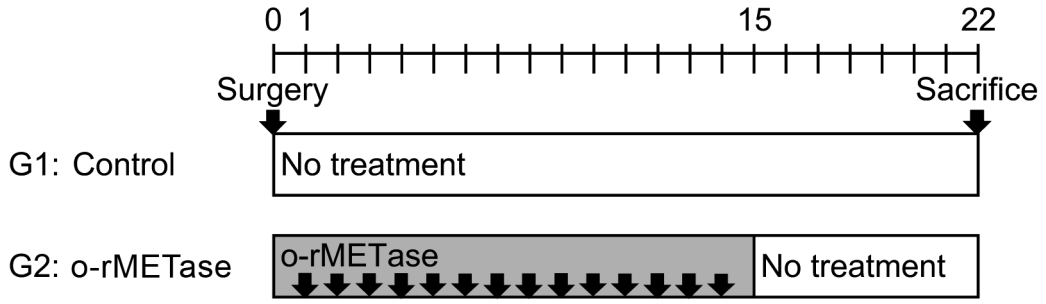


Figure 1. *Treatment protocol. The mice were randomized into 2 groups of 3 mice each.*

A

	Control	o-rMETase
Local recurrence		
(+)	**3**	**1**
(-)	**0**	**2**

B

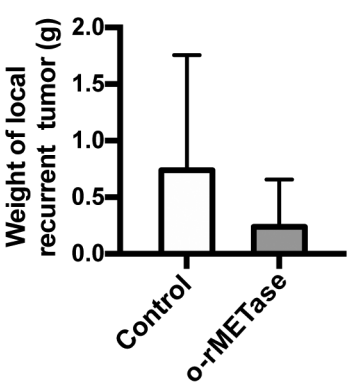


Figure 2. *Efficacy of treatment on local recurrence on day 22. (A) Frequency of local recurrence in each group. (B) Bar graphs show weight of local recurrent tumor. Error bars:±SD.*

Materials and Methods

Mice. BALB/c mice (AntiCancer, Inc., San Diego, CA, USA) were used in this study. Animal housing and their diet were as previously described (26). Mice were observed on a daily basis and humanely sacrificed by CO_2 inhalation if they met the humane endpoint criteria as previously described (26). The protocol was approved by an AntiCancer Inc. Institutional Animal Care and Use Committee (IACUC). All mice were handled according to the principles and procedures provided in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

Orthotopic implantation of 4T1 cells. The 4T1 murine breast cancer cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin at 37˚C in a 5% CO_2 incubator. 4T1 cells (1×10^6) in phosphate-buffered saline were implanted into the 2nd mammary fat pad of 6-week old BALB/c mice.

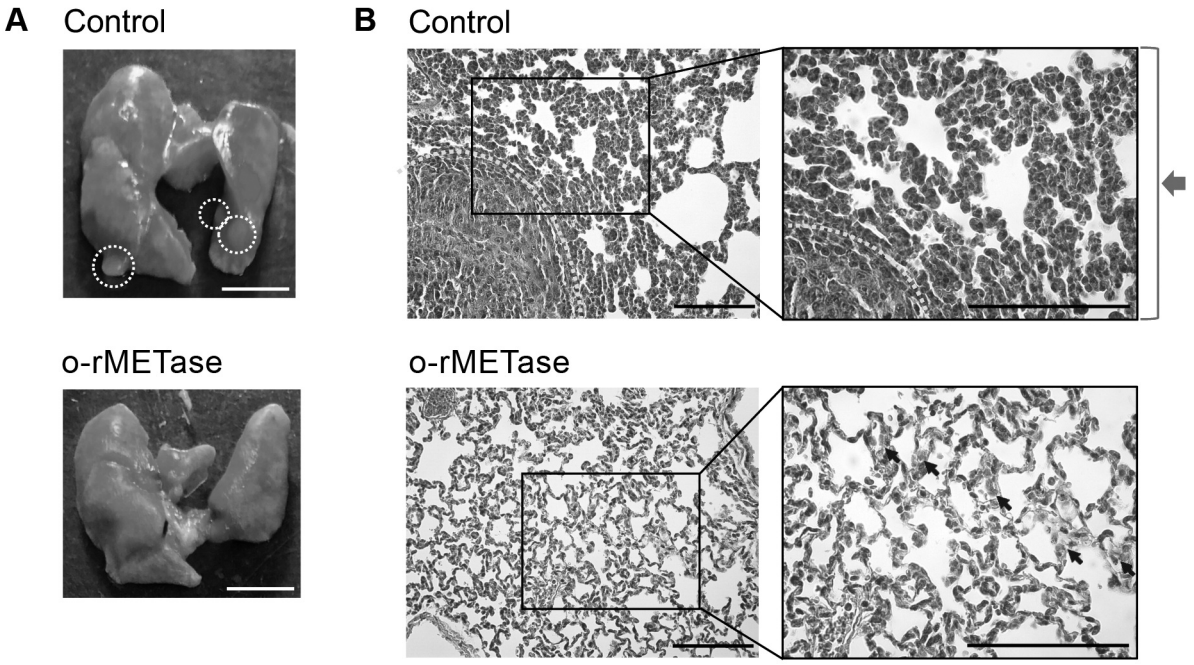
Resection of primary tumor. Primary tumor was resected under anesthesia 2 weeks after implantation. The main drainage vein flowing into the subclavian vein was ligated with a 7-0 polypropylene suture (AD Surgical, Sunnyvale, CA, USA) in order to avoid fatal bleeding. The wound was closed with a 6-0 nylon suture (AD Surgical) after confirming hemostasis.

rMETase production. The methioninase gene was originally cloned from *Pseudomonas putida* and inserted in *Escherichia coli,* which was used to produce rMETase as previously described (9). rMETase was dissolved in PBS for oral gavage at 500 units/ml.

Treatment of the 4T1 murine breast cancer model. The 4T1-bearing mice were randomized into 2 groups of 3 mice each after primary tumor resection at 2 weeks after tumor implantation (Figure 1): G1: untreated control; G2: oral rMETase (o-rMETase) (100 units, oral, daily, 2 weeks). Mouse body weight was measured twice a week. After 3 weeks, local recurrence was determined and the number of metastatic nodules on the lung surface was macroscopically counted.

Histological examination. Local recurrent primary tumors and metastatic lungs were fixed in 10% formalin. These tissue samples were embedded in paraffin, and then hematoxylin and eosin (H&E) staining was performed according to standard protocol. The H&E-stained slides were observed with a model BH2 microscope (Olympus Corp., Tokyo, Japan).

Statistical analysis. The data are presented as the mean±SD, and the Student's *t*-test was performed to evaluate the differences between the means. $p<0.05$ was considered to be statistically significant.



C

	Control	o-rMETase
Lung metastasis		
(+)	**3**	**2**
(-)	**0**	**1**

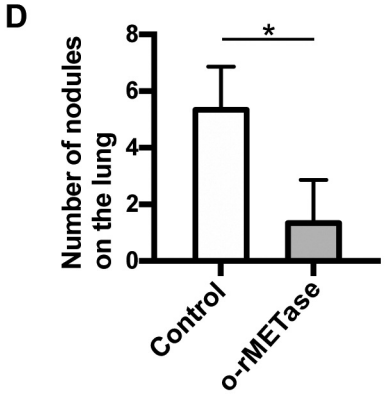


Figure 3. *Efficacy of treatment against post-resection lung metastasis on day 22. (A) Representative photos of resected lungs from each group. Metastatic nodules are shown in white dotted circles. Scale bars: 5 mm. (B) Representative images of histology with H&E staining of the lung of each group. Dotted line shows the margins of a metastatic nodule. Thick arrow indicates extensive cancer cells in the lung of an untreated control mouse. Small black arrows show individual cancer cells in the lung of the o-rMETase-treated mouse. Scale bars: 100 μm. (C) Frequency of lung metastasis in each group. (D) Bar graphs show the number of the nodules on the lung surface in each group. Error bars:±SD. *p<0.05.*

Statistical analyses were conducted with GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Efficacy of treatment on local recurrence. Although all three mice in the untreated control had local recurrence by day 22, only one mouse treated with o-rMETase for 2 weeks had local recurrence (Figure 2A). The weight of the local recurrent tumor tended to be less in the o-rMETase-treated mice [0.24 g±0.42 (mean±SD)] than in the untreated control [0.74 g±1.01 (mean±SD)] (p=0.47) (Figure 2B).

Efficacy of treatment on lung metastasis. Figure 3A shows representative photos of the lungs in each group. Figure 3B shows representative images of H&E-stained histological slides of the lungs in each group. In the untreated control, numerous cancer cells infiltrated into the pulmonary wall, which was replaced with cancer cells and became thickened, and subsequently metastatic nodules arose. In contrast, much fewer cancer cells infiltrated into the pulmonary wall in the o-rMETase-treated mice which had a much milder effect with only slight pulmonary-wall thickening. Lung metastasis was macroscopically detected on day 22 in all three mice in

the untreated control and in 2 of 3 (66.7%) mice in the o-rMETase-treated group (Figure 3C). However, the number of metastatic nodules on the lung surface in o-rMETase-treated mice [1.3±2.3 (mean±SD)] was significantly lower than that in the untreated control (5.3±1.5 (mean±SD)) (p=0.032) (Figure 3D).

Effect of treatment on body weight. All mice completed the treatment as scheduled, and there was no mortality. There was no significant difference in relative body weight on day 22 between the untreated control [1.13±0.02 (mean±SD)] and the o-rMETase-treated group [1.19±0.12 (mean±SD)] (p=0.46) (Figure 4).

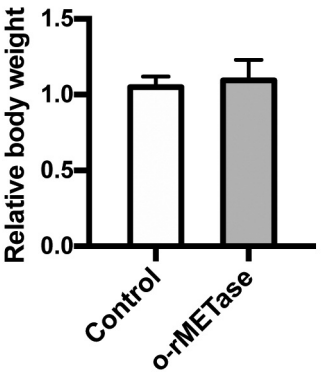


Figure 4. *Bar graphs showing relative body weight on day 22. Error bars:±SD.*

Discussion

In the present study, adjuvant o-rMETase significantly reduced the number of metastatic nodules on the lung surface of BALB/c mice, which were observed 22 days after surgical resection at the primary tumor. Histological examination showed that metastases occurred in both the untreated-control mice and o-rMETase-treated mice. However, a higher number of cancer cells infiltrated into the lung in the untreated control, and significantly more tumor nodules were observed in the lungs of the untreated-control mice than in the o-rMETase-treated mice. In addition, all 3 mice in the untreated control had local recurrence, while only one mouse treated with o-rMETase had local recurrence. In addition, the recurrent tumor in the o-rMETase-treated group tended to be smaller than those in the untreated control. No side effects, including body weight loss were observed in the o-rMETase-treated group. The present study suggests that adjuvant o-rMETase might be tolerable for long periods. Future studies will determine whether treatment with o-rMETase for 3 weeks or more will result in better control of lung metastasis and extend survival.

Methionine restriction targets the methionine addiction of cancer cells (27, 28). We discovered methionine addiction in cancer cells almost 50 years ago (1-5, 29-32), which was recently claimed as novel (33, 34). rMETase can be used clinically as an effective oral supplement (35). o-rMETase is very effective for methionine depletion in the plasma of patients (35) and mice (10, 13, 36), and has many benefits, especially the lack of immunological reactions, compared to injected rMETase (37).

Our previous studies showed that the efficacy of o-rMETase was greater when combined with chemotherapy drugs (10-16), a concept we demonstrated in 1986 (38). In the present study, adjuvant o-rMETase for 2 weeks was possibly not sufficient to totally inhibit micrometastasis. The present study was a proof of concept that o-rMETase can inhibit lung metastasis in aggressive breast cancer in the adjuvant setting. Therefore, we plan a future study where adjuvant chemotherapy will be combined with o-rMETase.

In conclusion, adjuvant o-rMETase inhibited local recurrence and lung metastasis after primary tumor resection without any side effects in a 4T1 murine breast cancer orthotopic syngeneic model. The results of the present study suggest the clinical potential of o-rMETase for aggressive breast cancers in the adjuvant setting.

Conflicts of Interest

The Authors declare that there are no potential conflicts of interest. AntiCancer, Inc. uses 4T1 cell syngeneic models for contract research. QH is an employee of AntiCancer, Inc. NS, KH, JY, YS, HN, and RMH are or were unsalaried associates of AntiCancer, Inc.

Authors' Contributions

Conception and design: NS and RMH. Acquisition of data: NS and KH. Analysis and interpretation of data: NS, KH, QH, JY, YS, HN, KK, MB, MU, and RMH. Writing, review, and/or revision of the manuscript: NS, MU, and RMH.

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