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Engineered Methioninase-expressing Tumor-targeting *Salmonella typhimurium* A1-R Inhibits Syngeneic-Cancer Mouse Models by Depleting Tumor Methionine

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Abstract

Background/Aim: We previously developed *Salmonella typhimurium* A1-R, which selectively targets and kills tumors. In the present study, we established recombinant methioninase (rMETase)-producing *Salmonella typhimurium* A1-R (A1-R-rMETase), by transfer of the *Pseudomonas putida methioninase* gene, to target methionine addiction of syngeneic-cancer mouse models.

Materials and Methods: A plasmid containing the *Pseudomonas putida methioninase* gene was extracted from METase-producing recombinant *E. coli* and inserted into *Salmonella typhimurium* A1-R using electroporation. Lewis Lung Carcinoma (LLC) cells (10^6) were injected subcutaneously in male C57BL/6 mice aged 4-6 weeks. We determined that 10^8 *Salmonella typhimurium* A1-R-rMETase administered iv was a safe dosage in C57BL/6 mice and was used for efficacy studies on LLC tumors in C57BL/6 mice. Tumor size was measured with calipers three times per week for 3 weeks. On day 22, tumor methionine levels were measured using HPLC in the control mice injected with phosphate-buffered saline (PBS) and the mice injected with *Salmonella typhimurium* A1-R-rMETase.

Results: The mean LLC tumor size of each group on day 22 was as follows: PBS control: 741.5 mm^3; mice injected with *Salmonella typhimurium* A1-R: 566.3 mm^3 (p=0.370); and mice injected with *Salmonella typhimurium* A1-R-rMETase: 198.8 mm^3 (p=0.0003 *vs.* control and p=0.0117 *vs. Salmonella* A1-R). The mice injected with *Salmonella typhimurium* A1-R-rMETase showed a significantly lower mean tumor methionine level than mice injected with PBS (5.9 nM/mg protein *vs.* 11.1 nM/mg protein, p=0.0095). *Salmonella typhimurium* A1-R-rMETase grew continuously in the tumors but not in the liver or spleen.

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Conclusion: Tumor-targeting *Salmonella typhimurium* A1-R engineered to express the *Pseudomonas putida methioninase* gene, inhibited LLC tumor growth in a syngeneic mouse model and reduced the methionine level in the tumor. *Salmonella typhimurium* A1-R-rMETase combines the tumor targeting and killing capability of *Salmonella typhimurium* A1-R plus rMETase which targets the methionine addiction of cancer.

Keywords: *Salmonella typhimurium* A1-R, methioninase-producing *Salmonella typhimurium* A1-R, tumor targeting, Lewis lung carcinoma, C57BL/6 mice, tumor inhibition, tumor methionine depletion.

Introduction

Methionine addiction of cancer is a general and fundamental hallmark of cancer and is termed the Hoffman effect (1-4). Methionine addiction has been targeted as a cancer vulnerability by methionine restriction (5-8). Recombinant methioninase (rMETase) (9-11), and most recently oral administration of rMETase (o-rMETase) have been shown to be effective for methionine restriction. o-rMETase does not enter the blood stream and therefore has not shown side-effects in preclinical mouse models or initial clinical studies (12-20). o-rMETase has shown efficacy on all types of cancer in various mouse models including patient derived orthotopic xenograft (PDOX) models (12, 13, 21-32). Initial clinical reports have demonstrated efficacy of o-rMETase, especially in combination with first-line therapy (14-20).

Methionine restriction may inhibit the activity of CD8-positive T-lymphocytes by lowering their intracellular levels of methionine and the methyl donor S-adenosylmethionine (SAM) resulting in a loss of dimethylation at lysine 79 of histone H3 (H3K79me2) (33). Therefore, it would be desirable to deplete methionine selectively in the tumor for greater efficacy. We previously showed the efficacy of bacterial therapy using rMETase-producing *Escherichia coli* (*E. coli*) in mouse models of cancer (34, 35). *Salmonella typhimurium* A1-R has been shown to selectively target tumors of all major types without infecting normal tissue (36-39).

Recently *Salmonella typhimurium* strain VNP20009, expressing a methionine-degrading enzyme, has shown efficacy in preclinical mouse models (40, 41). The present report describes the strong efficacy of tumor-targeting *Salmonella typhimurium* A1-R expressing methioninase on syngeneic mouse models of lung cancer.

Materials and Methods

Transformation of Salmonella typhimurium A1-R with the methioninase gene. A plasmid containing the *Pseudomonas putida methioninase* gene was extracted from rMETase-producing *E. coli* JM109 (AntiCancer Inc., San Diego, CA, USA) using ZR Plasmid Miniprep–Classic (ZYMO RESEARCH, Irvine, CA, USA). The rMETase gene is contained in plasmid pATG3131, which also includes the tetracycline (TC)-resistance gene. For further details regarding this plasmid, please refer to previous reports (9, 10). *Salmonella typhimurium* A1-R (AntiCancer Inc.) was cultivated at 37˚C until reaching the mid-logarithmic phase in Luria-Bertani (LB) liquid medium and then collected at 4˚C. 2.0×10^8 colony forming units (CFU) *Salmonella typhimurium* A1-R were combined with 2 μl of the rMETase plasmid in 40 μl 10% glycerol. The mixture was then placed on ice for 5 min before being electroporated using a Gene Pulser (Bio-Rad, Hercules, CA, USA), following the instructions provided by the manufacturer. The electroporation procedure was performed at a voltage of 1.8 kV, applying a pulse controller with a parallel resistance of 1,000-Ω. Subsequently, *Salmonella-Shigella* agar culture was used to identify bacteria as *Salmonella* by the presence of black colonies. The activity of rMETase produced by these bacteria was determined from α-ketobutyrate produced from L-methionine, as reported previously (34, 42).

Correlation of OD600 and colony-forming units of Salmonella typhimurium A1-R-rMETase. Salmonella typhimurium A1-R-rMETase was grown overnight in LB liquid medium and then diluted 1:100 in LB liquid medium the next morning. Appropriately-diluted culture liquid was re-cultured at 37˚C and harvested every hour until 8 h with O.D.600 measurements performed for each sample. Every sample was serially diluted 10-fold 4 to 7 times in PBS. Diluted culture liquid was placed on LB agar with 20 μg/ml TC at 5 locations, 20 μl each, and incubated at 37˚C overnight. Colonies were counted to determine colony-forming units (CFU/ml) at each time point.

Cancer-cell culture. Human colon-cancer cells (HT-29), prostate-cancer cells (PC-3), breast-cancer cells (MDA-MB-435), and mouse lung-cancer cells (Lewis Lung Carcinoma (LLC) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum (FBS) and 100 IU/ml of penicillin/streptomycin.

Infection assay of Salmonella typhimurium A1-R-rMETase on cancer cell lines. HT-29 colon-cancer, PC-3 prostate-cancer, MDA-MB-435 breast-cancer, and LLC lung-cancer (10^4 cells) were grown overnight in 24-well tissue-culture plates with 500 μl DMEM. *Salmonella typhimurium* A1-R-rMETase was grown to the late-log phase in LB liquid medium. The bacteria were diluted to 2×10^7 CFU/mL in DMEM, and 500 μl (1×10^7 CFU) were seeded on the cancer cells and the co-culture was placed in an incubator at 37˚C for 3 h. Gentamycin (500 μl of 200 μg/ml) was added to each well to kill the external, but not internal bacteria. After 1 h, the medium was removed and washed 4 times using PBS. Triton ×100 (200 μl) was added to the well for 10 min to break cells. LB (800 μl) broth was added to each well, and of these, 20 μl were cultured on LB agar with tetracycline (20 μg/ml) overnight. After culture, the colony number was counted. The assay was repeated at least 3 times, and average colony number was calculated.

Mice. C57BL/6 mice, aged 4-6 weeks (AntiCancer Inc., San Diego, CA, USA), were used in the present study. Mice were housed in a barrier facility with a HEPA-filtered rack and 12 h light/dark cycles. During this study, mice were fed an autoclaved laboratory rodent diet. The AntiCancer Institutional Animal Care and Use Committee's ethical committee approved the present mouse studies. All experiments were conducted according to Animal Research: Reporting of In Vivo Experiments (ARRIVE) 2.0 criteria (43).

Tolerability of Salmonella typhimurium A1-R-rMETase in C57BL/6 mice. Two C57BL/6 mice each were injected with 10^7, 10^8, and 10^9 CFU of *Salmonella typhimurium* A1-R-rMETase, respectively, through the tail vein to evaluate tolerability. The injections were administered twice a week for 2 weeks.

Establishment of subcutaneous tumors. Thirty C57BL/6 male mice were injected subcutaneously with LLC cells (10^6) in the right flank. By one week after injection, subcutaneous tumors were established.

Quantification of Salmonella typhimurium A1-R-rMETase in tumors and organs over time after tail-vein injection. Three mice were given of *Salmonella typhimurium* A1-R-rMETase (10^8 CFU) *via* tail-vein injection after the subcutaneous LLC tumors had grown. The subcutaneous tumor, liver, and spleen were removed from 3 mice each after 24 h, 72 h, and 168 h, respectively. Tumor, liver, and spleen were homogenized, and supernatants were plated on LB agar with TC (20 μg/ml). The *Salmonella* A1-R-rMETase colony number per gram of tumor or organ was calculated.

Treatment of LLC tumors in C57BL/6 mice with Salmonella A1-R vs. Salmonella A1-R-rMETase. After tumor growth, 18 mice were divided into three groups of 6 (Figure 1). One group was injected with PBS *via* the tail vein, twice per week as a control. Another group was injected with *Salmonella typhimurium* A1-R (10^8) *via* the tail vein, twice per week. Another group was injected with *Salmonella typhimurium*

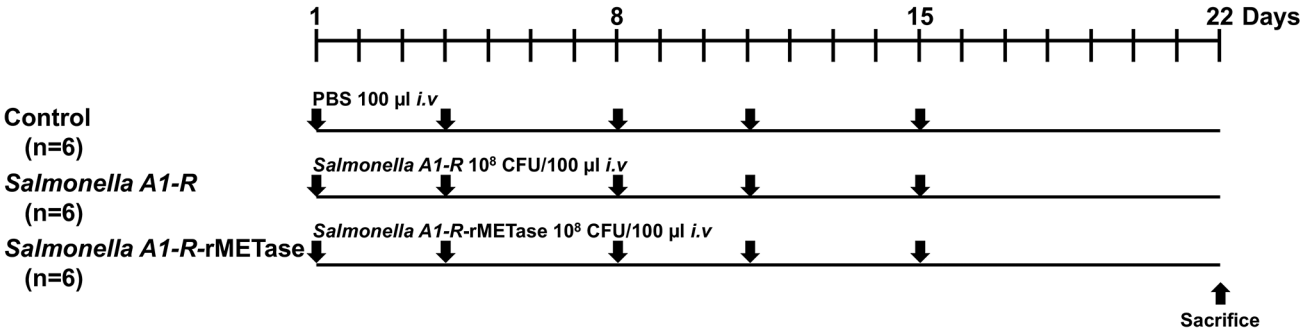

Figure 1. *Treatment protocol. The mice were randomized into 3 groups of 6 mice each: Group 1: control, treated with phosphate-buffered saline (PBS), 100 µl iv on days 1, 4, 8, 11, 15. Group 2: Treated with Salmonella typhimurium A1-R, 10^8 colony forming units (CFU)/100 µl PBS iv on days 1, 4, 8, 11, 15. Group 3: Treated with Salmonella typhimurium A1-R-rMETase, 10^8 colony forming units (CFU)/100 µl PBS iv on days 1, 4, 8, 11, 15. Please see Materials and Methods for details.*

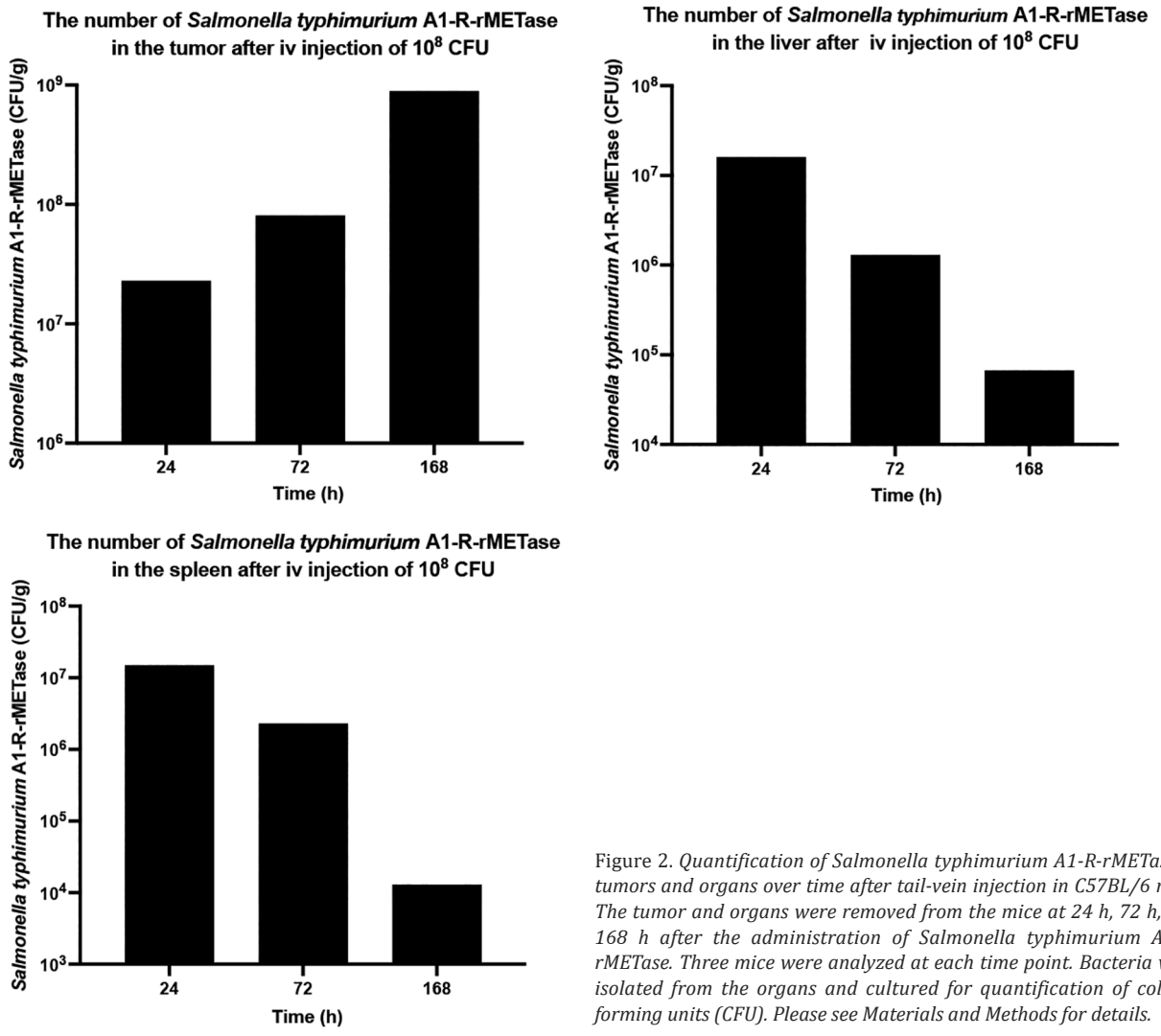

Figure 2. *Quantification of Salmonella typhimurium A1-R-rMETase in tumors and organs over time after tail-vein injection in C57BL/6 mice. The tumor and organs were removed from the mice at 24 h, 72 h, and 168 h after the administration of Salmonella typhimurium A1-R-rMETase. Three mice were analyzed at each time point. Bacteria were isolated from the organs and cultured for quantification of colony-forming units (CFU). Please see Materials and Methods for details.*

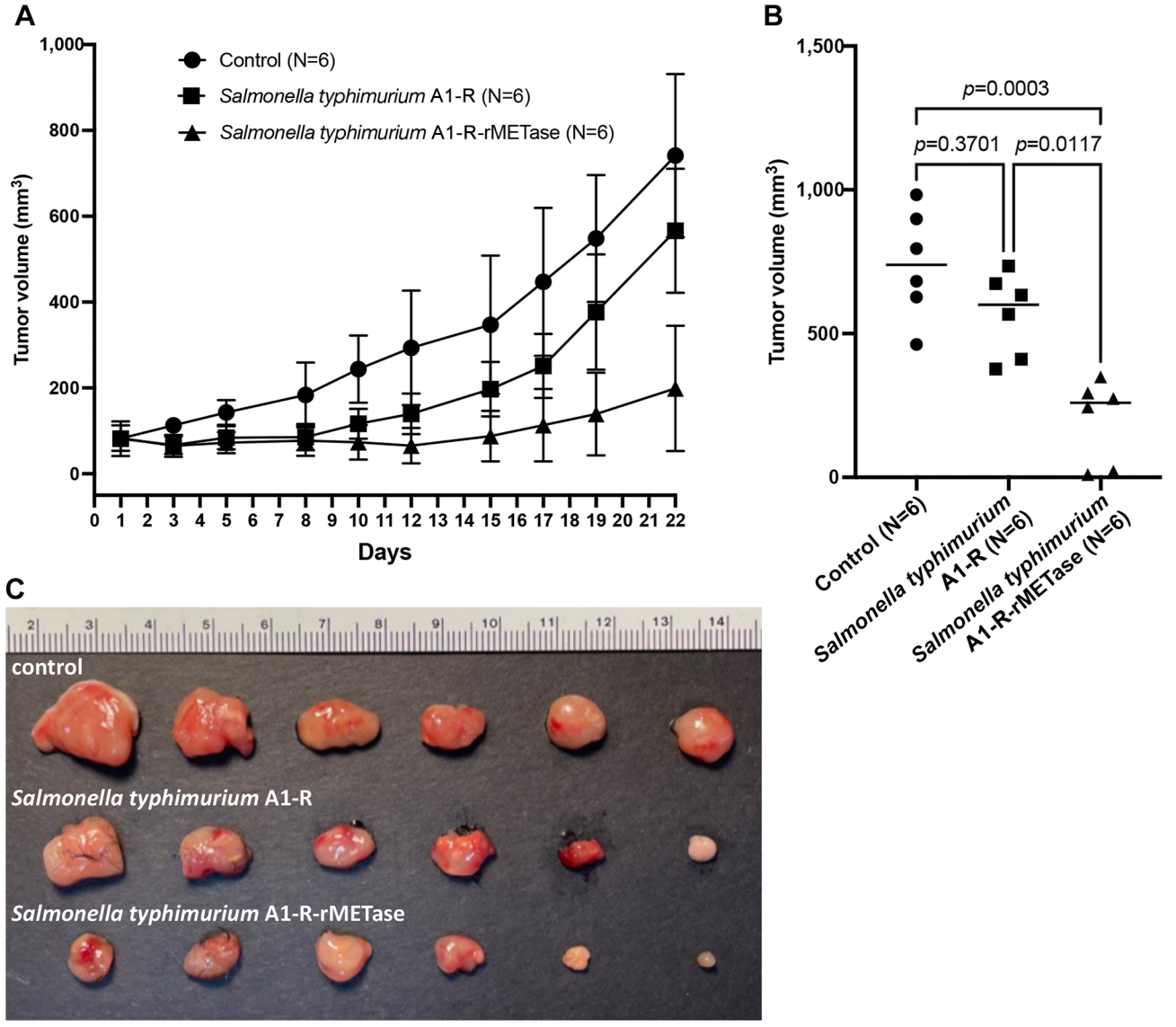

Figure 3. *Efficacy of Salmonella typhimurium A1R and Salmonella typhimurium A1-R-rMETase on the syngeneic LLC lung-cancer in C57BL/6 mice. (A) Tumor growth curves. (B) Tumor size on day 22. There were 6 mice in each group: Group 1: control treated with phosphate-buffered saline (PBS) 100 µl iv on days 1, 4, 8, 11, 15. Group 2: treated with Salmonella typhimurium A1-R, 10^8 colony forming units (CFU)/100 µl PBS iv on days 1, 4, 8, 11, 15. Group 3: treated with salmonella typhimurium A1-R-rMETase, 10^8 colony-forming units (CFU)/100 µl PBS iv on days 1, 4, 8, 11, 15. (C) Photographs of tumors excised from the mice of each group on day 22. Please see Materials and Methods for details.*

A1-R-rMETase (10^8) *via* the tail vein, twice per week for two weeks (Figure 1). Tumor size was measured with calipers three times per week for 3 weeks. On day 22, the intra-tumor methionine level was measured using HPLC in the PBS control and the mice injected with *Salmonella typhimurium* A1-R-rMETase, as previously demonstrated (13, 44).

Results

Comparison of OD_{600} and colony forming units (CFU) of Salmonella typhimurium A1-R-rMETase. The relationship between OD_{600} and *Salmonella typhimurium* A1-R-rMETase CFU had an r^2 of 0.77. An OD600 of 1.6, which

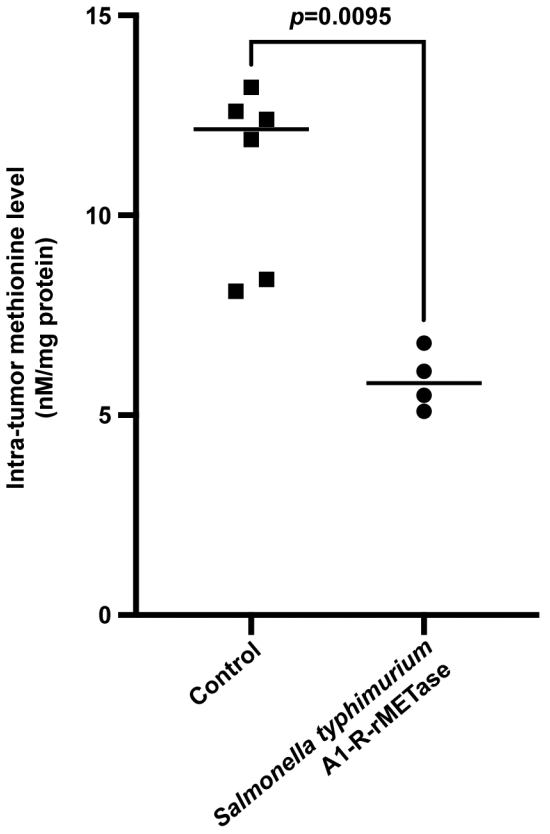

Figure 4. *Intra-tumor methionine level at day 22 in mice treated with PBS (6 mice) or Salmonella typhimurium A1-R-rMETase (4 mice). Please see Materials and Methods for details.*

corresponds to late-log phase bacterial growth, comprises approximately 3.0×10^9 CFU/ml *Salmonella typhimurium A1-R-rMETase.*

rMETase production by Salmonella typhimurium A1-R-rMETase. A total of 10^{10} CFU/ml of *Salmonella typhimurium* A1-R-rMETase produced 17.2 U/ml of rMETase after adding isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium. Interestingly, the bacteria produced rMETase without IPTG, at 17.4 U/ml (10^{10} CFU).

Selection of Salmonella typhimurium A1-R-rMETase that efficiently infects LLC in vitro. LLC was chosen for *in vivo* studies as it supported extensive growth of *Salmonella typhimurium* A1-R-rMETase *in vitro* (data not shown). *Salmonella typhimurium* A1-R-rMETase growing in LLC cells was collected. Then, LLC cells were infected with the collected *Salmonella typhimurium* A1-R-rMETase again. We repeated this three times and selected a specific *Salmonella typhimurium* A1-R-rMETase, which infected LLC cells at high levels. This *Salmonella typhimurium* A1-R-rMETase sub-strain was used for the subsequent *in vivo* study.

Tolerability of Salmonella typhimurium A1-R-rMETase in C57BL/6 mice. C57BL/6 mice injected with 1×10^7 or 1×10^8 CFU of *Salmonella typhimurium* A1-R-rMETase maintained their body weight for 2 weeks. Mice injected with 1×10^9 CFU of *Salmonella typhimurium* A1-R-rMETase lost approximately 20% of their body weight by day 3. According to this result, 1×10^8 CFU of *Salmonella typhimurium* A1-R-rMETase was injected twice a week, over 15 days, for the *in vivo* efficacy experiment.

Targeting specificity of Salmonella typhimurium A1-R-rMETase in the LLC tumor, liver, and spleen of C57BL/6 mice. Salmonella typhimurium A1-R-rMETase grew continuously in the LLC tumors for a week. The number of *Salmonella typhimurium* A1-R-rMETase CFU decreased in liver and spleen over the course of a week (Figure 2).

Efficacy of Salmonella typhimurium A1-R-rMETase on LLC tumor growth in C57BL/6 mice. The mean LLC tumor size of each group on day 22 was as follows: PBS control: 741.5 mm^3; mice injected with *Salmonella typhimurium* A1-R: 566.3 mm^3; and mice injected with *Salmonella typhimurium* A1-R-rMETase: 198.8 mm^3 (Figure 3A, B, C). Turkey's multiple comparisons test showed a significant difference between the PBS control and mice injected with *Salmonella typhimurium* A1-R-rMETase (p=0.0003) and between mice injected with *Salmonella typhimurium* A1-R and mice injected with *Salmonella typhimurium* A1-R-rMETase (p=0.0117). However, the PBS control and the mice injected with *Salmonella typhimurium* A1-R did not show a significant difference (p=0.370).

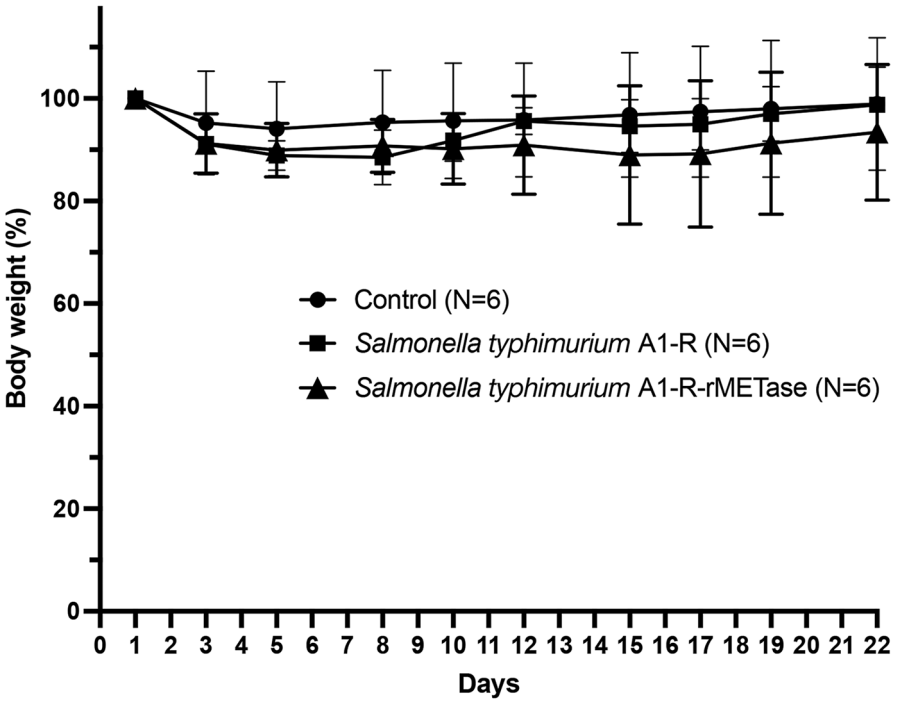

Figure 5. *Body weight change over treatment time with PBS, or Salmonella typhimurium A1-R, or Salmonella typhimurium A1-R-rMETase as described above. Data are shown as the mean±standard deviation. Mice were weighed every 2 days (n=6). Please see Materials and Methods for details.*

Effect of Salmonella typhimurium A1-R-rMETase on intra-tumor methionine levels. Mice injected with *Salmonella typhimurium* A1-R-rMETase showed a significantly lower tumor mean methionine level than mice injected with PBS 5.9 nM/mg protein *vs.* 11.1 nM/mg protein, respectively (p=0.0095, Mann Whitney test) (Figure 4).

Body weight changes. No significant change in mouse body weight for each group was found, confirming the tolerability of 10^8 CFU of *Salmonella typhimurium* A1-R-rMETase (Figure 5).

Discussion

Salmonella typhimurium A1-R-rMETase and *Salmonella typhimurium* VNP20009-SGN1 (40, 41, 45), express methioninase. A recombinant *E. coli* Nissle strain, SYNB1353, expresses a methionine decarboxylase (46). All three bacteria have shown promise for reducing methionine in vivo, including in humans. *Salmonella typhimurium* A1-R-rMETase and *Salmonella typhimurium* VNP20009-SGN1 target cancer (40, 41, 45), while SYNB1353 targets homocystinuria (46). We also previously developed a recombinant *E. coli* JM109 strain expressing rMETase at 7.9 U/ml per 10^{10} CFU which inhibited tumor growth (34), but less than the present *Salmonella typhimurium* A1-R-rMETase. All of these studies represent a new paradigm of targeting methioninase *in vivo,* including directly to the tumor or metastasis, through tumor-targeting bacteria. These bacteria grow, allowing for a continuous supply of methioninase within the tumor, primary or metastatic. It may be also possible for the methioninase-producing bacteria to become permanent components of the microbiome, thereby lowering total-body methionine.

In the present study, rMETase expressed by *Salmonella typhimurium* A1-R, which has been previously selected as a tumor-targeting strain (36-38), inhibited LLC tumor growth and decreased methionine in the tumor. Further studies are

necessary to maximize the potential of tumor targeting of methioninase with *Salmonella typhimurium* A1-R-rMETase.

rMETase is effective because it targets methionine addiction, the fundamental hallmark of cancer, termed the Hoffman effect (1-8, 47-58). Orally administered rMETase (o-rMETase) is showing clinical promise (14-20, 59-62).

Conflicts of Interest

The Authors declare no competing interests regarding this work.

Authors' Contributions

YK and RMH developed the concept of the study. YK performed experiments. YK and RMH wrote the article. KK supervised the total of this study. YA, NM, KO, SM, KM, MS, MB, HQ, ZM and TT reviewed the article.

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