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Recombinant Methioninase Combined With Tumor-targeting *Salmonella typhimurium* A1-R Induced Regression in a PDOX Mouse Model of Doxorubicin-resistant Dedifferentiated Liposarcoma

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Abstract. *Background/Aim: Dedifferentiated liposarcoma (DDLPS) is associated with a poor survival rate even with multi-modality treatment. In the present study, we evaluated the efficacy of recombinant methioninase (rMETase) combined with tumor-targeting Salmonella typhimurium (S. typhimurium) A1-R against a doxorubicin-resistant DDLPS in a patient-derived orthotopic xenograft (PDOX) mouse model. Materials and Methods: A recurrent high-grade DDLPS from the right retroperitoneum of a patient was grown orthotopically in the retroperitoneum of nude mice to establish a PDOX model. The PDOX models were randomly divided into the following groups: Control, no treatment; doxorubicin monotherapy; rMETase monotherapy; S. typhimurium A1-R monotherapy; S. typhimurium A1-R and rMETase combination therapy. Tumor length and width were measured before and after treatment. Results: On day 14 after treatment, all treatments significantly inhibited DDLPS PDOX tumor growth compared to the untreated control except for doxorubicin monotherapy. rMETase combined with S. typhimurium A1-R was significantly more effective and regressed tumor volume compared to either rMETase or S. typhimurium A1-R alone. The relative body weight did not significantly differ between days 0 and 14 for individual groups. Conclusion: The combination of rMETase and S. typhimurium A1-R has important clinical potential for this recalcitrant sarcoma.*

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Key Words: Dedifferentiated liposarcoma, PDOX, nude mice, recombinant methioninase, *S. typhimurium* A1-R, combination, tumor regression.

Methionine addiction is perhaps the most common and fundamental hallmark of cancer (1, 2). Methionine restriction specifically arrests cancer cells in the S/G_2 phase of the cell cycle (3-6). Cancer cells have a higher transmethylation rate compared to normal cells due to excessive use of and addiction to methionine (2, 7, 8). Methionine overuse in cancer cells is the "Hoffman effect" (9). The Hoffman effect for methionine is possibly stronger than the Warburg effect for glucose (10). Altered transmethylation in cancer may result in DNA hypomethylation, a phenomenon discovered in our laboratory (11-14). Methionine addiction is associated with other fundamental characteristics of cancer (15).

Methioninase (L-methioninedeamino-mercaptomethane-lyase) is a methionine-cleaving enzyme synthesized from Pseudomonas putida which was shown to cause methionine depletion in normal and nude mice. It has been cloned and expressed in *Escherichia coli* and termed recombinant methioninase (rMETase) (16).

rMETase was tested in several human cancer (lung, colon, kidney, melanoma, brain, and prostate) and non-cancer cell lines (17). The mean half-maximal inhibitory concentration (IC_{50}) of rMETase for cancer cells was much lower compared

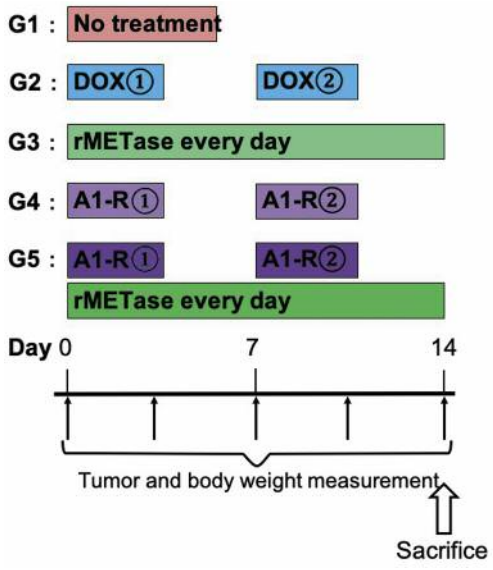

Figure 1. *Treatment schema. G1: No treatment; G2: doxorubicin (DOX); G3: recombinant methioninase (rMETase); G4: S. typhimurium A1-R (A1-R); G5: A1-R and rMETase.*

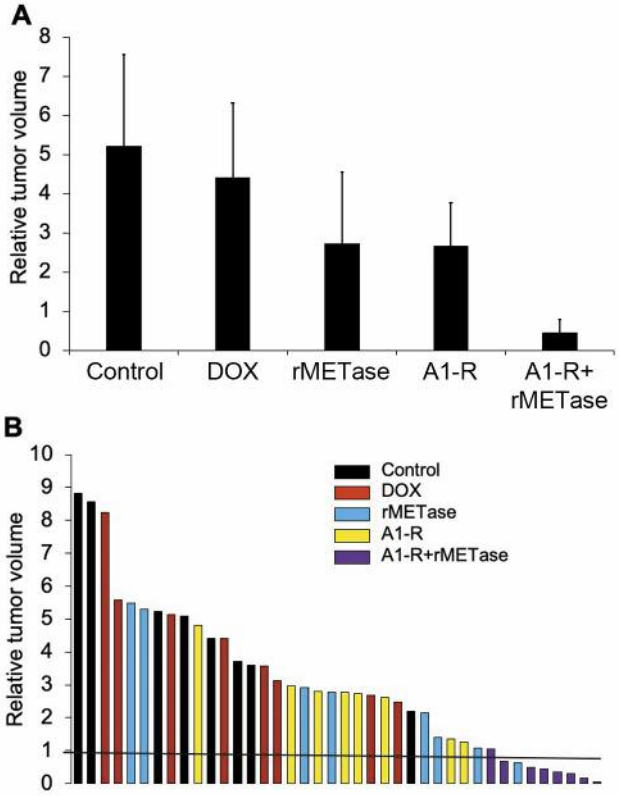

Figure 2. *Efficacy of treatment. A: Efficacy of doxorubicin (DOX), recombinant methioninase (rMETase), Salmonella typhimurium A1-R (A1-R) and S. typhimurium A1-R combined with rMETase on the dedifferentiated liposarcoma patient-derived orthotopic xenograft (PDOX). B: Waterfall plot of tumor volume at day 14 relative to the initial tumor volume for each mouse. Data are means±SD. N=8 mice/group.*

to normal cells (17). rMETase has been shown to arrest all cancer types tested in mouse models including patient-derived orthotopic xenograft (PDOX) models (18-39).

Previously, we developed tumor-targeting *Salmonella typhimurium* A1-R (40). *S. typhimurium* A1-R is auxotrophic for Leu-Arg that attenuates infection of normal tissue but allows high tumor virulence (40). *S. typhimurium* A1-R showed high effectiveness against mouse models of primary and metastatic cancer (41-57).

Soft-tissue sarcoma is a malignant tumor with high heterogeneity (58, 59). Currently, it has been divided into more than 50 subtypes, which are classified based on their tissue of origin (60). Liposarcoma is the most general type and is classified into four main groups: Well-differentiated and de-differentiated liposarcomas (DDLPS), myxoid/round-cell liposarcoma, and pleiomorphic liposarcoma (61). Among these, DDLPS is a recalcitrant sarcoma associated with poor survival (62), DDLPS has frequent recurrence, and metastasizes even with multi – modality treatment with surgery, radiation, or chemotherapy (63). Since response rates to chemotherapeutics are low (64) effective new therapy is warranted.

Previously, we demonstrated that rMETase combined with palbociclib caused regression in a PDOX model of DDLPS (26). We also demonstrated that the combination of rMETase and *S. typhimurium* A1-R was also effective against Ewing's sarcoma (25), osteosarcoma (33), and melanoma (28) PDOX models.

The present study investigated the efficacy of recombinant methioninase in combination with *S. typhimurium* A1-R on a PDOX model of doxorubicin-resistant DDLPS.

Materials and Methods

Animal care. Nude mice (4-6 weeks, athymic nu/nu; AntiCancer Inc., San Diego, CA, USA) were used. All studies were conducted with an AntiCancer Institutional Animal Care and Use Committee protocol specifically approved for this study and followed the principles and procedures in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873-1 (26). Animal suffering was reduced by using anesthesia and analgesics during surgical procedures (26). Procedures for mouse housing, handling, anesthesia, feeding, and humane endpoint criteria have been described elsewhere (26).

Patient-derived tumor. A 69-year-old male with DDLPS of the right retroperitoneum underwent radical resection with en bloc right nephrectomy (26). Two years after surgery, there was local recurrence and the patient underwent surgical resection at the Department of Surgery, University of California, Los Angeles (26). Written informed consent was obtained from the patient as part of a University of California, Los Angeles Institutional Review Board (IRB #10-001857)-approved protocol (26).

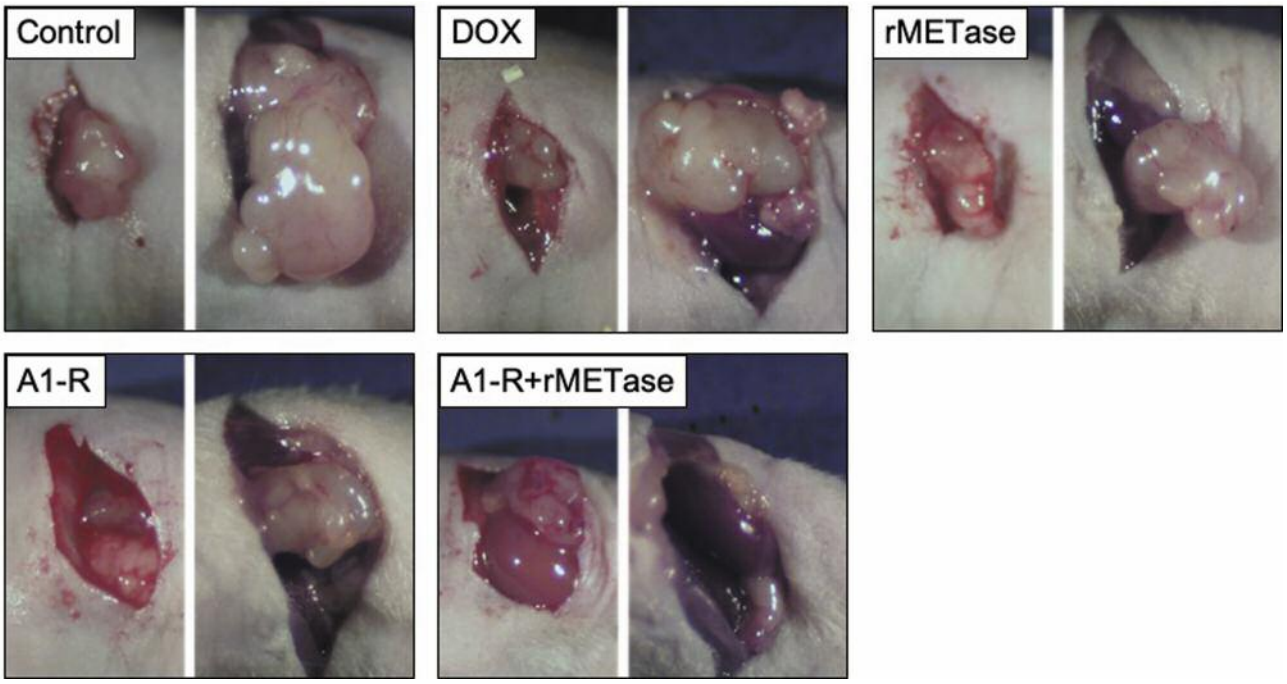

Figure 3. *Photographs of tumors in representative untreated and treated patient-derived orthotopic xenograft models of dedifferentiated liposarcoma. Treatments comprised doxorubicin (DOX), L-methionine α-deamino-γ-mercaptomethane lyase (rMETase), Salmonella typhimurium A1-R (A1-R) and A1-R combined with rMETase. For each group left is at the beginning of treatment period, while right is at the end.*

Establishment of PDOX model. Sample collection, preparation of recurrent high-grade DDLPS tumor fragments (5-mm each) and their implantation and growth in nude mice were performed as previously described (26). All experiments were carried out in the laboratory at AntiCancer, Inc. The grown tumors were cut into small fragments (3-4 mm). A 20 mm skin incision was made on the left flank of nude mice, then the obliquus externus abdominis muscle was split to reach the retroperitoneum (26). A single tumor fragment was implanted orthotopically into the space between the left kidney and retroperitoneal fat tissue to establish a PDOX model and the wound was closed with a nylon suture (26).

rMETase production. The procedures of rMETase production and purification are described elsewhere (16). rMETase was administered by intra-peritoneal injection.

Preparation and administration of S. typhimurium A1-R. Detailed procedures for culture of green fluorescent protein (GFP)-expressing *S. typhimurium* A1-R bacteria (AntiCancer Inc.) and their processing have previously been described (41, 42). *S. typhimurium* A1-R was intra-venously injected into mice with a total of 5×10^7 colony-forming units (CFU) of *S. typhimurium* A1-R in 100 μl phosphate-buffered saline for each mouse (41, 42).

Treatment study design for the DDLPS PDOX model. The DDLPS PDOX models were randomly assigned into the following groups once the tumor volume became 100 mm^3 in size: Control group; doxorubicin

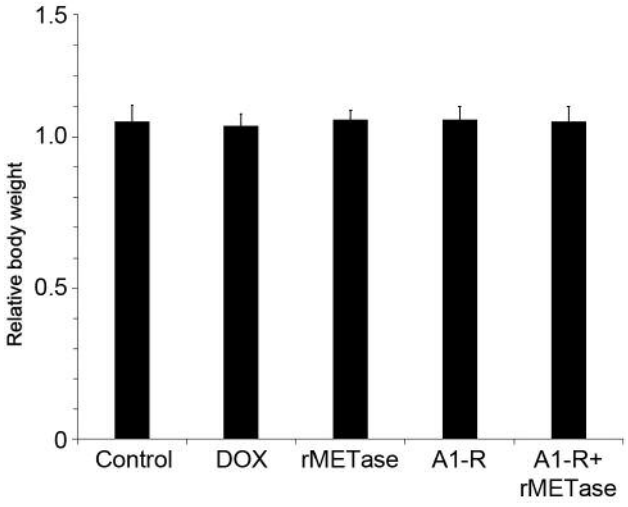

Figure 4. *Safety evaluation of doxorubicin (DOX), L-methionine α-deamino-γ-mercaptomethane lyase (rMETase), Salmonella typhimurium A1-R (A1-R) and A1-R combined with rMETase in treatment of dedifferentiated liposarcoma patient-derived orthotopic xenograft. Bar graphs show mean body weight in each group 2 weeks after drug administration relative to the initial body weight. Data are means±SD. N=8 mice/group. There were no significant differences between days 0 and 14 for any group.*

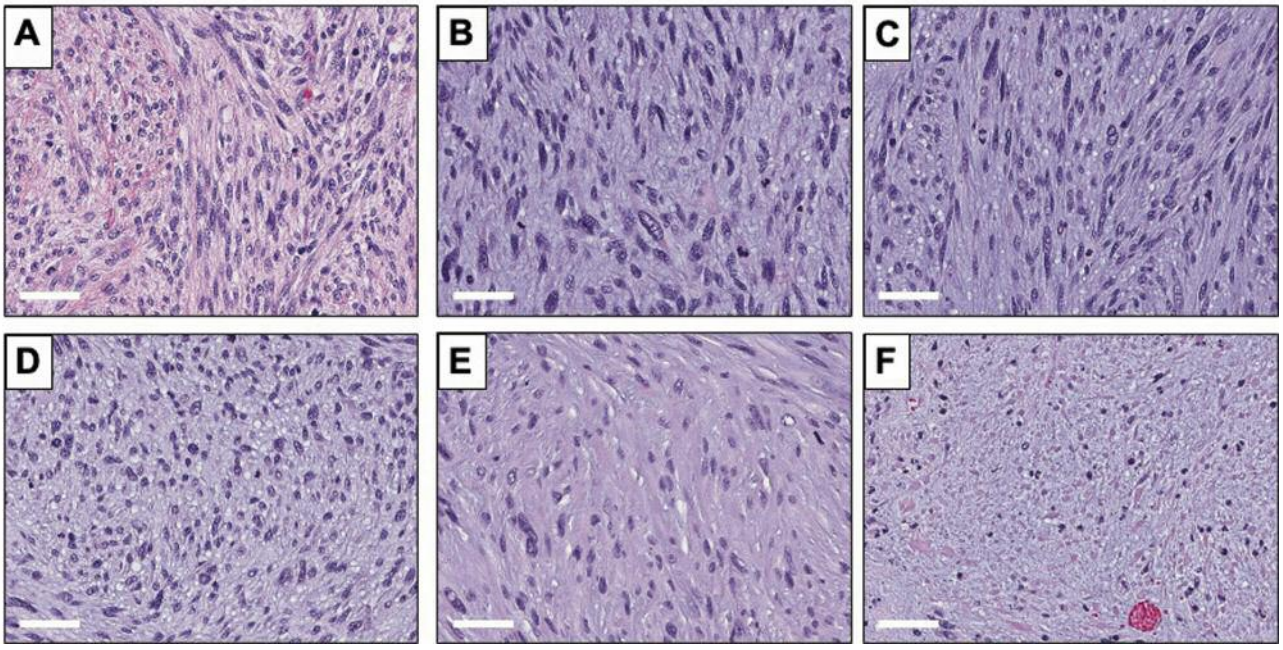

Figure 5. *Tumor histology of untreated and treated dedifferentiated liposarcoma as shown by hematoxylin and eosin (HE) staining. Section of the original patient tumor (A), untreated patient-derived orthotopic xenograft (PDOX) tumor (B), PDOX tumor treated with doxorubicin (C), PDOX tumor treated with recombinant methioninase (rMETase) (D), PDOX tumor treated with Salmonella typhimurium A1-R (A1-R) (E) and PDOX tumor treated with both A1-R and rMETase (F). Bars: 50 μm.*

monotherapy group (3 mg/kg intra-peritoneal injection weekly for 2 weeks); rMETase monotherapy group (100 units/mouse intra-peritoneal injection daily for 2 weeks). *S. typhimurium* A1-R monotherapy group (5×10^7 CFU/100 μl intra-venously weekly for 2 weeks); combination therapy of *S. typhimurium* A1-R (5×10^7 CFU/100 μl intra-venously weekly for 2 weeks) and rMETase (100 units/mouse intra-peritoneal injection daily, for 2 weeks) group (Figure 1). Tumor size and body weight were measured twice a week. Tumor size was calculated based on a previously-described formula (34). The body weight of each mouse was measured with a digital balance (34).

Histological examination. All histological procedures were performed as previously described (34). Hematoxylin and eosin staining were performed according to standard protocols. Histological examination was performed with a BHS system microscope (34). Images were acquired with INFINITY ANALYZE software (Lumenera Corporation, Ottawa, Canada) (34).

Statistical analysis. All statistical analyses were performed as previously described (34). Student's t-test was used to determine the significant differences for continuous variables. Data are presented as the mean±standard deviation (SD). Differences with a probability value of $p\leq0.05$ are considered statistically significant.

Results

Treatment efficacy. At the end of treatment period (day 14), all treatments except doxorubicin monotherapy ($p=0.469$) significantly inhibited DDLPS PDOX tumor growth compared with the untreated control group: (rMETase: $p=0.03$; *S. typhimurium* A1-R: $p=0.02$; rMETase combined with *S. typhimurium* A1-R: $p<0.001$). rMETase in combination with *S. typhimurium* A1-R was significantly more effective compared to both rMETase alone ($p=0.006$) and *S. typhimurium* A1-R alone ($p<0.001$) (Figures 2 and 3). There were no significant changes found in the relative body weight between days 0 and 14 for any group (Figure 4).

Histology. The patient's original DDLPS displayed sarcoma cells characterized by spindle-shaped cells with hyperchromatic, enlarged tapering nuclei. Mitosis and atypical cells were present (Figure 5A). The PDOX tumor displayed similar characteristics, including spindle-shaped cells with hyperchromatic and enlarged tapering nuclei. Mitosis and atypical cells were also present (Figure 5B). The PDOX tumor treated with doxorubicin monotherapy had viable cells without obvious necrosis or morphological changes (Figure 5C). The PDOX tumor treated with rMETase monotherapy or *S. typhimurium* A1-R monotherapy showed changes in sarcoma cell shape (Figure 5D and E). *S. typhimurium* A1-R and rMETase combination therapy resulted in the PDOX tumor with reduced cellularity and the presence of necrotic areas (Figure 5F).

Discussion

Tumor-targeting *S. typhimurium* A1-R showed effectiveness against primary and metastatic tumors in cell lines and PDOX models of cancer, indicating *S. typhimurium* A1-R is a general cancer therapeutic (41-57). We have also shown that rMETase appears to be a general therapeutic in cancer. Previously we demonstrated *S. typhimurium* A1-R could decoy quiescent cancer cells into undergoing cell-cycle progression to S/G_2 phase and subsequent treatment with rMETase specifically trapped the cancer cells in S/G_2 phase resulting in apoptosis of the cancer cells (38).

Several bacterial species have been shown to target and kill tumors. Among these, various strains of Salmonella colonize tumors and induce antitumor immunity (65). Yoon et al. demonstrated that attenuated *S. typhimurium* expressing recombinant interferon-γ invaded and directly killed melanoma cells (66). We previously showed that *S. typhimurium* A1-R is able to kill cancer cells directly *in vitro* (41). *S. typhimurium* A1-R destroys tumor vessels (47) and also induces infiltration of $CD8^+$ T-cells into tumors (67). As stated above, *S. typhimurium* A1-R decoys quiescent cancer cells to cycle from G_0/G_1 to S/G_2 in the cell cycle and makes cancer cells more sensitive to chemotherapy (54). It has been reported that colonization of *S. typhimurium* at the tumor site caused an elevation of interleukin (IL)-1β and tumor necrosis factor-α within the tumor mass (68). It was demonstrated that a systemic injection of IL-2-expressing *S. typhimurium* inhibited angiogenesis and increased necrosis within tumor tissues (69). We also demonstrated that *S. typhimurium* combined with rMETase was effective against sarcomas (25, 28, 33). These studies suggest that tumor-targeting bacteria combined with rMETase (70) have great potential for cancer treatment.

In this study, the combination of *S. typhimurium* A1-R and rMETase led to regression of a doxorubicin-resistant DDLPS in a PDOX model, which is consistent with our previous results that this combination caused regression of PDOX models of Ewing's sarcoma (25), osteosarcoma (33), and melanoma (28). Thus, the combination of rMETase and S. typhimurium A1-R has important clinical potential for this recalcitrant sarcoma.

Recently papers have come out claiming novelty regarding methionine addiction (71, 72) about which we published long ago (1-5, 7, 8, 73-75). Targeting a central aspect of metabolism as methionine has much more potential for cancer therapy than targeting peripheral metabolism (76).

Conflicts of Interest

MZ, QH and YT are employees of AntiCancer Inc., KI, KK, TK, KM, TH, NY, KH, HK, SM and RMH are or were unsalaried members of AntiCancer Inc., which uses PDOX models for contract research. There are no other competing financial interests.

Authors' Contributions

Conception and design: KI and RMH. Acquisition of data: KI, KK, MZ, QH, YT, TK, KM and TH. Analysis and interpretation of data: KI, KK, MZ, QH, YT, TK, KM, TH, NY, KH, HK, SM, SRS, HT and RMH. Writing, review, and/or revision of the article: KI, RMH and SRS. SRS contributed to this article in his personal capacity.

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This paper dedicated to the memory of A. R. Moossa MD., Sun Lee MD., Professor Li Jiaxi and Masaki Kitajima MD.

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