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Combination of oral recombinant methioninase and decitabine arrests a chemotherapy-resistant undifferentiated soft-tissue sarcoma patient-derived orthotopic xenograft mouse model

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ABSTRACT

Cancer cells are methionine (MET) and methylation addicted and are highly sensitive to MET restriction. The present study determined the efficacy of oral-recombinant methioninase (o-rMETase) and the DNA methylation inhibitor, decitabine (DAC) on restricting MET in an undifferentiated-soft tissue sarcoma (USTS) patient-derived orthotopic xenograft (PDOX) nude-mouse model. The USTS PDOX models were randomized into five treatment groups of six mice: Control; doxorubicin (DOX) alone; DAC alone; o-rMETase alone; and o-rMETase-DAC combination. Tumor size and body weight were measured during the 14 days of treatment. Tumor growth was arrested only in the o-rMETase-DAC condition. Tumors treated with the o-rMETase-DAC combination exhibited tumor necrosis with degenerative changes. This study demonstrates that the o-rMETase-DAC combination could arrest the USTS PDOX tumor suggesting clinical promise.

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1. Introduction

Soft-tissue sarcoma (STS) is a rare type of malignancy that is usually treated with a combination of chemotherapy and surgery, with or without radiotherapy [1]. Doxorubicin (DOX)-containing regimens are usually used as a first-line therapy for STS but show limited efficacy [2–4]. Undifferentiated/unclassified soft tissue sarcoma (USTS) is a common form of STS in adults [5] and is often resistant to radiotherapy and most chemotherapies [3,4].

Methionine (MET) addiction [6–14] is possibly the only general hallmark of cancer [6,7]. MET addiction in cancer cells involves an elevation in MET flux [6–9] due to increased transmethylation reactions [7,9] known as the Hoffman effect, which is analogous to the Warburg effect of glucose overuse by cancer cells. MET restriction (MR) causes severe MET and S-adenosylmethionine (SAM) depletion [6,7,12], as well as selective cell cycle arrest in the S/G_2 phase in cancer cells [13,14]. MET addiction is linked to other characteristics of cancer [10], and as a general phenomenon in cancer, it is an important target for cancer treatment [11].

Recombinant methioninase (rMETase), a *Pseudomonas putida* enzyme cloned in *Escherichia coli*, targets MET-addicted cancer cells by severely depleting MET [15,16]. We have reported the efficacy of oral administrations of rMETase (o-rMETase) on various chemo-resistant cancers grown in patient derived orthotopic xenograft (PDOX) models [17–20]. Decitabine (5-Aza-2′-deoxycytidine, DAC), a deoxy-derivative of azacitidine, is an epigenetic drug that binds irreversibly to DNA methyltransferase and causes DNA

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hypomethylation when incorporated into newly synthesized DNA [21]. Both DAC and azacitidine are used together in the treatment of some forms of myelodyxplastic syndrome and leukemia [22]. However, studies have reported only limited efficacy when these drugs were used alone [23–27]. We recently reported that the combination of azacitidine and o-rMETase arrested a chemotherapy-resistant osteosarcoma PDOX model [28].

In the present report, we determined if the o-rMETase-DAC combination could overcome drug-resistance in a PDOX model of USTS.

2. Materials and methods

2.1. Mice

Athymic nu/nu nude mice (AntiCancer, Inc., San Diego, CA, USA) were used. The investigations presented here were carried out at AntiCancer Inc., Institutional Animal Care and Use Committee (IACUC) protocol specifically approved for this study and 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 [29,30]. To minimize any suffering of the animals, anesthesia and analgesics were used for all surgical experiments.

2.2. Patient-derived tumor

A fresh surgical sample of 62-year-old female with undifferentiated sarcoma not otherwise specified in the left upper arm was previously obtained and transported immediately to the laboratory at AntiCancer Inc., on wet ice [31]. The patient did not receive any chemotherapy or radiotherapy prior to surgery. Written informed consent was obtained from the patient as part of a UCLA Institutional Review Board approved protocol (IRB#10-001857) [31]. The sample was cut into 5-mm fragments and implanted subcutaneously in nude mice.

2.3. Surgical orthotopic implantation for establishment of an USTS PDOX model

Mice were anesthetized before the following procedures: after the subcutaneously-implanted tumors grew to more than 10 mm in diameter, the tumors were harvested and cut into small fragments (3-4 mm^3). 5-mm skin incisions were made on the right thigh of nude mice. The biceps femoris was split, and a single tumor fragment was implanted orthotopically into the space to establish the USTS PDOX model. The muscle and wound were closed with a 6–0 nylon suture (UNIFY, AD Surgical, Sunnyvale, CA) [6–9,31].

2.4. Recombinant methionase (rMETase) production

Recombinant L-metionine α-deamino-γ-mercaptomethane lyase (recombinant methioninase, [rMETase]) [EC 4.4.1.11] from *Pseudomonas putida* has been previously cloned and was produced in *Escherichia coli* (AntiCancer Inc., San Diego, CA). rMETase is a homotetrameric PLP enzyme of 172-kDa molecular mass [32].

2.5. Treatment study design in the USTS-PDOX model

The USTS PDOX mouse models were randomized into 5 groups of 6 mice each and treated with the following drug regiment for 2 weeks (Fig. 1A): G1, control without treatment; G2, DOX (3 mg/kg, intraperitoneal [i.p.] injection, once a week) alone; G3, DAC (50 mg/kg, i.p., daily) alone; G4, o-rMETase (50 units/day, oral, twice daily); G5, DAC (50 mg/kg, i.p., daily) + o-rMETase (50 units/day, oral, twice daily). Treatment started when all tumors reached 100–200 mm^3. Tumor length, width and mouse body weight was measured twice a week. Tumor volume was calculated with the following formula: Tumor volume (mm^3) = length (mm) × width (mm) × width (mm) × 1/2. Data are presented as mean ± standard error of the mean (SEM).

2.6. Histological analysis

Fresh tumor samples were fixed in 10% formalin and embedded in paraffin before sectioning and staining. Tissue sections were deparaffinized in xylene and rehydrated in an ethanol series. Hematoxylin and eosin staining was performed according to standard protocols.

2.7. Statistical analysis

All statistical analyses were performed with statistical software EZR (Saitama Medical Center, Jichi Medical University). A normal distribution was assessed with the Shapiro-Wilk test. The Bartlett's test was used to verify the homogeneity of variances across groups. One-way ANOVA with Tukey HSD for post hoc analysis was used for the parametric test for inter-group comparison. The paired *t*-test was used for the parametric test to compare the means between two related groups. All *p*-values were two-sided and *p*-values of less than 0.05 are considered statistically significant.

3. Results

3.1. Efficacy of DOX, DAC, o-rMETase, and the o-rMETase-DAC combination on the USTS-PDOX

The o-rMETase-DAC combination significantly inhibited the USTS PDOX tumor compared to the control ($p < 0.001$), DOX alone ($p = 0.002$), DAC alone ($p = 0.001$), or rMETase alone ($p < 0.001$). There was no statistical difference in USTS PDOX growth between the control and DOX alone ($p = 0.61$), DAC alone ($p = 0.68$), or rMETase alone ($p = 1.00$). (Fig. 1B and C, 2A,B).

3.2. Effect of treatment on body weight

Mouse body weight was measured pre-treatment and post-treatment. The final body weight of control mice ($p < 0.001$), DOX alone ($p = 0.007$), and DAC alone ($p = 0.02$) significantly increased from their initial body weights. There was no significant difference in body weight change among o-rMETase alone ($p = 0.59$) or the o-rMETase-DAC combination ($p = 0.49$) treated groups (Fig. 2C). There were no other observable side effects or animal deaths in any group.

3.3. Histology of the USTS PDOX

The control tumor mainly comprised viable highly-dense cancer cells characterized by pleomorphic spindle-shaped cells. PDOX tumors treated with administration of o-rMETase, DOX, or DAC alone comprised viable tumor cells, although the cancer-cell density was lower than that of control. PDOX tumors treated with the o-rMETase-DAC combination had the lowest cancer-cell density. These tumors had degenerative scars that replaced necrotic cancer cells, matching the anti-tumor efficacy of the combination (Fig. 3).

4. Discussion

Aberrant DNA methylation is an important modification in cancer cells that can lead to abnormal transcription, cell proliferation, and differentiation [21]. Various hypomethylating drugs have

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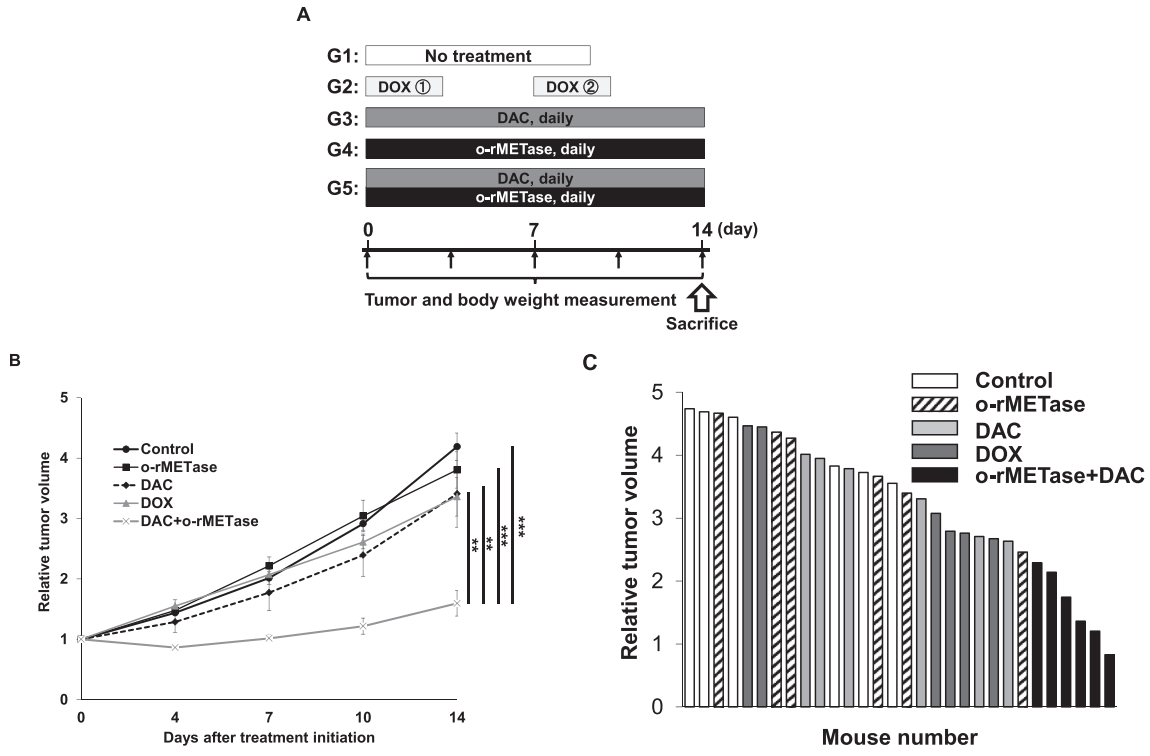

Fig. 1. Treatment regime and tumor efficacy. (A) Treatment schema. (B) Quantitative efficacy of drugs on the tumor volume of USTS-PDOX model. Line graphs indicate tumor volume at each time point after the onset of treatment relative to the initial tumor volume for each treatment and control group. (C) Waterfall plot of relative tumor volume at day 14 relative to the initial tumor volume for each mouse. N = 6 mice/group. $^{**}p < 0.01$; $^{***}p < 0.001$. Error bars: ± SEM.

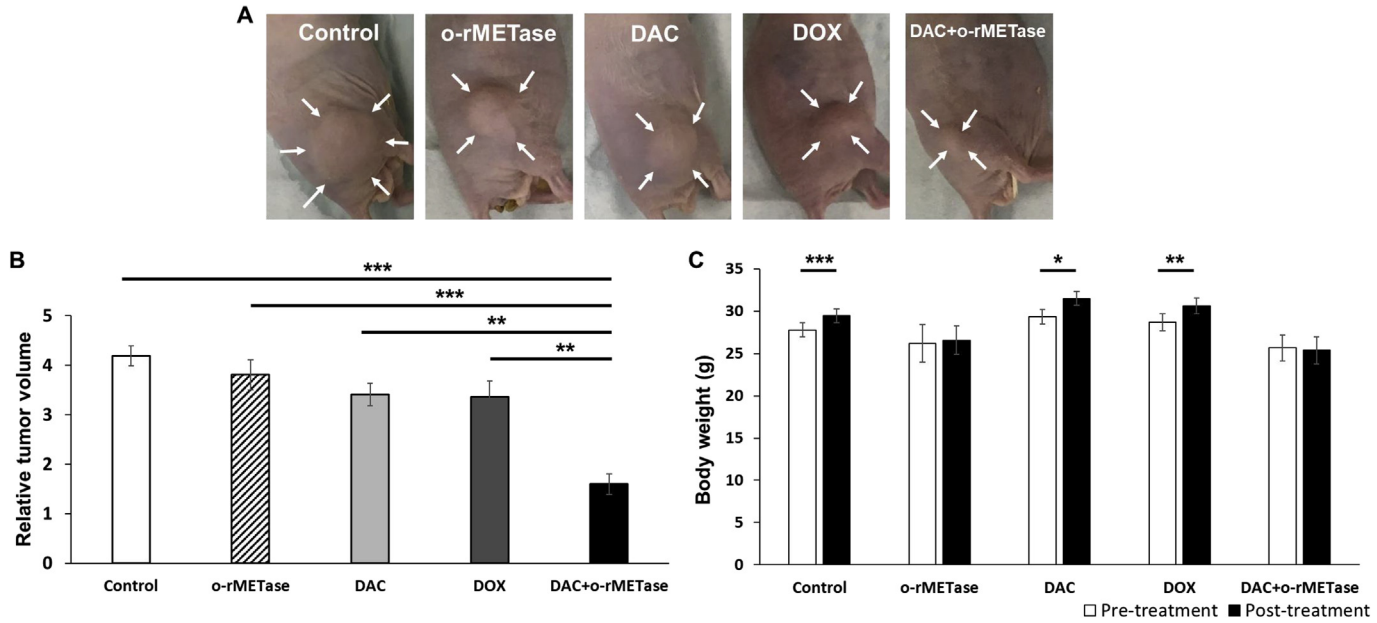

Fig. 2. Efficacy of drugs on the undifferentiated-soft tissue sarcoma-PDOX at the end point (A) Photographs of representative USTS PDOX mouse models from each treatment group at the end of treatment. Arrows indicate the apparent margin of the tumors. (B) Bar graphs show relative tumor volume of each treatment group on day 14. N = 6 mice/group. $^{**}p < 0.01$; $^{***}p < 0.001$. Error bars: ± SEM. (C) Mouse body weight. Bar graphs show mouse body weight in each treatment or control group at pre- and post-treatment times. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Error bars: ± SEM.

been developed to prevent cancer progression [22,33]. DAC and azacitidine, which are clinically used nucleoside-based DNA methyltransferase inhibitors, have been reported to have anti-tumor activity through two mechanisms: by inhibiting DNA methyltransferase resulting in the hypomethylation of DNA, and by direct cytotoxicity to cancer cells through its incorporation into DNA and RNA [34]. However, these drugs alone have shown only limited efficacy in the clinic [26,33]. Because targeting DNA

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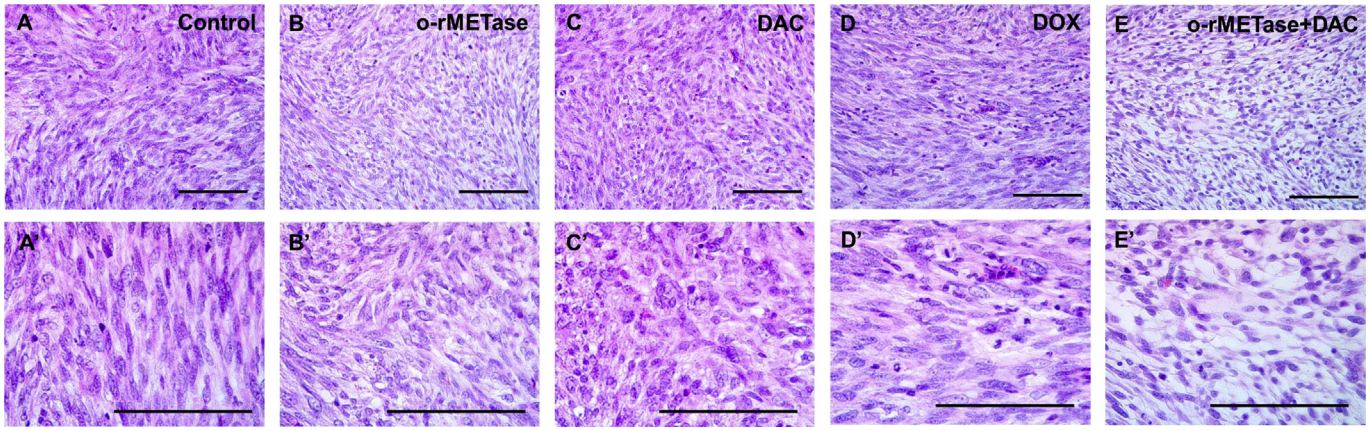

Fig. 3. Tumor histology. (A, A′) Control. (B, B′) o-rMETase alone. (C, C′) DAC alone. (D, D′) DOX alone. (E, E′) o-rMETase + DAC. Scale bars: 100 μm.

methylation in cancer cells using DNA hypomethylating drugs also sensitizes them to chemotherapy, combination therapy with other drugs should be studied [21]. Our laboratory was the first to discover DNA hypermethylation in human cancer cells [35].

S-adenosylmethionine (SAM) is synthesized from MET in the MET metabolic pathway and is essential for all cellular methylation reactions [6]. MR with rMETase depletes SAM which can result in the reduction of DNA methylation in the tumor [7,12]. Therefore, rMETase itself may restrict DNA methylation and enhance the DNA hypomethylation effect of DAC. In the present study, DAC alone had only limited efficacy on the USTS PDOX tumor. Importantly, the combination of o-rMETase-DAC could arrest recalcitrant USTS PDOX tumor growth, suggesting clinical importance. Future studies should introduce a SAM synthetase inhibitor to the treatment regimen of the present report to effect total methylation blockage.

In conclusion, the present study determined that o-rMETase-DAC combination could arrest the USTS PDOX tumor suggesting clinical promise. The approach of the present report to target the fundamental alteration of MET addiction in cancer seems a more promising approach to sarcoma therapy than targeting secondary alternations which has shown to be ineffective [36].

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Declaration of competing interest

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

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