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Growth of doxorubicin-resistant undifferentiated spindle-cell sarcoma PDOX is arrested metabolic targeting with recombinant methioninase†

Running title: Recombinant methioninase arrests a doxorubicin-resistant spindle-cell sarcoma

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Abstract
Undifferentiated spindle-cell sarcoma (USCS) is a recalcitrant cancer in need of individualized therapy. A high-grade USCS from a striated muscle of a patient was grown orthotopically in the right biceps femoris muscle of nude mice to establish a patient-derived orthotopic xenograft (PDOX) model. In a previous study, we evaluated the efficacy of standard first-line chemotherapy of doxorubicin (DOX), gemcitabine (GEM) combined with docetaxel (DOC), compared to pazopanib (PAZ), a multi-targeting tyrosine-kinase inhibitor, in an USCS PDOX model. In the present study, animal-bearing the USCS PDOX tumors were randomized into the following groups when tumor volume reached 100 mm$^3$: G1, untreated control without treatment; G2, DOX (3 mg/kg, intraperitoneal (i.p.) injection, weekly, for 2 weeks); G3, L-methionine $\alpha$-deamino-$\gamma$-mercaptomethane lyase (recombinant methioninase [rMETase]) (100 unit/mouse, i.p., daily, for 2 weeks). Tumor sizes and body weight were measured with calipers and digital balance twice a week and methionine level of supernatants derived from sonicated tumors were measured. rMETase inhibited tumor growth compared to untreated controls and the DOX treated group on day 14 after initiation of treatment: control (G1): 347.6 ± 88.2 mm$^3$; DOX (G2): 329.5 ± 78.8 mm$^3, p=0.670$; rMETase (G3): 162.6 ± 51.2 mm$^3, p=0.0003$. The mouse body weight of the treated mice was not significantly different from the untreated controls. Tumor L-methionine levels were reduced after the rMETase-treatment compared to untreated control and pre-rMETase treatment. We previously reported efficacy of rMETase against Ewing’s sarcoma in a PDOX model. These studies suggest clinical development of rMETase, especially in recalcitrant cancers such as sarcoma. This article is protected by copyright. All rights reserved.

Key words: undifferentiated spindle-cell sarcoma, doxorubicin, resistant, patient-derived orthotopic xenograft, PDOX, recombinant methioninase
**Introduction**

Undifferentiated spindle-cell sarcoma (USCS) is a recalcitrant cancer sarcoma which has predominant spindle-shaped cells which can originate in nerve sheaths, layers of connective tissue such as that under the skin, in muscles, and other organs. Spindle cell sarcomas have been characterized as fibromyxoid with characteristics of both mesenchymal and neuroendocrine differentiation [Shaikh et al., 2014]. A balanced translocation between chromosomes 7 and 16 has been identified in a case of spindle-cell carcinoma [Reid et al., 2003; Igarashi et al., 2017a]. A high-grade USCS from a striated muscle of the patients was grown orthotopically in the right biceps femoris muscle of nude mice to establish a PDOX model. In a previous study, we evaluated the efficacy of standard first-line chemotherapy of doxorubicin (DOX), gemcitabine (GEM) combined with docetaxel (DOC), compared to pazopanib (PAZ), a multi-targeting tyrosine-kinase inhibitor, in an USCS PDOX model. The USCS first line therapy was resistant to DOX but significantly inhibited by the combination of gemcitabine (GEM) and docetaxel (DOC) and pazopanib (PAZ). PAZ showed significantly more efficacy compared to GEM+DOC. These results demonstrated that the PDOX model of USCS can identify a promising novel agent with significantly greater efficacy than first-line therapy for this recalcitrant disease [Igarashi et al., 2017a].

Methionine dependence is due to the overuse of methionine for aberrant transmethylation reactions in cancer. In order to exploit methionine dependence for therapy, our laboratory previously cloned L-methionine α-deamino-γ-mercaptopemethane lyase (recombinant methioninase [rMETase]). We previously reported efficacy of rMETase against Ewing’s sarcoma in a PDOX model [Murakami et al., 2017a].

The present report demonstrates the efficacy of recombinant methioninnase on the PDOX model of USS compared to conventional DOX therapy.
Materials and Methods

Mice

Athymic \textit{nu/nu} female nude mice (AntiCancer Inc., San Diego, CA), 4–6 weeks old, were used in this study. Animals were housed in a barrier facility on a high efficiency particulate arrestance (HEPA)-filtered rack under standard conditions of 12-hour light/dark cycles. The animals were fed an autoclaved laboratory rodent diet. All animal studies were conducted with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specifically approved for this study and in accordance with the principals and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1. In order to minimize any suffering of the animals the use of anesthesia and analgesics were used for all surgical experiments. Animals were anesthetized by subcutaneous injection of a 0.02 ml solution of 20 mg/kg ketamine, 15.2 mg/kg xylazine, and 0.48 mg/kg acepromazine maleate. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. The animals were observed on a daily basis and humanely sacrificed by CO\textsubscript{2} inhalation when they met the following humane endpoint criteria: severe tumor burden (more than 20 mm in diameter), prostration, significant body weight loss, difficulty breathing, rotational motion and body temperature drop [Igarashi et al., 2017a].

Patient-derived tumor

A 56-year-old male diagnosed with USCS at primary right shoulder previously underwent surgical resection at Department of Surgery, University of California, Los Angeles (UCLA). He 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 (IRB #10-001857)-approved protocol [Igarashi et al., 2017a].

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Surgical orthotopic implantation (SOI) for establishment of PDOX model

A single USCS tumor fragment, previously grown subcutaneously in nude mice, was implanted orthotopically into the right thigh to establish a PDOX model. The wound was closed with 6-0 nylon suture (Ethilon, Ethicon, Inc., NJ, USA) [Igarashi et al., 2017a].

Recombinant methioninase (rMETase) production

The pAC-1 rMETase high expression clone was used for rMETase production. The fermentation procedure for host E.coli cells and the purification protocol for rMETase were the same as previously described: rMETase was purified by 3 different steps using columns of DEAE Sepharose FF and Sephacryl S-200HR, and ActiClean Etox, which is designed for eliminating endotoxin [Tan et al., 1997].

Treatment study design

The USCS PDOX mouse models were randomized into 4 groups of 8 mice each: G1, untreated control without treatment; G2, DOX (3 mg/kg, intraperitoneal (i.p.) injection, weekly, for 2 weeks); G3, rMETase (100 unit/mouse in 250 µl phosphate buffered saline [PBS], i.p., daily, for 2 weeks). Tumor length, width and mouse body weight were measured twice in a week. Tumor volume was calculated by following formula: Tumor volume (mm$^3$) = length (mm) × width (mm) × width (mm) × 1/2. Data was presented as mean ± SD.

Tumor protein level measurement

To standardize tumor L-methionine measurements, tumor protein levels were measured. Briefly, each PDOX tumor was placed in phosphate buffered saline (PBS) (1 ml). Tumors were sonicated on ice for 30 seconds and subsequently centrifuged at 12,000 rpm for 10 minutes.
Supernatants were diluted to the concentration ranging from 200 to 1500 μl/ml. Protein assay reagent (Bio-Rad, Hercules, CA) was prepared as a 4-fold dilution and added to each tube with the sonicated tumor supernatents, then absorbance of 595 nm was measured by the microprocessor controlled microplate reader (Sunrise™; TECAN, San Joes, CA, USA). Protein levels were calculated from the standard curve obtained by protein standard, bovine serum albumin (BSA) [Murakami et al., 2017a].

**Tumor L-methionine level analysis at termination**

Tumor supernatants obtained from the above-described sonication procedure were precipitated by acetonitrile. L-methionine levels were measured with an HPLC (Hitachi L-6200A Intelligent pump; Hitachi, Ltd., Tokyo, Japan) after derivatization of supernatants amino acids with the fluoraldehyde reagent OPA as described previously [Tan et al., 1997; Sun et al., 2005]. L-methionine levels were determined as nmol/ml with the HPLC procedure described above. Standardized L-methionine levels were calculated using the following formula: standardized L-methionine level (nmol/mg protein) = L-methionine level (nmol/ml) / protein level (mg protein/ml) [Murakami et al., 2017a].

**Histological examination**

Fresh tumor samples were fixed in 10% formalin and embedded in paraffin before sectioning and staining. Tissue sections (3 μm) were deparaffinized in xylene and rehydrated in an ethanol series. Hematoxylin and eosin (H&E) staining was performed according to standard protocol. Histological examination was performed with a BHS system microscope. Images were acquired with INFINITY ANALYZE software (Lumenera Corporation, Ottawa, Canada) [Murakami et al., 2017a].

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Result and Discussion

rMETase inhibited tumor growth compared to untreated control and DOX-treated mice. At day 14 after treatment initiation, tumor volumes were as follows: control (G1): 347.6±88.2 mm$^3$; DOX (G2): 329.5±78.8 mm$^3$, $p=0.670$; rMETase (G3): 162.6±51.2 mm$^3$, $p=0.0003$ (Figures 1-3). We have previously published correlations of tumor volume to tumor weight [Katz et al., 2003; Hiroshima, et al. 2014a]. The body weight of the treated mice was not significantly different from the untreated control (Figure 4). Tumor L-methionine levels were significantly reduced after the rMETase-treatment compared to untreated control and pre-rMETase treatment (Figure 5).

Figure 5 shows methionine (MET) levels at 2 time-points: pre-and post-treatment for the untreated control and the recombinant methioninase (rMETase)-treated groups. In the untreated control, the tumor MET increased by the end of the treatment group. The larger untreated tumor probably required more methionine in order to increase in size, as tumors are highly methionine-dependent for growth [Hoffman, 2015]. However, this increase in MET was not statistically significant. In contrast, the rMETase-treated tumors at the end of the experiment had a statistically-significant decrease in MET, with respect to the control tumors at the beginning and end of the treatment period and from the rMETase-treated-tumors at the beginning of the experiment. This experiment shows, with statistical-significance, that the rMETase-treatment reduced tumor MET levels, which is the probable mechanism for the tumor growth inhibition observed in Figure 3.

DOX was chosen as a treatment control because DOX is first-line therapy of sarcoma [Murakami et al., 2016a] and we wished to learn if rMETase treatment would be an improvement over
first-line therapy for this recalcitrant disease. The other control was no treatment. Therefore, we could compare the efficacy of DOX and rMETase against an untreated control.

**Histology**

High power photomicrographs of the original patient tumor showed cancer cells characterized by spindle shaped with hyperchromatic, enlarged tapering nuclei. Mitotic figures and atypical forms are present (Figure 6A). A high power view of the orthotopically implanted tumor showed similar features including spindle-shaped cells with hyperchromatic, enlarged tapering nuclei. Numerous mitotic figures, including atypical forms are also present (Figure 6B). Tumors treated with DOX were comprised of spindle shaped viable cells without apparent necrosis or inflammatory changes (Figure 6C). Tumors treated with rMETase showed changes in tumor cell shapes with apparent tumor necrosis (Figure 6D).

The first hint that methionine metabolism is perturbed in cancer came almost 60 years ago when Sugimura et al. observed that tumor growth in rats was slowed a defined diet depleted in methionine [Sugimura et al., 1959]. Approximately 45 years ago, it was observed that L5178Y mouse leukemia cells in culture required abnormally high levels of methionine to proliferate [Chello et al., 1973]. Subsequently, most cancer cell lines were found to be methionine dependent [Mecham et al., 1983; Tan et al., 2010; Hoffman 2017].


However, the amount of endogenous methionine, the level of free methionine and S-adenosylmethionine (AdoMET), the universal methyl donor, were very low in
methionine-dependent cancer cells in methionine limiting conditions, despite normal amounts of methionine synthesized [Coalson et al., 1982; Stern et al., 1983].

Cancer cells have enhanced overall rates of transmethylation compared with normal cells. The enhanced transmethylation rates may be the basis of the methionine dependence of cancer cells which explains the low levels of free methionine and the low AdoMET/AdoHCY ratio in cancer cells under methionine deprivation, despite high rates of methionine synthesis [Stern et al., 1984]. The elevated methionine use in cancer cells has been termed the “Hoffman effect.” [Murakami et al., 2017a].

Growth arrest of methionine-dependent cancer cells under conditions of methionine restriction arrest was in the S/G2 phases of the cell cycle [Hoffman and Jacobsen 1980; Yano et al., 2014].

**Methionine independent revertants**

Rare cells from methionine-dependent cancer cell lines regained the normal ability to grow under methionine restriction. These lines were termed methionine-independent revertants [Hoffman et al., 1978]. Methionine-dependent revertants also had much lower basal transmethylation rates than parental methionine-dependent cell lines [Judde et al., 1989]. These results further suggested that methionine dependence is due to an increase in the rate of transmethylation reactions. We then demonstrated that methionine-independent revertants cells simultaneously reverted for characteristics associated with cancer and became less malignant. Thus, the methionine independent revertants become more normal-like indicating further a relationship between altered methionine metabolism and oncogenic transformation [Hoffman et al., 1979].

Toward the goal of precision personalized oncology, our laboratory pioneered the
patient-derived orthotopic xenograft (PDOX) nude mouse model with the technique of surgical orthotopic implantation (SOI), including pancreatic [Hiroshima et al., 2014b,c, 2015a; Fu et al., 1992; Kawaguchi et al., 2017a], breast [Fu et al., 1993a], ovarian [Fu and Hoffman 1993], lung [Wang et al., 1992], cervical [Hiroshima et al., 2015b; Murakami et al., 2017b], colon [Hiroshima et al., 2014d; Fu et al., 1991; Metildi et al., 2014], stomach [Furukawa et al., 1993], sarcoma [Murakami et al., 2016a, b, 2017c; Igarashi et al., 2017ab, Hiroshima et al., 2015c, Kawaguchi et al., 2017b; Kiyuna et al., 2016] and melanoma [Yamamoto et al., 2016; 38-42].

The present report indicates the PDOX models of USCS could identify rMETase as an effective therapy of this recalcitrant disease and suggest its clinical development.

Acknowledgement

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References


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Figure Legends

Figure 1. Treatment schema.

Figure 2. Efficacy of doxorubicin (DOX) and L-methionine α-deamino-γ-mercaptopemethane lyase (recombinant methioninase [rMETase]) on an undifferentiated spindle-cell sarcoma (USCS) PDOX. A USCS from a patient was grown orthotopically in the right biceps femoris muscle of nude mice and allowed to form tumors. Mice were treated with DOX (3 mg/kg/week, i.p., for 2 weeks) or rMETase (100 U/mouse/day, i.p., for 14 days). Representative photographs of mice from each group at day-14.

Figure 3. Growth curves of the untreated and DOX and rMETase-treated USCS PDOX. Tumor volume was measured at the indicated time points after the onset of treatment. N=8 mice/group. * p< 0.001

Figure 4. Body weight of untreated control, DOX and rMETase-treated groups. Bar graph shows body weight in each group at pre-treatment and 2 weeks after drug administration. There were no significant differences between each group.

Figure 5. L-methionine levels in pre- and post-rMETase treatment of the USCS PDOX. Bar graphs show tumor L-methionine level normalized by tumor protein concentration. Tumor L-methionine levels in the post-rMETase-treated animals were lower than control. Error bars: ± SD. N = 4 for each group.

Figure 6. Tumor histology. Hematoxylin and eosin (H&E)-stained section of the original
patient’s tumor (A), untreated PDOX tumor (B), PDOX tumor treated with DOX (C), PDOX tumor treated with rMETase (D). Scale bars: 80µm.
Figure 1

G1: No treatment
G2: DOX①, DOX②
G3: rMETase every day

0 7 14 (day)

Tumor and body weight measurement

Sacrifice
Figure 2
There were no significant differences between each group.

There were no significant differences between each group.

Figure 4
Figure 5

![Bar chart showing standardized tumor L-methionine (nmol/mg protein) for control and rMETase groups. The chart includes error bars and p-values: p=0.04, p=0.03, and p=0.05. The chart compares pre-treatment (red bars) and post-treatment (purple bars).]