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Original Articles

Oral recombinant methioninase (o-rMETase) is superior to injectable rMETase and overcomes acquired gemcitabine resistance in pancreatic cancer

Kei Kawaguchi[a,b,c], Kentaro Miyake[a,b], Qinghong Han[a], Shukuan Li[a], Yuying Tan[a], Kentaro Igarashi[a,b], Tasuku Kiyuna[a,b], Masuyo Miyake[a,b], Takashi Higuchi[a,b], Hiromichi Oshiro[a,b], Zhiying Zhang[a], Sahar Razmjooei[a], Sintawat Wangsiricharoen[a], Michael Bouvet[b], Shree Ram Singh[d,***], Michiaki Unno[c,**], Robert M. Hoffman[a,b,*]

[a] AntiCancer, Inc., San Diego, CA, USA
[b] Department of Surgery, University of California, San Diego, CA, USA
[c] Department of Surgery, Graduate School of Medicine, Tohoku University, Sendai, Japan
[d] Basic Research Laboratory, National Cancer Institute, Frederick, MD, USA

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ABSTRACT

Recombinant methioninase (rMETase) was previously administered as an injectable drug to target methionine dependence of cancer. Recently, we observed that rMETase could be administered orally (o-rMETase) in a patient-derived orthotopic xenograft (PDOX) mouse model of melanoma. Here, we determined the efficacy of o-rMETase on a pancreatic cancer PDOX model. Forty pancreatic cancer PDOX mouse models were randomized into four groups of 10 mice each. o-rMETase was significantly more effective than i.p.-rMETase, but the combination of both was significantly more effective than either alone. Acquired gemcitabine resistance is a major factor in the recalcitrance of pancreatic cancer. We tested a human pancreatic cancer cell line, which has acquired > 100-fold GEM-resistance (PK-9R) than its parental cell line PK-9. In contrast to GEM, both cell lines were very sensitive to rMETase. In orthotopic nude mouse models of PK-9 and PK-9R, GEM inhibited tumor growth in PK-9 but not PK-9R. In contrast, o-rMETase could inhibit both tumors. The combination of GEM + o-rMETase could regress the PK-9 tumor and inhibit PK-9R tumor growth. The present study shows that o-rMETase is effective and overcomes acquired GEM resistance in pancreatic cancer and demonstrates the clinical potential of this strategy.

1. Introduction

The elevated methionine (MET) requirement of cancer cells to proliferate is termed MET dependence and may be the only known general metabolic defect in cancer [1,2]. Cancer cells utilize excess methionine for aberrant transmethylation reactions [3], a metabolic reprogramming in cancer known as the "Hoffman Effect" and analogous to the Warburg effect of over-utilization of glucose in cancer [2]. In head-to-head comparisons in PET imaging, [^{11}C] MET-PET shows a more stronger signal than [^{18}F] fluorodeoxyglucose [4], suggesting that the Hoffman effect is more extensive in cancer than the Warburg effect. Targeting MET by recombinant methioninase (rMETase) can arrest the growth of cancer cells *in vitro* and *in vivo* [1–4].

METase has been used as a therapeutic strategy for multiple type of cancers [4–11]. Song et al. [12] reported that MET starvation therapy using a MET-free diet or total parenteral nutrition (TPN) extends the survival time of high-stage gastric carcinoma patients.

Previously rMETase was administrated by intra-peritoneal injection (ip-rMETase) and was effective against patient-derived orthotopic xenograft (PDOX) models of recalcitrant cancer [13–17]. Recently, we compared ip-rMETase and oral rMETase (o-rMETase) for efficacy on the melanoma PDOX [13]. o-rMETase was significantly more effective than ip-rMETase [13], indicating the potential widespread use of rMETase for acute and chronic cancer treatment.

Pancreatic cancer is the fourth leading causes of cancer-related deaths in the Western world because of low-response to radio- and

* Corresponding author. AntiCancer, Inc., San Diego, CA, USA.
** Corresponding author.
*** Corresponding author.
E-mail addresses: singhshr@mail.nih.gov (S.R. Singh), m_unno@surg1.med.tohoku.ac.jp (M. Unno), all@anticancer.com (R.M. Hoffman).

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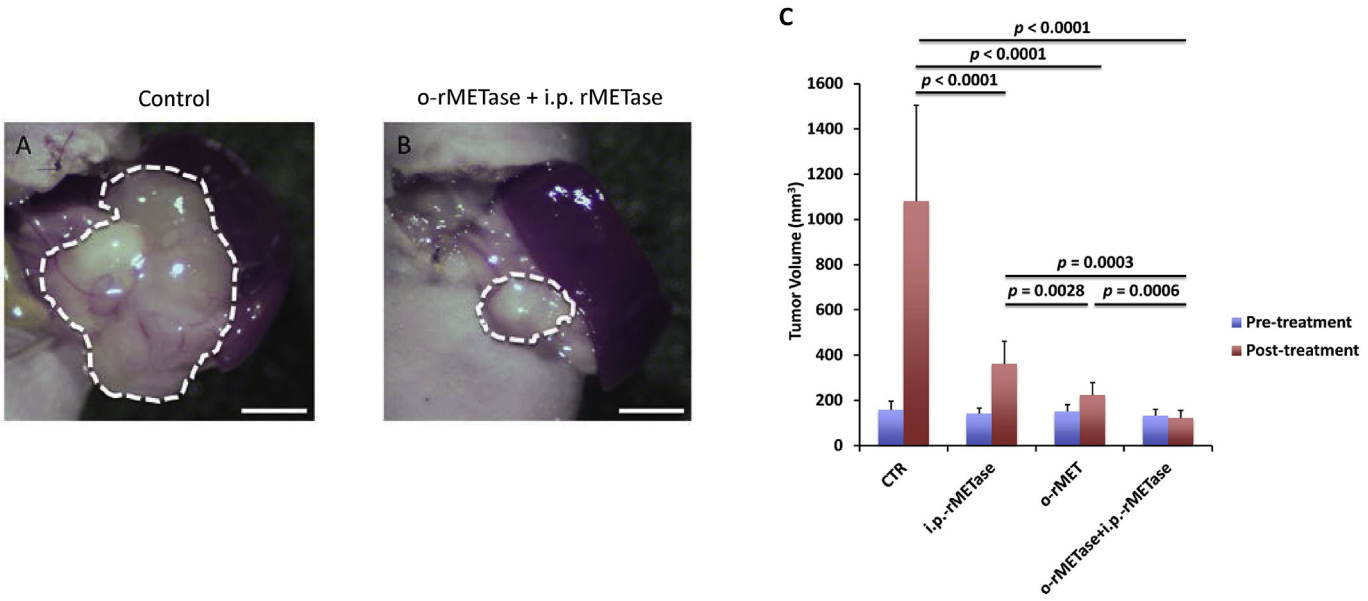

Fig. 1. Photographs of a representative untreated control (**A**) and a tumor treated with the combination of o-rMETase and ip-rMETase (**B**) in the pancreatic cancer PDOX. Tumors were resected on day 15. Scale bar: 5 mm (**C**) Quantitative efficacy ip-rMETase, o-rMETase and their combination on the pancreatic cancer PDOX. Bar graphs show tumor volume at pre- and post-treatment. Error bars: ± SD.

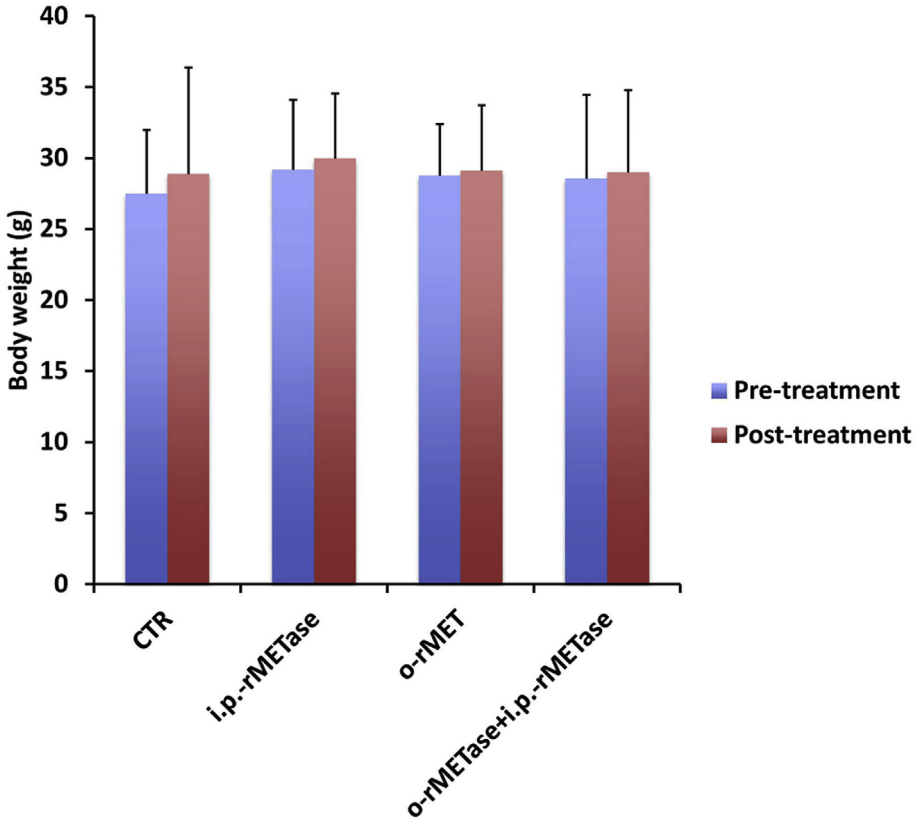

Fig. 2. Effect of ip-rMETase, o-rMETase and their combination on mouse body weight. Bar graphs show mouse body weight in each treatment group at pre- and post-treatment.

chemo-therapies. The American Cancer Society estimates that in 2018, about 55,440 new cased will be diagnosed with pancreatic cancer and about 44,330 people will die of this disease in the USA alone [18]. Despite extensive research to improve detection techniques, surgical methods and developing better chemotherapy drugs, the prognosis of pancreatic cancer patients remains poor.

Gemcitabine (GEM) is the standard first line treatment for patients with advanced pancreatic cancer; however, it is ineffective because of acquired resistance [19,20]. Acquired GEM resistance is a major factor in the recalcitrance of pancreatic cancer. Various genetic and/or epigenetic changes have been proposed that causes GEM resistance [20–25]; but how to overcome this resistance remains unclear.

We recently reported that intra-peritoneal rMETase (i.p.-rMETase) combined with GEM could regress a partially GEM-resistant pancreatic

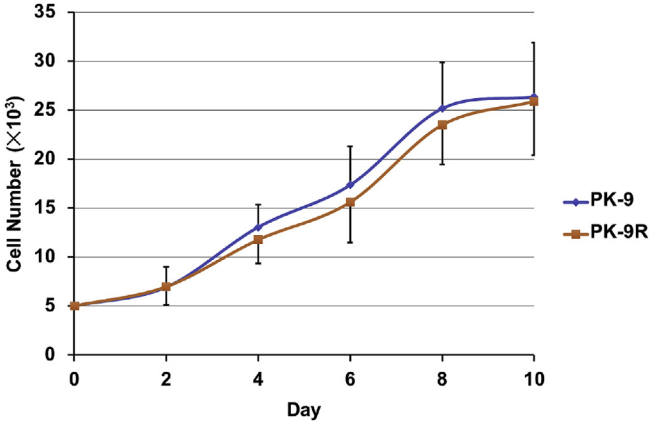

Fig. 3. Cell proliferation of PK-9 and PK-9R.

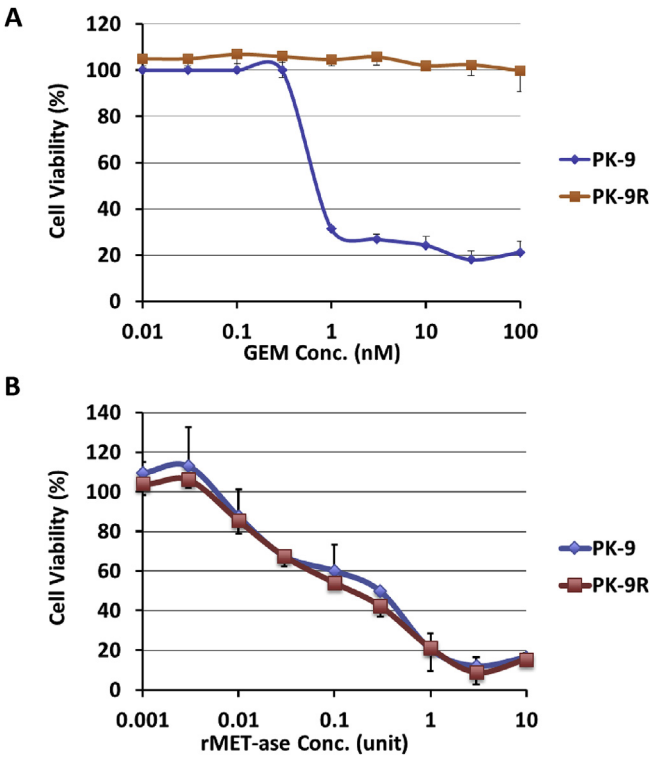

Fig. 4. Inhibition of cell proliferation. **(A)** Gemcitabine (GEM); **(B)** Recombinant methioninase (rMETase).

Table 1
Comparison of IC_{50} values of GEM and rMETase on PK-9 and PK-9R cells.

	PK-9	PK-9R	p-value
GEM (nM)	0.85 ± 0.05	> 100	P < 0.0001
rMETase (units)	0.21 ± 0.01	0.14 ± 0.01	P = 0.0809

cancer PDOX [26]. In the present study, we compared the efficacy of o-rMETase and i.p.-rMETase in a pancreatic-cancer PDOX. Acquired GEM resistance is a major factor in the recalcitrance of pancreatic cancer. In addition, we describe a human pancreatic cancer cell line which acquired greater than 100-fold GEM resistance. We evaluated the efficacy of o-rMETase and o-rMETase in combination with GEM against the GEM-resistant human PK-9R pancreatic cancer cell line *in vitro* and orthotopic mouse models compared to its parental GEM-sensitive line PK-9. We found that o-rMETase can overcome GEM resistance.

2. Methods

2.1. Mice

Athymic *nu/nu* nude mice (AntiCancer Inc., San Diego, CA), 4–6 weeks old, were used in this study. All experimental protocols and data collection were as previously described [13–17]. All mouse surgical procedures and imaging were performed with the animals anesthetized by subcutaneous injection of a ketamine mixture were as previously described [13–17]. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. The animals were observed daily and humanely sacrificed by CO_2 inhalation if 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 or body temperature drop. Animals were housed in a barrier facility on a high-efficacy particulate arrestance (HEPA)-filtered rack under standard conditions of 12-h light/dark cycles. The animals were fed an autoclaved laboratory rodent diet. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

2.2. Patient-derived tumor

The pancreatic cancer was previously resected and established in nude mice in the MD Anderson Cancer Center. All experimental protocols and data were collected as described [13–17,27–32]. Written informed consent was provided by the patient and the Institutional Review Board (IRB) of MD Anderson Cancer Center approved this experiment.

2.3. Establishment of PDOX models of pancreatic cancer by surgical orthotopic implantation (SOI)

After nude mice were anesthetized with the ketamine solution described above, a 1–2 cm skin incision was made on the left side abdomen through the skin, fascia and peritoneum and the pancreas was exposed. Surgical sutures (8–0 nylon) were used to implant tumor fragments onto the tail of pancreas to establish the PDOX model. The wound was closed with a 6–0 nylon suture (Ethilon, Ethicon, Inc., NJ, USA). All experimental protocols and data were collected as described [13–17,27–32].

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 [33].

2.5. Formulation of o-rMETase and pyridoxal L-phosphate (PLP) supplement

Mouse drinking water contained 100 μmol/l PLP. rMETase was administered twice daily by gavage using a stainless feeding needle (50 units, [1 mg], twice a day) in phosphate-buffered saline (PBS).

2.6. Treatment study design in the PDOX model of pancreatic cancer

Pancreatic cancer PDOX nude mice were randomized into four groups of 10 mice each: untreated control; i.p.-rMETase (50 units, i.p., twice a day, 14 consecutive days); o-rMETase (50 units, p.o., twice a day, 14 consecutive days); o-rMETase + i.p.-rMETase (50 units, p.o. +50 units i.p., twice a day, 14 consecutive days). Tumor length and width were measured on day 0 and 14. Tumor volume was calculated

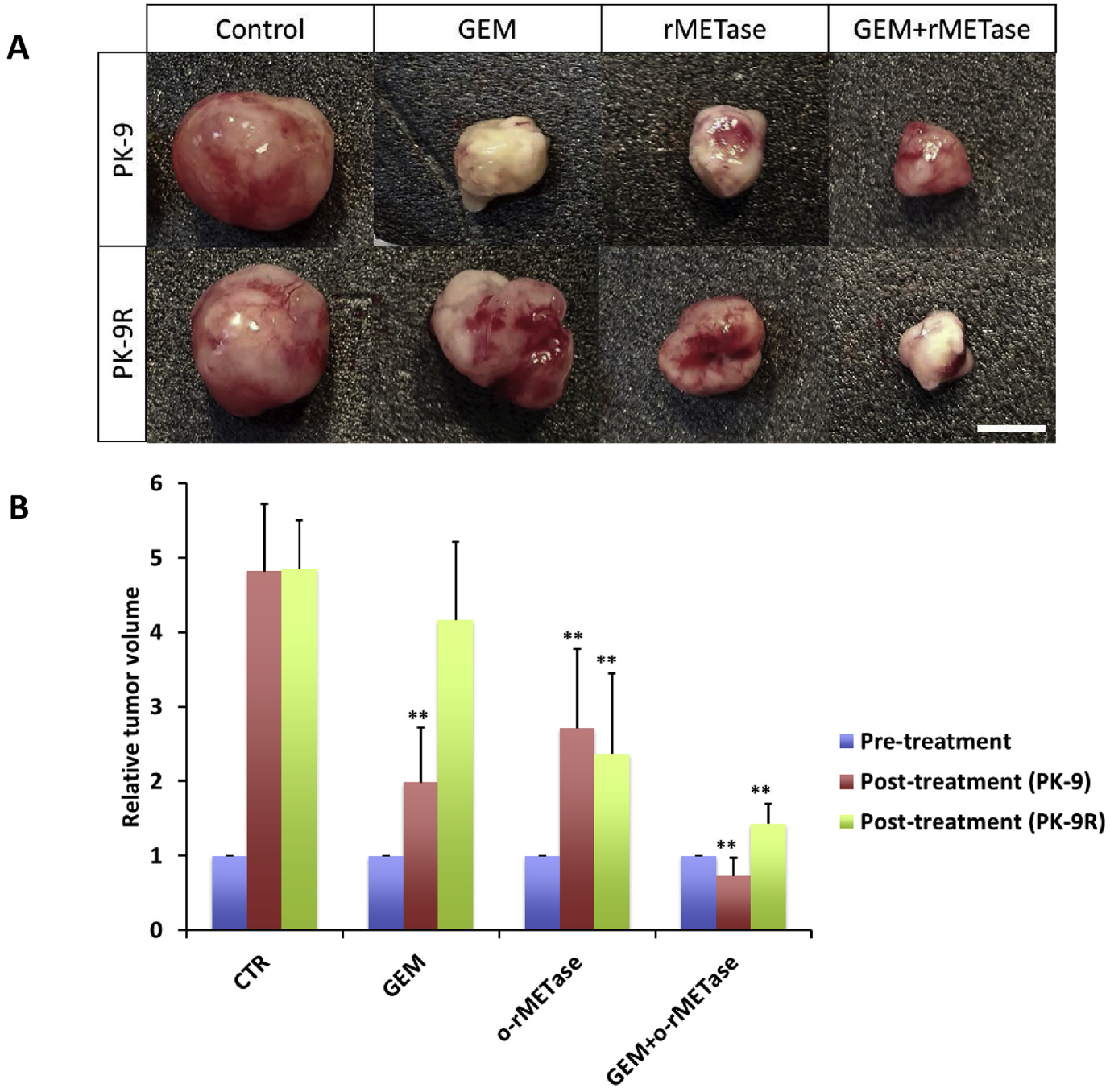

Fig. 5. (A) Macroscopic evaluation of therapeutic efficacy of GEM, o-rMETase, and GEM + o-rMETase. **(B)** Quantitative efficacy of GEM and rMETase. Bar graphs demonstrate tumor volume at post-treatment relative to the pre-treatment tumor volume. **p < 0.01, compared to each control. Error bar: ± SD.

with the following formula: Tumor volume (mm^3) = length (mm) × width (mm) × width (mm) × 1/2. All experimental protocols and data were collected as described [13–17,27–32]. Data are presented as mean ± SD.

2.7. Cell culture

Two human pancreatic cancer cell lines, PK-9 and PK-9R, were established in the Department of Surgery, Graduate School of Medicine, Tohoku University (Sendai, Japan). PK-9R was developed by continuously exposing PK-9 cells to GEM, with a starting concentration of 1 ng/ml (IC_{50} value for PK-9), followed by stepwise increases to 1000 ng/ml. These cells were seeded onto non-coated tissue culture dishes and cultured with RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The culture medium was exchanged every 2 days.

2.8. Cell growth assay

Briefly, the cells were seeded on 96-well plates (5000 cells/well) in culture medium containing 10% FBS. At day 2, 4, 6, 8 and 10, the cells were counted using trypan blue from triplicate culture.

2.9. Cytotoxicity assay

The growth-inhibitory efficacy of GEM on each cell line was assessed by means of a colorimetric assay using a Cell Counting Kit

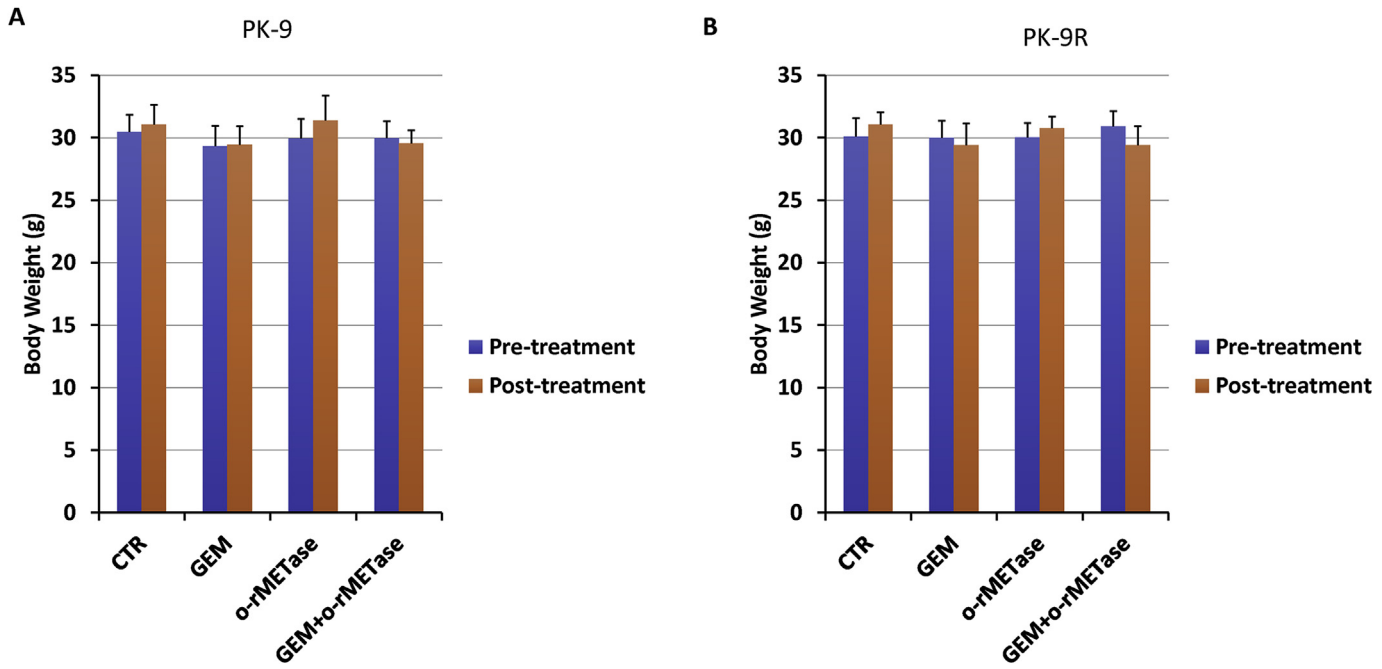

Fig. 6. Effect of GEM, rMETase, and GEM-rMETase on mouse body weight. Bar graph shows body weight at both pre- and post-treatment. **(A)** PK-9; **(B)** PK-9R. Error bar: ± SD.

(Dojindo, Kumamoto, Japan). Briefly, the cells were seeded on 96-well plates (5000 cells/well) in culture medium containing 10% FBS. After 24 h, the cells were incubated with GEM (0.01–100 nM) or rMETase (0.001–10 units) for 72 h, and then cell viability was determined according to the manufacturer's instructions. Absorbance was measured at 450 nm with a microplate reader. The IC_{50} value was estimated by plotting cell viability versus drug concentration.

2.10. Establishment of subcutaneous tumor

PK-9 and PK-9R cells were harvested by trypsinization and washed twice with serum-free medium. Cells of each subline (5×10^5 in 50 µl serum-free medium with 50% Matigel) were injected subcutaneously in nude mice. Three weeks after injection, subcutaneous tumors were established.

2.11. Surgical orthotopic implantation of pancreas

Subcutaneous pancreatic-cancer tumors, as described above, were resected and cut into blocks (5 mm^3). After nude mice were anesthetized with the ketamine solution described above, a 1–2 cm skin incision was made on the left side abdomen through the skin, fascia and peritoneum and pancreas was exposed. Surgical sutures (8–0 nylon) were used to implant tumor fragments onto the tail of pancreas. The wound was closed with a 6–0 nylon suture (Ethilon, Ethicon, Inc., NJ, USA) [34,35], all as described above.

2.12. Treatment study design for PK-9 or PK-9R cell lines

Mice implanted othotopically with PK-9 or PK-9R were randomized into four groups of 10 mice each: untreated control; GEM (1000 mg/kg, intra-peritoneal injection (i.p.), once a week for 2 weeks); o-rMETase (50 units, p.o., twice a day for 2 weeks); GEM + i.p.-rMETase (GEM: 1000 mg/kg, i.p., once a week for 2 weeks + o-rMETase: 50 units, p.o., twice a day for 2 weeks). Tumor length and width were measured both before and after treatment. 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 ± SD. The tumor volume ratio is defined at the tumor volume at each point relative to pre-treatment tumor volume as described above.

2.13. Statistical analysis

JMP version 11.0 was used for all statistical analyses. Significant differences for continuous variables were determined using the Mann-Whitney *U* test. Line graphs expressed average values and error bars showed SD. A probability value of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Efficacy of rMETase on a PDOX model of pancreatic cancer

To test which rMETase (o-rMETase or i.p.-rMETase) can effectively inhibit a PDOX model of pancreatic cancer, we treated mice with i.p.-rMETase, or o-rMETase alone or the combinations of o-rMETase + i.p.-rMETase twice a day for 14 consecutive days and compared them with untreated control group. All treatments significantly inhibited tumor growth compared to the untreated control (i.p.-rMETase: $p < 0.0001$; o-rMETase: $p < 0.0001$; o-rMETase + i.p.-rMETase: $p < 0.0001$); on day 14 after initiation of treatment. o-rMETase was significantly more effective than i.p.-rMETase ($p = 0.0028$). However, the combination of o-rMETase + i.p.-rMETase was significantly more effective than ip-rMETase alone or o-rMETase alone (i.p.-rMETase: $p = 0.0003$, o-rMETase: $p = 0.0006$) (Fig. 1).

3.2. Effect of rMETase treatment on body weight

We also compared the body weight between individual treatment group (i.p.-rMETase, or o-rMETase) and the combination group (o-rMETase + i.p.-rMETase) and found no significant body weight loss in any treatment group compared to the untreated control (Fig. 2). Furthermore, there were no animal deaths in any group. These results showed the safety of o-rMETase as well as i.p.-rMETase.

3.3. In vitro efficacy of rMETase and GEM against GEM-resistant pancreatic cancer cells

The PK-9R GEM-resistant cell line and its parental GEM-sensitive cell line (PK-9) had comparable cell-proliferation kinetics (Fig. 3). The IC_{50} value of GEM for PK-9R was more than 100 times greater than that of the parental PK-9 ($p < 0.0001$) (Fig. 4A). In contrast, the IG_{50} of rMETase was similar (PK-9: 0.21 ± 0.01 unit, PK-9R: 0.14 ± 0.01 unit, $p = 0.0809$) for both lines (Fig. 4B, Table 1). Thus, rMETase could overcome GEM resistance.

3.4. Efficacy of oral rMETase on GEM-resistant pancreatic cancer in orthotopic mouse models

Next, we compared the efficacy of oral rMETase on GEM-resistant pancreatic cancer using orthotopic mouse models. We found that the parental PK-9 tumor was inhibited by GEM ($p = 0.0002$), and the PK-9R tumor was resistant ($p = 0.0890$) compared to the untreated control (Fig. 5A and B). In contrast, oral rMETase (o-rMETase) significantly inhibited both tumor types (PK-9: $p = 0.0008$; PK-9R: $p = 0.0003$) compared to the untreated control (Fig. 5B). However, the combination of GEM + o-rMETase regressed the PK-9 tumor ($p = 0.0002$) and almost arrested the PK-9R tumor growth ($p = 0.0002$) compared to the untreated case (Fig. 5B). These results showed that rMETase therapy was effective alone and overcame GEM resistance in a pancreatic cancer orthotopic model.

3.5. Effect of rMETase and GEM treatment on body weight

We also measured the body weight between rMETase and GEM treatment groups. Body weight loss was observed in the treatment groups including GEM; however, there were no significant differences between any group compared to the untreated control (Fig. 6). There were no animal deaths in any group.

4. Discussion

In the present study using a PDOX model, we show that o-rMETase is more effective than injectable rMETase for pancreatic cancer. In addition, we show that o-rMETase can overcome acquired GEM resistance in pancreatic cancer and demonstrates the clinical potential of this strategy. We have previously shown the effect of injectable rMETase on tumor histology [14–16]. Detailed histological effects of o-rMETase will be part of future studies.

We have developed PDOX mouse models of cancer for discovery of transformative therapy for recalcitrant cancer. The PDOX nude mouse model is established with the technique of surgical orthotopic implantation (SOI). These models include breast cancer [36], ovarian cancer [37], lung cancer [38], cervical cancer [39], colon cancer [40–42], as well as pancreatic cancer [43–47] and stomach cancer [48], melanoma [14,27–29,43,49,50] as well as sarcoma [15–17,51–69]. Our PDOX model has many advantages over subcutaneous-transplant models which grow ectopically under the skin and rarely can metastasize [70].

We have recently demonstrated that rMETase is effective against a PDOX model of Ewing's sarcoma [51]. We have also found l-rMETase to be effective in a PDOX model of DOX-resistant spindle-cell sarcoma [15] and to overcome DOX-resistance in the spindle-cell sarcoma PDOX [16]. rMETase was also effective against a BRAF-V600E melanoma PDOX and a non-BRAF-V600E melanoma [14].

Extensive safety tests were performed with rMETase in primates [71,72] and initial testing in humans [73,74] indicated minimal toxicity of rMETase. Very recently we have shown that rMETase can be administered orally in a melanoma PDOX [49] and in the present study on a pancreatic cancer PDOX, suggesting the near-future widespread use of o-rMETase for all types of cancer in the clinic.

MET dependence is due to the overuse of MET for aberrant transmethylation reactions in cancer and is possibly the only known general metabolic defect in cancer [1,16,75–80]. The overuse of MET by cancer cells for enhanced and unbalanced transmethylation may be the basis of the methionine dependence of cancer cells and is termed the “Hoffman effect”, analogous to Warburg effect of glucose overuse in cancer [3,75–80]. The Hoffman effect can be observed clinically in [^{11}C] MET-PET imaging which gives a much stronger signal [81] than fluorodeoxyglucose (FDG)-PET [82].

The present study reports that o-rMETase is effective against the pancreatic cancer PDOX and is more effective than i.p.-rMETase. The use of o-rMETase opens many possibilities for chronic cancer treatment, for example, of recalcitrant cancers such as pancreatic as well as melanoma. O-rMETase can also be used for cancer prevention and for general life span extension of healthy people since MET is also a target of aging [72,83]. We have previously definitively shown that o-rMETase targets and lowers plasma MET to a greater extent than ip-rMETase [49]. We have also previously shown that rMETase lowers tumor MET [13,14,26,51]. Future experiments will focus on details of MET lowering by o-rMETase, such as the effect of combination therapy. The point of the present study is that o-rMETase, an easily administered oral drug, overcomes GEM-resistance in pancreatic cancer. The present study demonstrates the potential of o-rMETase to revolutionize pancreatic cancer therapy and overcome the lethal aspect of this disease, GEM-resistance.

Several mechanisms have been suggested to overcome GEM resistance to pancreatic cancer [84–88]. Amponsah et al. [89] demonstrated that miR-210 may be able to reverse GEM resistance and restore GEM-induced cell death of pancreatic cancer tissue possibly through inhibition of drug efflux by ABCC5. Jung et al. [90] reported that oncolytic adenovirus expressing relaxin (YDC002) could effectively sensitize chemo-resistant pancreatic tumors toward GEM treatment by dismantling the tumor extracellular matrix (ECM) that resulted in dramatic increase in GEM-induced tumor cell death with no side effects. Borsoi et al. [91] found that nanoparticle-albumin-bound paclitaxel (nAb-PTX) treatment of GEM-resistant pancreatic cancer can be enhanced by GEM through up-regulation of caveolin-1 and multistage nanovectors (MSV) by increasing the quantity of nAb-PTX in the tumor. A recent study suggests that microRNA (miR)-1266 promotes pancreatic cancer cells resistance to GEM by targeting multiple negative regulators of the STAT3 and NF-κB pathways and inhibition of miR-1266 sensitizes pancreatic cancer cells to GEM [92].

Many pancreatic cancer patients are initially sensitive to GEM therapy but acquire resistance over the treatment period and eventually succumb to their disease [93,94]. Therefore, more effective approaches to GEM acquired-resistance in pancreatic cancer are urgently needed to improve pancreatic cancer outcome.

Industrial GMP-like production scale of rMETase has been achieved [95–97]. It has been demonstrated that MET starvation induces a tumor-selective G_2 cell-cycle arrest of a cancer cells [98–101], which sensitizes the cancer cells to drugs that interfere with DNA synthesis, such as GEM.

Altogether, our results presented here demonstrate that o-rMETase is superior to injectable rMETase and can overcome acquired GEM resistance of pancreatic cancer. o-rMETase, used in the present study, has important clinical potential to overcome GEM resistance and can be used chronically.

Conflicts of interest

The authors declare that they have no competing interests.

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This paper is dedicated to the memory of A. R. Moossa, M.D., Sun Lee, M.D. and Shigeo Yagi, Ph.D.

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