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# Oral Recombinant Methioninase Sensitizes a Bladder Cancer Orthotopic Xenograft Mouse Model to Low-dose Cisplatinum and Prevents Metastasis

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**Abstract.** *Background/Aim: The aim of the study was to determine if oral recombinant methioninase (o-rMETase) can sensitize an orthotopic bladder tumor in nude mice to low-dose cisplatinum (CDDP). Materials and Methods: The green fluorescent protein (GFP)-expressing UM-UC-3-GFP bladder cancer was surgically orthotopically implanted (SOI) to the bladder in nude mice. The treatment was initiated when the primary tumor volume reached 100 mm$^3$. Mice were assigned to 3 groups: G1: Saline vehicle (0.1 ml per mouse, oral, twice per day); G2: low-dose CDDP (0.5 mg/kg, intraperitoneal twice per week); G3: o-rMETase + low-dose CDDP (100 units per mouse, oral, twice per day + 0.5 mg/kg, intraperitoneal twice per week, respectively). Tumor volume and body weight were measured twice per week. The expression of Ki-67 was detected by immunohistochemistry to evaluate cell proliferation. Results: The combination of o-rMETase and low-dose CDDP increased inhibition efficacy compared to low-dose CDDP monotherapy, on primary-tumor growth (p=0.032) and metastasis (p=0.002). Conclusion: The combination of o-rMETase with low-dose CDDP has future clinical potential for bladder cancer.*

Bladder cancer is a frequently-occurring and fatal malignancy worldwide (1-3). Cisplatinum (CDDP) has been widely used in the clinic as an effective chemotherapy agent for bladder cancer (4, 5). However, CDDP is dose-limited by its toxicity (6). High-dose CDDP demonstrates different types of side-effects including nephrotoxicity, hematotoxicity, ototoxicity, hepatotoxicity, testicular toxicity, *etc*. (7-10).

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*Key Words:* Bladder cancer, orthotopic, nude mice, GFP, cisplatinum, oral recombinant methioninase, combination, sensitize, efficacy.

Methionine restriction (MR), targeting methionine dependence/addiction of cancer, using the recombinant L-methionine α-deamino-γ-mercapto methane-lyase (recombinant methioninase, r-METase), inhibits tumor growth (11-13). Oral recombinant methioninase (o-rMETase) has shown equivalent efficacy as injectable r-METase (11, 14-20). Our previous studies have shown that r-METase combined with CDDP had a synergistic inhibition efficacy on primary tumor growth and metastasis in a variety of cancers, but this has not been reported in bladder cancer (19, 21-25). We previously established an imageable GFP-expressing orthotopic nude-mouse model of human bladder cancer (26). In the present study, we evaluated the inhibition efficacy of low-dose CDDP in combination with o-rMETase on the primary tumor and metastasis of the orthotopic bladder-cancer model.

## Materials and Methods

*Animals*. A total of 15 female athymic nude (nu/nu) mice, 4-6 weeks old (AntiCancer Inc., San Diego, CA, USA) were used in the study. All the mice were obtained and bred at AntiCancer Inc. All animals were maintained in a HEPA-filtered environment at a constant temperature of 24-25˚C and humidity of 50-60%. Cages, food, water and bedding were autoclaved.

*Cell lines*. Human bladder cancer cell line UM-UC-3-GFP (AntiCancer Inc, San Diego, CA, USA) labeled with green fluorescent protein (GFP) was maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin, and cultured at 37˚C in a 5% $CO_2$ incubator.

*Test agent preparation*. rMETase from *Pseudomonas putida* has been previously cloned and produced in *Escherichia coli* (AntiCancer, Inc., San Diego, CA, USA), rMETase was purified as previously described (27). CDDP was purchased from WG Critical Care, LLC (Paramus, New Jersey, USA). Saline was purchased from Nurse Assist, Inc. (Haltom City, TX, USA).

*Subcutaneous injection of cancer cells in nude mice*. Tumor stock was grown subcutaneously (*s.c.*) by injecting 5×10$^6$ UM-UC-3-GFP

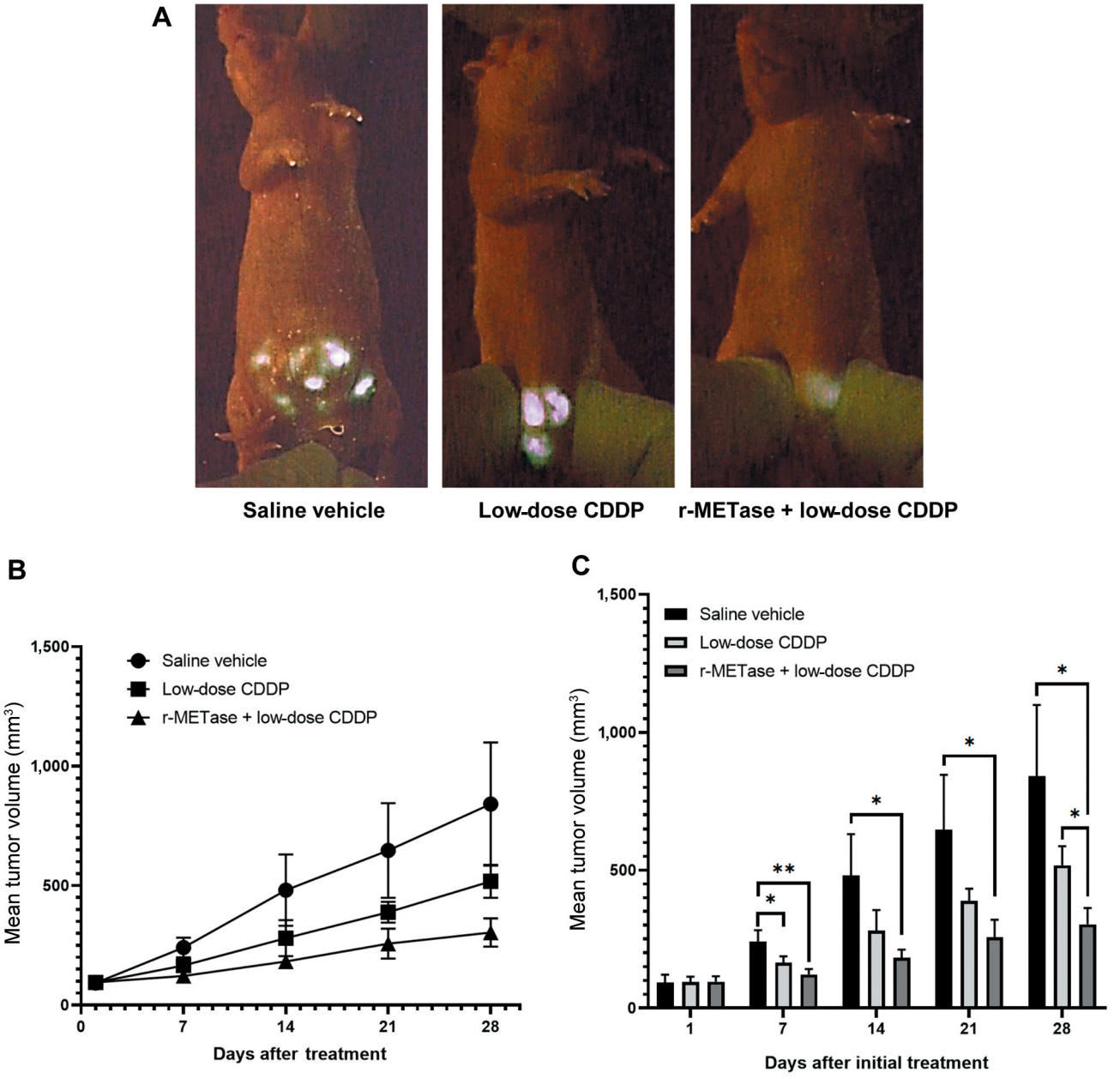


Figure 1. *Comparison of control; and low-dose CDDP; and low-dose CDDP + o-rMETase on tumor growth. (A) Tumor growth monitored by fluorescence in each group; (B) Tumor growth curve from treatment initiation to the end of the study. (C) Comparison of tumor volume between the three groups at each measurement point. *p<0.05; **p<0.01. All data were analyzed using one-way ANOVA followed by Tukey's correction.*

cells in 100 μl PBS into the flank of nude mice. The strong GFP expression in the tumors grown in the subcutis of mice was demonstrated using the FluorVivo fluorescence-imaging system and its software (INDEC Systems, Santa Clara, CA, USA) when the tumor volume reached 500 $mm^3$. The tumor stock was harvested, inspected, and any suspected or grossly-necrotic tissues or non-GFP-expressing tumor tissues were removed. Healthy tumor tissues were subsequently cut into small fragments of approximately 1 $mm^3$ and used for surgical orthotopic implantation (SOI).

*Orthotopic bladder-cancer mouse model.* The UM-UC-3-GFP tumor fragments harvested from the stock subcutaneous tumors were transplanted by SOI to the bladder in nude mice. All procedures of the surgery were performed under an 8x magnification microscope in a HEPA-filtered laminar flow hood. Animals were anesthetized by intramuscular injection of a ketamine mixture. The surgical area was sterilized using iodine and alcohol. An incision of approximately 1 cm long was made in the lower abdomen of the nude mouse using surgical scissors. The bladder was exposed and

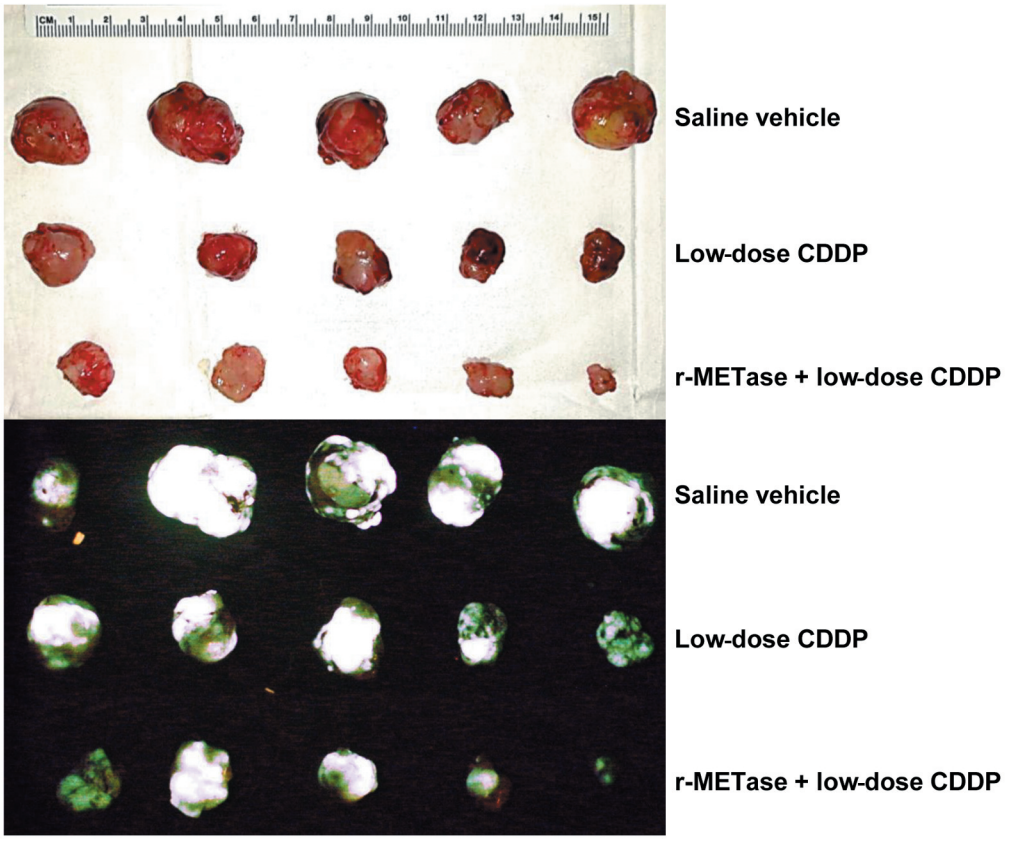


Figure 2. *Images of excised primary tumors shown under bright light (upper panel) and fluorescence (lower panel).*

one UM-UC-3-GFP tumor fragment (1 $mm^3$) was transplanted on the bladder and secured with an 8-0 surgical nylon suture. The abdomen was closed with a 5-0 surgical suture.

*Treatment and monitoring*. All mice bearing tumors were randomized and assigned to 3 groups (n=5) based on tumor size. Group 1: Saline vehicle (0.1 ml per mouse, intraperitoneal, every 3 days + 0.1ml per mouse, oral, twice per day); Group 2: Low-dose CDDP (0.5 mg/kg, intraperitoneal, twice per week); Group 3: Low-dose CDDP + o-rMETase (0.5 mg/kg, intraperitoneal, twice per week + 100 units per mouse, oral, twice per day, respectively). Mouse food was removed every night and refilled every morning to further restrict methionine, which was started when the tumor reached 100 $mm^3$. Primary tumor volume was measured twice per week using the FluorVivo fluorescence-imaging system. Body weight was recorded using an electronic scale. Primary tumor volume was estimated by measuring the perpendicular minor dimension (W) and major dimension (L). Approximate tumor volume was calculated by the formula $(W^2 \times L)/2$.

The study was terminated 28 days after the initiation of treatment. All animals were euthanized, and necropsy was performed. Potential metastasis to local and distant organs including the lymph nodes, spleen, lung, prostate, diaphragm, abdominal cavity was carefully explored. The images of GFP-expressing tumors and metastasis were acquired using the FluoVivo fluorescence-imaging system. Images were processed for contrast and brightness and analyzed with the use of Image-Pro Plus 6.1 software (Media Cybernetics, Silver Springs, MD, USA). The primary tumor was completely resected and weighed using the electronic scale.

*Immunohistochemistry*. Immunohistochemical staining of Ki-67 was performed on the primary tumor of each mouse to compare the difference in cell proliferation between treatment groups and the vehicle-control group. Primary rabbit polyclonal anti-mouse Ki-67 antibody was purchased from Abcam (Abcam Inc., Cambridge, MA, USA). All steps were performed in accordance with the protocol of the rabbit-specific HRP/DAB (ABC) detection IHC kit (Abcam) using 10% formalin-fixed, paraffin-embedded, tissue sections (4 μm) with antigen retrieval, immunostaining, mounting, visualization and counterstaining. The tissue sections, prepared as described above, were examined and representative images were captured using a microscope (IX2-ILL100, Olympus Corp., Tokyo, Japan) and its software (PictureFrame™ , Optronics Inc., Goleta, CA, USA) (28).

*Statistical analysis*. The GraphPad Prism 8.0 software (GraphPad Software, Inc. La Jolla, CA, USA) was used to analyze the data. The significant differences in mean body weight, tumor volume and tumor weight between all groups were calculated using one-way variance (ANOVA) followed by Tukey's correction. The difference in metastasis among the groups was calculated using Chi-square

Table I. *Efficacy of treatment on mean tumor volume at each measurement point.*

| Group | Mean tumor volume ($mm^3$) at each measurement point | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 |
| G1 Saline-vehicle control | 92.5±28.7 | 240.9±41.6 | 481.3±149.3 | 647.5±198.4 | 841.5±257.6 |
| G2 Low-dose CDDP | 94.9±19.6 | 165.5±22.6 | 280.5±75.3 | 389.1±43.6 | 518.0±69.1 |
| G3 o-rMETase + low-dose CDDP | 95.4±20.1 | 122.0±19.8 | 182.5±29.4 | 257.6±63.0 | 303.9±59.0 |
| *p*-Value | G1 *vs*. G2: 0.971 | G1 *vs*. G2: **0.046** | G1 *vs*. G2: 0.095 | G1 *vs*. G2: 0.101 | G1 *vs*. G2: 0.138 |
| | G1 *vs*. G3: 0.976 | G1 *vs*. G3: **0.004** | G1 *vs*. G3: **0.035** | G1 *vs*. G3: **0.043** | G1 *vs*. G3: **0.032** |
| | G2 *vs*. G3: 0.999 | G2 *vs*. G3: 0.128 | G2 *vs*. G3: 0.163 | G2 *vs*. G3: 0.087 | G2 *vs*. G3: **0.013** |

Data are expressed as mean±standard deviation (SD) and analyzed using one-way ANOVA followed by Tukey's correction. A *p*-value of ≤0.05 is considered significantly different; A *p*-value of <0.01 is considered to be extremely significant. Bold values indicate statistical significance.

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## Results

*Establishment of the orthotopic human bladder-cancer mouse model*. Twenty-two animals were used for surgical orthotopic implantation (SOI) with UM-UC-3-GFP tumor fragments harvested from subcutaneous tumors. Fluorescence whole-body imaging was used to monitor tumor growth. Seventeen of 22 animals were found to develop visible tumors.

*o-rMETase combined with low-dose CDDP is effective on the primary and metastatic tumors*. Treatment was initiated 12 days after tumor transplantation when the mean tumor size reached approximately 5-6 mm in diameter (100 $mm^3$ in volume). Fluorescence *in vivo* imaging was used to monitor and measure primary tumor volume (Figure 1A). Mean tumor volume±standard deviation (SD) was calculated for each group and a tumor growth curve was plotted as a function of time (Figure 1B). As shown in Figure 1C, in the early stage of treatment, the two treatment groups showed significantly lower tumor growth compared to the saline-vehicle control ($p<0.05$). At the end of the study, the combination of o-rMETase + low-dose CDDP inhibited primary tumor growth compared to both the saline control ($p=0.032$) and low-dose CDDP monotherapy ($p=0.013$). Although the low-dose-CDDP treatment group showed less primary growth than the saline control, it was not a significant difference ($p>0.05$).

At necropsy, the primary bladder tumor of each mouse was excised and weighed (Figure 2). The mean tumor weight of each group at the end point is shown in Figure 3. The combination of o-rMETase + low-dose CDDP treatment significantly reduced primary tumor weight compared to both the saline-vehicle control ($p=0.045$) and low-dose CDDP monotherapy ($p=0.036$).

The data for mean tumor volume at each measurement point are listed in Table I; The data for mean tumor weight at the end of study are listed in Table II.

Table II. *Efficacy on mean tumor weight at the end of study.*

| Group | Tumor weight (g) Mean±SD | *p*-Value |
|---|---|---|
| G1 Saline-vehicle control | 1.24±0.58 | G1 *vs*. G2: 0.118 |
| G2 Low-dose CDDP | 0.54±0.19 | G1 *vs*. G3: **0.046** |
| G3 o-rMETase + low-dose CDDP | 0.29±0.08 | G2 *vs*. G3: **0.036** |

Data are expressed as mean±standard deviation (SD) and analyzed using one-way ANOVA followed by Tukey's correction. A *p*-value ≤0.05 was considered statistically significant. Bold values indicate statistical significance.

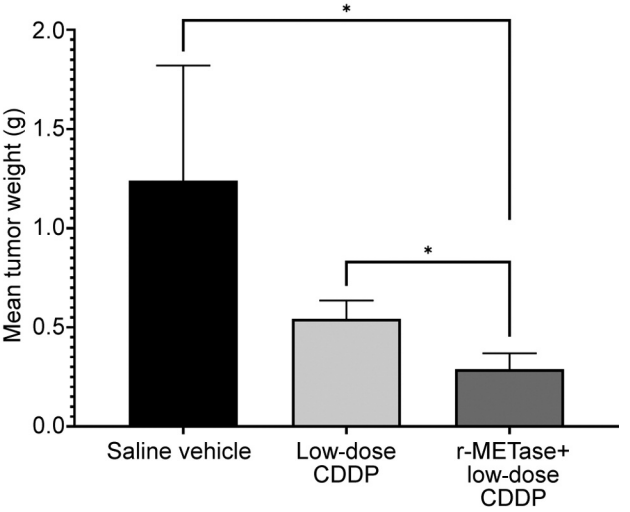


Figure 3. *Final tumor weight. o-rMETase + low-dose CDDP compared to saline vehicle, p=0.045. o-rMETase + low-dose CDDP compared to low-dose CDDP monotherapy, p=0.036). All data were analyzed using one-way ANOVA followed by Tukey's correction.*

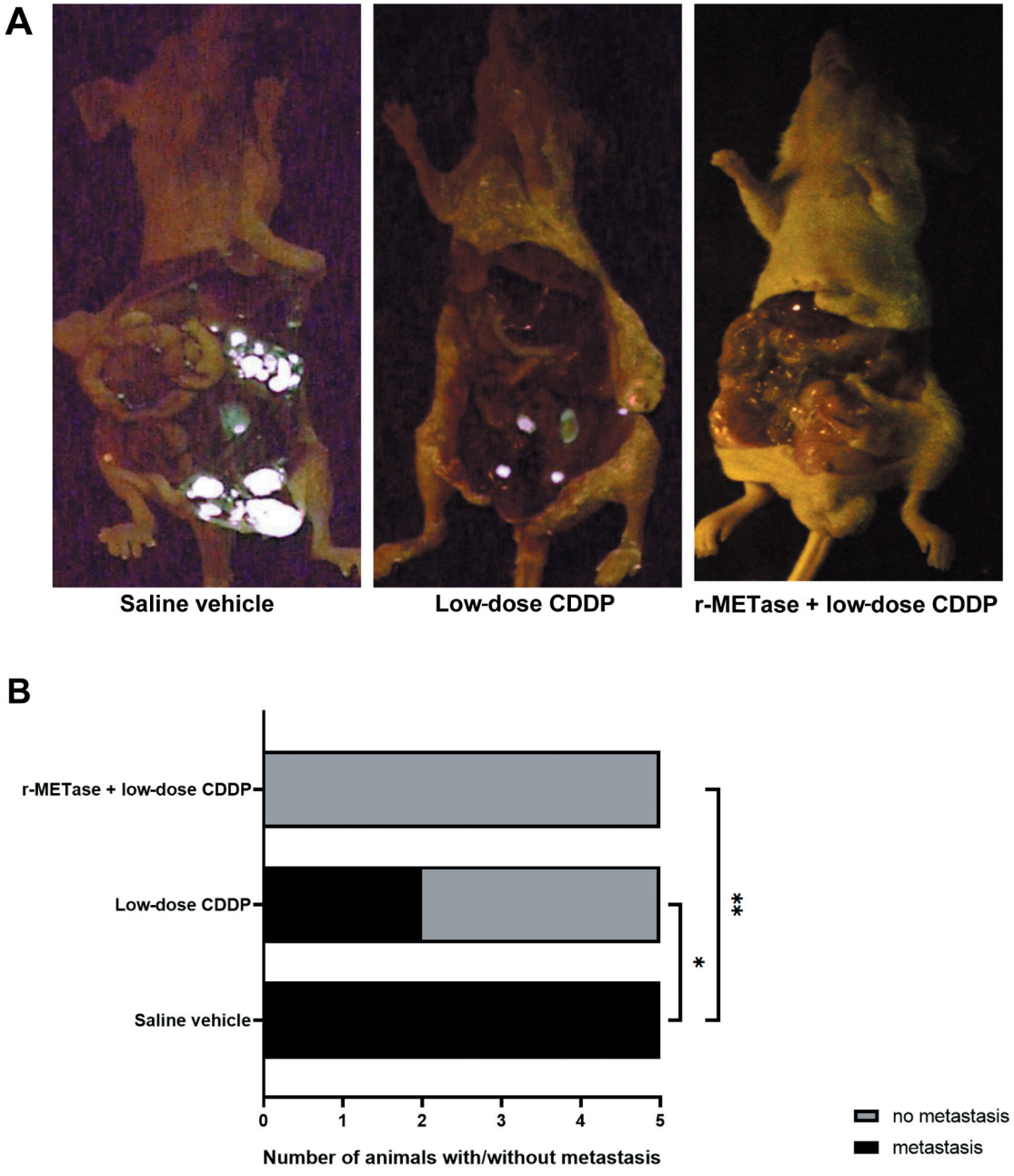


Figure 4. *Comparison of control, low-dose CDDP and the combination of low-dose CDDP and o-rMETase on metastasis incidence. (A) Representative images of metastasis incidence in each group. Wide-spread metastases, including lymph nodes and distant organs, were observed in the saline-control group. In contrast, few lymph-node metastases were found in the low-dose CDDP monotherapy group, and no metastasis in the combination of low-dose CDDP + o-rMETase group, *p<0.05; (B) Metastasis incidence analysis between each group. Low-dose CDDP compared to the saline-vehicle *p<0.05); o-rMETase + low-dose CDDP compared to saline vehicle, *p<0.01. All data were calculated using Chi-square statistical analysis.*

*Effect of o-rMETase and low-dose CDDP on metastasis.* Necropsy was performed in each mouse at the end of the study. All mice had metastasis occurrence in the saline-vehicle control. In contrast, both low-dose CDDP monotherapy and the combination of o-rMETase + low-dose CDDP had significantly less metastasis compared to the vehicle control. In addition, the o-rMETase + low-dose CDDP combination (0 of 5, $p$=0.002 compared to the control) was more effective on metastasis than low-dose CDDP monotherapy (2 of 5, $p$=0.038 compared to the control) (Figure 4A and B).

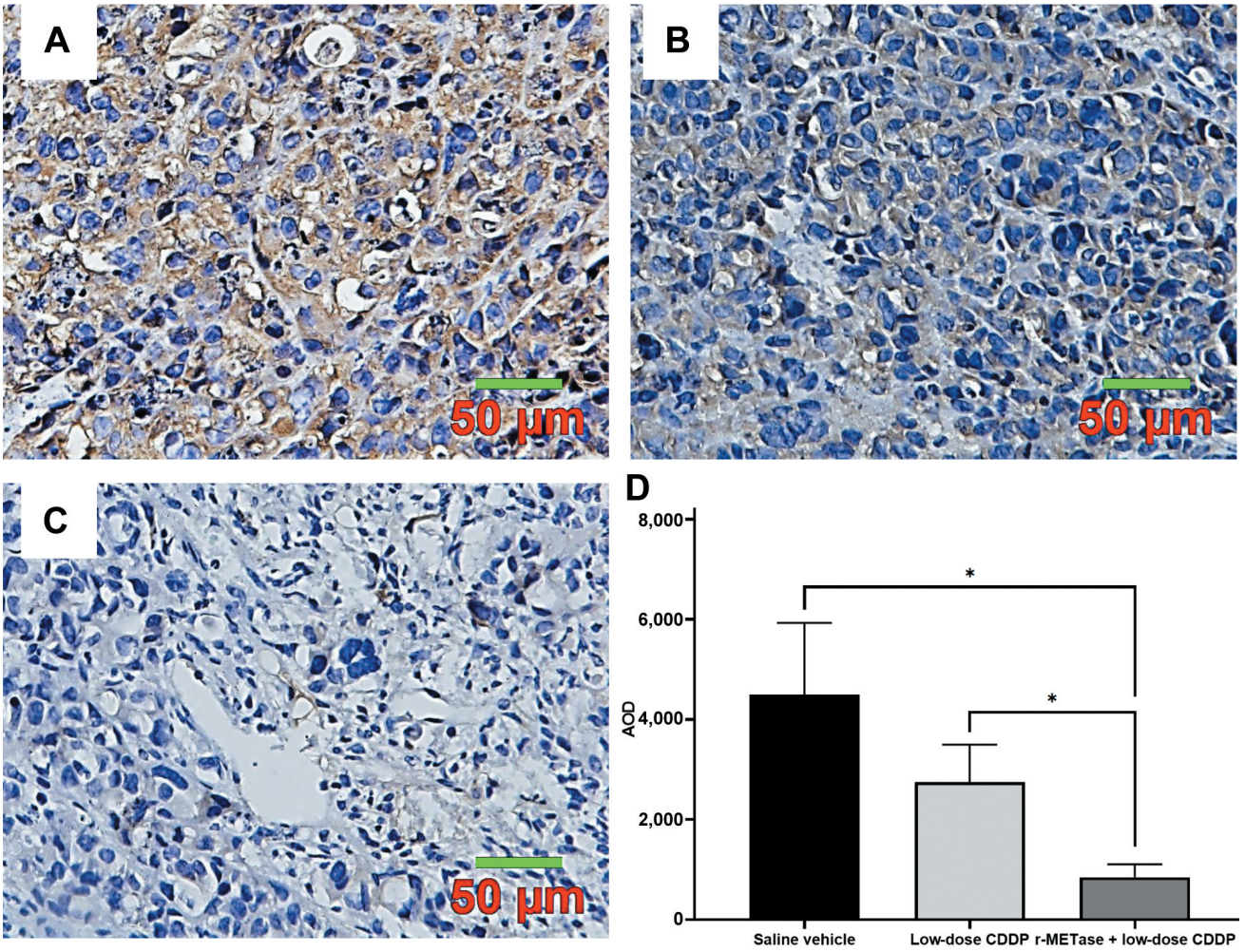


Figure 5. *Comparison of control, low-dose CDDP and the combination of low-dose CDDP and o-rMETase on cancer-cell proliferation within the tumors. (A-C) Representative immunohistochemistry-stained images for Ki-67 expression in each group (200x magnification). (A) Saline vehicle. (B) Low-dose CDDP monotherapy. (C) o-rMETase + low-dose CDDP. (D) Mean average optical density (AOD) in each group. The combination of low-dose CDDP and 0-rMETase showed a significant lower cell proliferation compared to both the saline-vehicle group and low-dose CDDP monotherapy group ($p<0.05$). All data were analyzed using one-way ANOVA followed by Tukey's correction.*

*o-rMETase combined with low-dose CDDP increased the efficacy on cancer-cell proliferation compared to low-dose CDDP monotherapy.* Expression levels of Ki-67 were quantified by the average optical density (AOD) of the positive cells in 5 fields/sample with Image-Pro Plus 6.1 software. As shown in Figure 5, the o-rMETase + low-dose CDDP combination demonstrated a significant reduction in cell proliferation compared to both the saline vehicle ($p$=0.011) and low-dose-CDDP monotherapy group ($p$=0.017). Low-dose-CDDP monotherapy also showed lower cell proliferation than the saline control, although there was not a significant difference ($p$=0.1148).

## Discussion

Methionine dependence in cancer cells was first reported by Sugimura *et al.* in 1959 (29). Methionine dependence is due to the methionine addiction of cancer cells (30). Methionine addiction is caused by methionine over-use for transmethylation reactions which deplete endogenous free methionine and S-adenosylmethionine under methionine restriction (31, 32). There are two strategies to restrict methionine levels in cancer. One is a methionine-free diet (33), but a long-term-low protein diet cannot maintain basic nutritional needs. Another way to deplete methionine is with methioninase

(11-25, 34). Our laboratory has focused on using o-rMETase to treat malignant cancer. In our previous reports, rMETase was shown to selectively trap cancer cells in S/$G_2$-phase of the cell cycle, where they are susceptible to most cytotoxic chemotherapy and can be successfully eradicated (12, 24, 25, 35-37). In our subsequent studies, we found that combination of o-rMETase and chemotherapeutic drugs can reverse drug resistance in some recalcitrant cancers (11-19). Drugs combined with o-rMETase in a variety of cancers, including tigatuzumab in pancreatic cancer (14), azacytidine in osteosarcoma (11), oxaliplatinum in colon cancer (15), have been shown to be effective.

In the present study, we used a GFP-expressing orthotopic bladder-cancer mouse model, in which tumor growth and especially metastasis can be imaged non-invasively and rapidly (26). In the present study, we demonstrated that o-rMETase can significantly enhance the inhibition of low-dose CDDP on primary tumor growth, cancer-cell proliferation and metastasis of bladder cancer.

In conclusion, the combination of o-rMETase and low-dose CDDP has potential for the clinical treatment of bladder cancer, especially for patients with strong side effects from CDDP. o-rMETase is widely effective (38) since methionine addiction, discovered by us (30), is a general phenomenon in cancer (31) and known as the Hoffman effect (39-41). o-rMETase is safe and effective (11, 13) compared to injectable rMETase (42). Therefore, o-rMETase should be an effective agent for clinical bladder cancer in general.

## Conflicts of Interest

The Author's declare no conflicts of interest in regards to the study.

## Authors' Contributions

YS and HN designed the study; NS, JY, KH and HL performed the experiments; YS and GZ analyzed the data; RMH revised the manuscript and supervised the study.

## Acknowledgements

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

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