Abstract. Background/Aim: The fundamental and general hallmark of cancer cells, methionine addiction, termed the Hoffman effect, is due to overuse of methionine for highly-increased transmethylation reactions. In the present study, we tested if the combination efficacy of recombinant methioninase (rMETase) and a methionine analogue, ethionine, could eradicate osteosarcoma cells and down-regulate the expression of c-MYC. Materials and Methods: 143B osteosarcoma cells and Hs27 normal human fibroblasts were tested. The efficacy of rMETase alone and ethionine, alone and in their combination, on cell viability was determined with the WST-8 assay on 143B cells and Hs27 cells. c-MYC expression was examined with western immunoblotting and compared in 143B cells treated with/without rMETase, ethionine, or the combination of both rMETase and ethionine. Results: 143B cells were more sensitive to both rMETase and ethionine than Hs 27 cells, with the following IC50: rMETase (143B: 0.22 U/ml; Hs27: 0.82 U/ml); ethionine (143B: 0.24 mg/ml; Hs27: 0.42 mg/ml). The combination of rMETase and ethionine synergistically eradicated 143B cells, lowering the IC50 for ethionine 14-fold compared to ethionine alone (p<0.001). In contrast, Hs27 fibroblasts were relatively resistant to the combination. The expression of c-MYC was significantly down-regulated only by the combination of rMETase and ethionine in 143B cells (p<0.001). Conclusion: In the present study, we showed, for the first time, the synergistic combination efficacy of rMETase and ethionine on osteosarcoma cells in contrast to normal fibroblasts, which were relatively resistant. The combination of rMETase and ethionine down-regulated c-MYC expression in the cancer cells. The present results indicate the combination of rMETase and ethionine may reduce the malignancy of osteosarcoma cells and can be a potential future clinical strategy.

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Methionine addiction of cancer is due to overuse of exogenous methionine for highly-increased transmethylation reactions (14-23). We have previously reported that all types of cancer cells, including osteosarcoma, are addicted to methionine (18, 21-25).

In the present study, we targeted methionine addiction of osteosarcoma cells with the combination of rMETase and the methionine-analogue ethionine (Figure 1), a competitive inhibitor of reactions involving methionine (26), on cell viability of osteosarcoma cells in comparison with normal fibroblasts, and c-MYC expression in the osteosarcoma cells.

Materials and Methods

Cell culture. The 143B human osteosarcoma cell line and Hs27 normal human foreskin fibroblasts were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Corning Inc., Corning, NY, USA), supplemented with 1 IU/ml penicillin/streptomycin and 10 % fetal bovine serum (FBS).

Reagents. DL-Ethionine was purchased from Sigma-Aldrich, Inc. (#5139-5G, St. Louis, MO, USA). Recombinant methioninase (rMETase) (AntiCancer Inc., San Diego, CA, USA) was produced by fermentation of E.coli, engineered with the Pseudomonas putida methioninase gene, as previously reported (27).

Drug sensitivity assay. Cell viability was determined with the WST-8 reagent (Dojindo Laboratory, Kumamoto, Japan). Cells were cultured on 96-well plates (143B: 7.5×10^2 cells/well; Hs27: 2×10^3 cells/well) in DMEM (100 μl/well), incubated overnight at 37˚C. Cell culture medium was then replaced with medium containing increasing concentrations of rMETase (0; 0.025; 0.05; 0.1; 0.2; 0.4;

Figure 1. Structural formulas of methionine and ethionine.

Figure 2. rMETase and ethionine sensitivity of 143B osteosarcoma cells (mean±SD, n=3). (A) Sensitivity to rMETase. (B) Ethionine Sensitivity to ethionine. (C) Comparison of the efficacy of control, rMETase (0.15 U/ml), ethionine (0.05 mg/ml), and the combination of rMETase (0.15 U/ml) and ethionine (0.05 mg/ml). (D) Sensitivity to ethionine in combination with 0.15 U/ml rMETase. **p<0.001. IC_{50}: Half-maximal inhibitory concentration.
0.8; 1.6; 3.2; 6.4; 12.8 U/ml), ethionine (0; 0.03125; 0.0625; 0.125; 0.25; 0.5; 1; 2; 4; 8 mg/ml), or both of rMETase and ethionine. The cells were incubated at 37˚C for 72 h. After the 72 h incubation period, 10 μl of the WST-8 solution was added to each well, followed by an additional 1 h incubation at 37˚C. Absorbance was measured with a microplate reader (SUNRISE: TECAN, Männedorf, Switzerland) at 450 nm. Drug-sensitivity curves were constructed with Microsoft Excel for Mac ver. 16.74 (Microsoft, Redmond, WA, USA) and half-maximal inhibitory concentration (IC\textsubscript{50}) values were calculated with ImageJ ver. 1.53k (National Institutes of Health, Bethesda, MD, USA). Experiments were performed in triplicate, repeated at least twice.

**Western immunoblotting.** Protein extraction of 143B osteosarcoma cells, treated with/without rMETase (0.15 U/ml), ethionine (0.05 mg/ml), or the combination of rMETase (0.15 U/ml) and ethionine (0.05 mg/ml), was performed as previously reported (22), as follows: RIPA Lysis and Extraction buffer (Thermo Fisher Scientific, Waltham, MA, USA) with 1% Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) were used for extraction of total protein from cells. 10% SDS-PAGE gels were used for electrophoresis, and 0.45 μm polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Chicago, IL, USA) were used for transfer of proteins after gel electrophoresis. Bullet Blocking One for Western Blotting (Nakalai Tesque, Inc., Kyoto, Japan) was used for blocking membranes. Anti-c-MYC antibody (1:2,000, #10828-1-AP, Proteintech, Rosemont, IL, USA) and anti-β actin antibody (1:1,500, #20536-1-AP, Proteintech) were used. β-actin was used as internal loading control. For the secondary antibody, horseradish-peroxidase-conjugated anti-rabbit IgG (1:5,000, #SA00001-2, Proteintech) was used. A UVP ChemStudio (Analytik Jena, Upland, CA, USA) was used to image the western blot, using the Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). Quantification of western immunoblotting was performed with ImageJ ver. 1.53k (National Institutes of Health).

**Statistical analysis.** Tukey’s honest significant difference (Tukey’s HSD) test was performed to statistically compare the means of four treatment groups: control; rMETase; ethionine; combination of rMETase and ethionine, with JMP pro ver. 15.0.0 (SAS Institute, Cary, NC, USA). Bar graphs indicate the mean, error bars indicate standard deviation of the mean. A statistically-significant difference was defined with a probability value ≤0.05.

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Figure 3. rMETase and ethionine sensitivity of Hs27 normal-human fibroblasts (mean±SD, n=3). (A) Sensitivity to rMETase. (B) Sensitivity to ethionine. (C) Comparison of the efficacy of control, rMETase (0.15 U/ml), ethionine (0.05 mg/ml), and the combination of rMETase (0.15 U/ml) and ethionine (0.05 mg/ml). (D) Sensitivity to ethionine combined with 0.15 U/ml rMETase. IC\textsubscript{50}: Half-maximal inhibitory concentration.
Results

Combination of recombinant methioninase and ethionine synergistically eradicated 143B osteosarcoma cell, but Hs27 normal-human-foreskin fibroblasts were relatively resistant. 143B osteosarcoma cells were more sensitive to both rMETase alone and ethionine alone than Hs27 fibroblasts, with the following IC50s: rMETase (143B: 0.22 U/ml; Hs27: 0.82 U/ml) (Figure 2A, Figure 3A, and Figure 4A); ethionine (143B: 0.24 mg/ml; Hs27: 0.42 mg/ml) (Figure 2B, Figure 3B, and Figure 4B). The combination of rMETase and ethionine eradicated 143B cells with the IC50 for ethionine lowered to 0.017 from 0.24 for ethionine alone, 14-fold reduction (p<0.001). In contrast, the Hs27 fibroblasts were relatively resistant to the combination treatment (Figure 2C and D, Figure 3C and D, Figure 4C and D).

Combination of recombinant methioninase and ethionine synergistically down-regulates c-MYC expression. The protein expression of c-MYC was evaluated in 143B osteosarcoma cells treated with/without rMETase, ethionine, or the combination of rMETase and ethionine, with western immunoblotting, at the 15% inhibitory concentrations (IC15) of rMETase (0.15 U/ml) and ethionine (0.05 mg/ml). This combination of rMETase and ethionine significantly down-regulated c-MYC expression (compared to control: p<0.001; compared to rMETase alone: p=0.0057; compared to ethionine alone: p=0.0065). rMETase alone (IC15) or ethionine alone (IC15) did not decrease the expression of c-MYC (p=0.11, 0.37, respectively) (Figure 5).

Discussion

In the present study, the combination of rMETase (0.15 U/ml) and ethionine (0.05 mg/ml) eradicated osteosarcoma cells and down-regulated c-MYC oncogene expression. In contrast, this combination of rMETase and ethionine, at the same concentrations, inhibited Hs27 normal-fibroblast viability by only 30%.
Ethionine is a structural analog of methionine, with a methyl group replaced by an ethyl group. Ethionine is an anti-metabolite of methionine and cells synthesize S-adenosylethionine from ethionine, instead of S-adenosylmethionine, which is the main methyl donor for proteins, DNA, and RNA (26). The synergistic efficacy of rMETase and ethionine on cell viability may be due to the combination of the mimetic effect of ethionine on methionine and depletion of exogenous methionine itself by rMETase. The synergy was strong: rMETase lowered the IC50 for ethionine 14-fold compared to ethionine alone.

The results of the present study are consistent with previous reports showing that the combination of methionine starvation and ethionine inhibited the proliferation of prostate cancer cells (28) and glioma cells (29), and the growth of the Yoshida sarcoma in mice (30). The present results show that the combination of rMETase and ethionine is highly effective for osteosarcoma, and may be highly effective on other types of sarcomas and carcinomas.

The combination of rMETase and ethionine is much more toxic to cancer cells than normal cells, because it attacks methionine addiction, the fundamental basis of cancer (8-13).
c-MYC is one of the most commonly activated oncoproteins in osteosarcoma. c-MYC is related to cancer-cell proliferation and tumor growth. Expression of the c-MYC has been reported to be closely related to prognosis of osteosarcoma patients (31-34). c-MYC is linked to both osteosarcoma malignancy and methionine addiction (23).

In the present study, the combination of rMETase and ethionine synergistically down-regulated c-MYC expression. These results indicate the combination of rMETase and ethionine has reduced osteosarcoma cell viability, at least in part, by down-regulating c-MYC.

In the present study, we showed for the first time, the combination of rMETase and ethionine selectively eradicated osteosarcoma cells, while down-regulating c-MYC expression, indicating the combination of rMETase and ethionine is a potential future clinical strategy for osteosarcoma.

Conflicts of Interest

The Authors declare that there are no conflicts of interest to declare in relation to this study.

Authors’ Contributions

YA, YT and RMH were involved in study conception and design. YA, YK, NM, KO, and RMH analyzed and interpreted data. YA, YT and RMH wrote the manuscript. All Authors reviewed and approved the manuscript.

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