

Extensive DNA Damage and Loss of Cell Viability Occur Synergistically With the Combination of Recombinant Methioninase and Paclitaxel on Pancreatic Cancer Cells which Report DNA-Damage Response in Real Time

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Abstract. Background/Aim: Methionine restriction selectively arrests cancer cells during the S-phase of the cell cycle. We hypothesized that DNA damage may occur in S-phase in cancer cells during methionine restriction. To determine if this occurs, we used MiaPaCa-2^{Tet-On} 53BP1-green fluorescent protein (GFP) pancreatic cancer cells, which report GFP fluorescence in real time after DNA-damage response (DDR) in these cells. We also determined whether a chemotherapy drug in combination with methionine restriction increases the rate of DNA damage. Materials and Methods: MiaPaCa-2^{Tet-On} 53BP1-GFP cells were used for in vitro experiments. The 25% and 50% inhibitory concentrations (IC₂₅ and IC₅₀, respectively) of recombinant methioninase (rMETase) and paclitaxel on MiaPaCa-2^{Tet-On} 53BP1-GFP pancreatic cancer cells were determined. Cell viability and DDR with rMETase alone, paclitaxel alone, and their combination were measured in MiaPaCa-2^{Tet-On} 53BP1-GFP cells. Results: The IC₂₅ of rMETase on MiaPaCa-2^{Tet-On} 53BP1-GFP cells

was 1.66 U/ml. The IC₂₅ for paclitaxel on MiaPaCa-2^{Tet-On} 53BP1-GFP cells was 3.31 nM. The combination of rMETase and paclitaxel synergistically reduced the viability of MiaPaCa-2^{Tet-On} 53BP1-GFP cells. The IC₅₀ of paclitaxel on MiaPaCa-2^{Tet-On} 53BP1-GFP cells was 5.1 nM. The IC₅₀ of rMETase on MiaPaCa-2^{Tet-On} 53BP1-GFP cells was 2.3 U/ml. The combination of rMETase (IC₅₀) plus paclitaxel (IC₅₀) on MiaPaCa-2^{Tet-On} 53BP1-GFP cells also caused more DNA damage than either agent alone. Conclusion: The present study suggests the synergy of methionine restriction and chemotherapy is due, at least in part, to DNA damage of cancer cells.

We discovered methionine addiction, which is a fundamental and general hallmark of cancer (1). Methionine addiction of cancer is termed the Hoffman Effect (2). Our laboratory has developed a recombinant methioninase (rMETase) to specifically restrict the availability of methionine and target the methionine addiction of cancer (3). Multiple studies have shown that combining chemotherapy with methionine restriction, achieved through the use of either rMETase, a methionine-free medium, or a methionine-depleted diet, results in a synergistic effect (4).

Methionine restriction selectively arrests cancer cells in the S-phase of the cell cycle during DNA replication (5, 6). We hypothesized that during selective S-phase arrest of cancer cells by methionine restriction, DNA damage and repair may occur.

Efimova *et al.* (7) linked the green fluorescent protein (GFP) with the DNA damage-response (DDR) protein 53BP1 to establish a reporter for live-cell imaging of DDR. In the MiaPaCa-2^{Tet-On} 53BP1-GFP pancreatic-cancer cell line, GFP expression in the cells reports DNA damage and repair in real time.

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Key Words: DNA damage, synergy, rMETase, paclitaxel, methionine addiction, Hoffman effect, methionine restriction.

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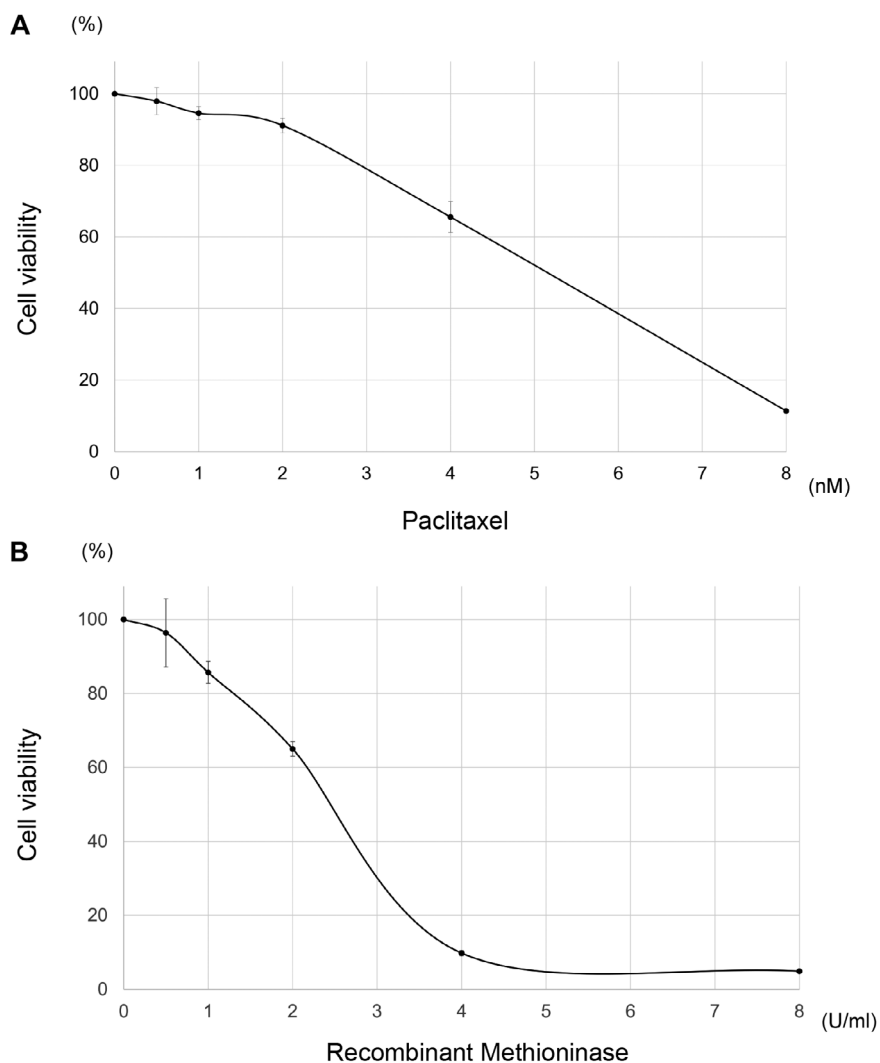


Figure 1. Paclitaxel and recombinant methioninase (rMETase) treatment of MiaPaCa-2^{Tet-On} 53BP1-GFP cells. (A) Sensitivity of MiaPaCa-2^{Tet-On} 53BP1-GFP cells to paclitaxel. (B) Sensitivity of MiaPaCa-2^{Tet-On} 53BP1-GFP cells to rMETase (mean±standard deviation, n=3).

Paclitaxel is widely used in oncology and it induces mitotic arrest in cancer cells by interfering with the normal functioning of microtubules (8).

The present study aimed to demonstrate if rMETase alone or paclitaxel alone or their combination are synergistic on MiaPaCa-2^{Tet-On} 53BP1-GFP cells to induce DNA damage and repair, as well as reduce cell viability.

Materials and Methods

Cell culture. The MiaPaCa-2^{Tet-On} 53BP1-GFP cell line (Clontech, Carlsbad, CA, USA) was previously genetically modified by introducing a fusion of 53BP1 and GFP using a lentiviral vector (Clontech) which is controlled by a tetracycline- or a doxycycline-responsive promoter (7). MiaPaCa-2^{Tet-On} 53BP1-GFP cells were

cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with high glucose (Thermo Fisher Scientific, Waltham, MA, USA) and fetal bovine serum-tet-system approved (Thermo Fisher Scientific) (10%) that is compatible with tet-responsive promoters (7).

Reagents. Paclitaxel was obtained from FRESINIUS KABI (Lake Zurich, IL, USA). rMETase was produced in AntiCancer Inc. (San Diego, CA, USA). The method for producing rMETase has been previously reported (9).

Determination of IC₂₅ and IC₅₀ of rMETase and paclitaxel on MiaPaCa-2^{Tet-On} 53BP1-GFP cells. Cell viability was assessed using the WST-8 reagent (Dojindo Laboratory, Kumamoto, Japan). MiaPaCa-2^{Tet-On} 53BP1-GFP cells were cultured in 96-well plates (3×10³ cells/well) in DMEM (100 µl/well) and incubated at 37°C overnight. MiaPaCa-2^{Tet-On} 53BP1-GFP cells were treated with increasing

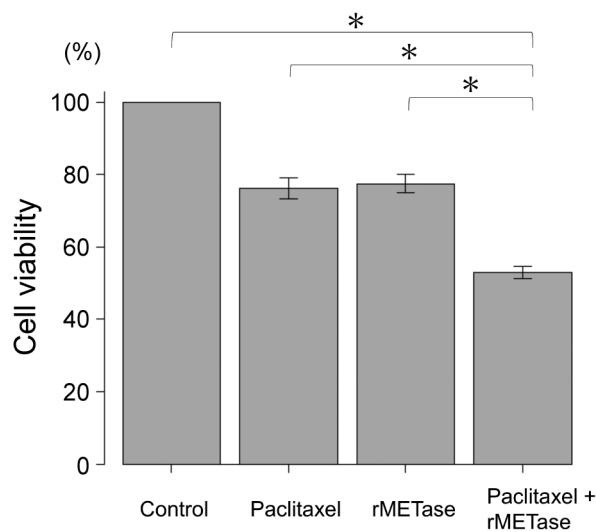


Figure 2. Efficacy of the combination of paclitaxel and rMETase on MiaPaCa-2^{Tet-On} 53BP1-GFP cells. The combination of recombinant methioninase (rMETase) (1.66 U/ml [IC₂₅]) and paclitaxel (3.31 nM [IC₂₅]) was synergistic for MiaPaCa-2^{Tet-On} 53BP1-GFP cells. $n=3$ * $p<0.05$ using Tukey-Kramer analysis.

concentrations of paclitaxel alone, between 0.5 nM and 8 nM for 72 h; or MiaPaCa-2^{Tet-On} 53BP1-GFP cells were treated with increasing concentrations of rMETase alone between 0.5 U/ml and 8 U/ml for 72 h. At the end of the culture period, 10 μ l of the WST-8 solution was added to each well and the plate was additionally incubated for 1 h at 37°C. Absorption was measured with a microplate reader (Sunrise; Tecan, Mannedorf, Switzerland) at 450 nm. Drug-sensitivity curves were obtained with Microsoft Excel ver. 15.52 (Microsoft, Redmond, WA, USA) and one-quarter- and half-maximal inhibitory concentration (IC₂₅ and IC₅₀, respectively) values were calculated using ImageJ ver. 1.53 (National Institutes of Health, Bethesda, MD, USA). Experiments were performed twice, each in triplicate.

Determination of synergy to reduce cell viability. MiaPaCa-2^{Tet-On} 53BP1-GFP cells were seeded at 3×10^3 cells/well in 96-well plates. Twenty-four hours later, four treatment groups were established Control (DMEM); paclitaxel (3.31 nM [IC₂₅]); rMETase (1.66 U/ml [IC₂₅]); and paclitaxel (3.31 nM) plus rMETase (1.66 U/ml). Seventy-two hours later, cell viability was measured in triplicate as described above. In the present study, we defined synergy as an effective combination greater than either component alone.

DNA damage-response assay. MiaPaCa-2^{Tet-On} 53BP1-GFP cells were used to report nuclear DNA-damage response treated either with paclitaxel alone, rMETase alone, or their combination. The cells were seeded at 6×10^5 cells per well in 6-well plates. To observe DNA damage response with GFP, 1mg/ml of doxycycline was added to the culture wells in all groups. Treatments were as following: control (DMEM); paclitaxel (5.1 nM [IC₅₀]); rMETase (2.3 U/ml [IC₅₀]); and paclitaxel (5.1 nM [IC₅₀]) plus rMETase (2.3 U/ml [IC₅₀]). After 48 hours, the cells were examined for GFP fluorescence using an IX71 fluorescence microscope (Olympus, Tokyo, Japan) at a magnification of $\times 200$. DNA-damage response was visualised by GFP fluorescence and was quantified per visual field for six separate fields.

Statistical analysis. All statistical analyses were conducted using the EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan). Associations between variables were tested using Tukey-Kramer analysis. Results with p -values ≤ 0.05 were considered statistically significant.

Results

Determination of synergy of paclitaxel and rMETase to reduce viability of MiaPaCa-2^{Tet-On} 53BP1-GFP cells. The IC₂₅ value of paclitaxel on MiaPaCa-2^{Tet-On} 53BP1-GFP cells was 3.31 nM. The IC₂₅ for rMETase on MiaPaCa-2^{Tet-On} 53BP1-GFP cells was 1.66 U/ml (Figure 1). The combination of paclitaxel (3.31 nM) and rMETase (1.66 U/ml) was synergistic to reduce the viability of MiaPaCa-2^{Tet-On} 53BP1-GFP cells to a greater extent than either agent alone ($p<0.05$) (Figure 2).

Determination of synergy of paclitaxel and rMETase to cause DNA damage response in MiaPaCa-2^{Tet-On} 53BP1-GFP cells. The combination of paclitaxel (5.1 nM [IC₅₀]) plus rMETase (2.3 U/ml [IC₅₀]) caused significantly more DNA-damage response, as observed by GFP fluorescence in MiaPaCa-2^{Tet-On} 53BP1-GFP cells, than either agent alone ($p<0.05$) (Figure 3).

Discussion

Genomic instability is a fundamental feature of cancers and is linked to an increased susceptibility to accumulate DNA damage (10, 11).

rMETase specifically targets the methionine addiction of cancer cells, by methionine restriction. Methionine restriction leads to the selective arrest of cancer cells in the late-S/G₂ phase of the cell cycle while leaving normal cells unaffected (3, 5, 6). Methionine restriction and chemotherapy are synergistic due to the selective S-phase arrest by methionine restriction which chemotherapy targets (4-6). Examples of the synergy of methionine restriction and chemotherapy include rMETase combined with oxaliplatin plus 5-fluorouracil or ethionine or rapamycin or irinotecan or eribulin (12-16). We hypothesized that DNA damage is occurring during S-phase arrest in cancer cells induced by methionine restriction. The selection of paclitaxel and METase was based on the ability of METase to arrest cancer cells in the late-S/G₂ phase, and if they attempt to enter mitosis, they become susceptible to paclitaxel, an antimetabolic agent. The G₂/M checkpoint becomes significantly more crucial in cancer cells after S-phase arrest, due to increased DNA damage. Sufficient DNA damage can surpass the threshold at which cancer cells can survive, even when the G₂/M checkpoint is functioning properly (11).

In the present study, the combination of rMETase and paclitaxel was synergistic to induce DNA damage response on MiaPaCa-2^{Tet-On} 53BP1-GFP cells resulting in loss of cell viability. We have previously shown that rMETase and taxanes are synergistic in both mouse models of cancer and in the clinic (17-20).

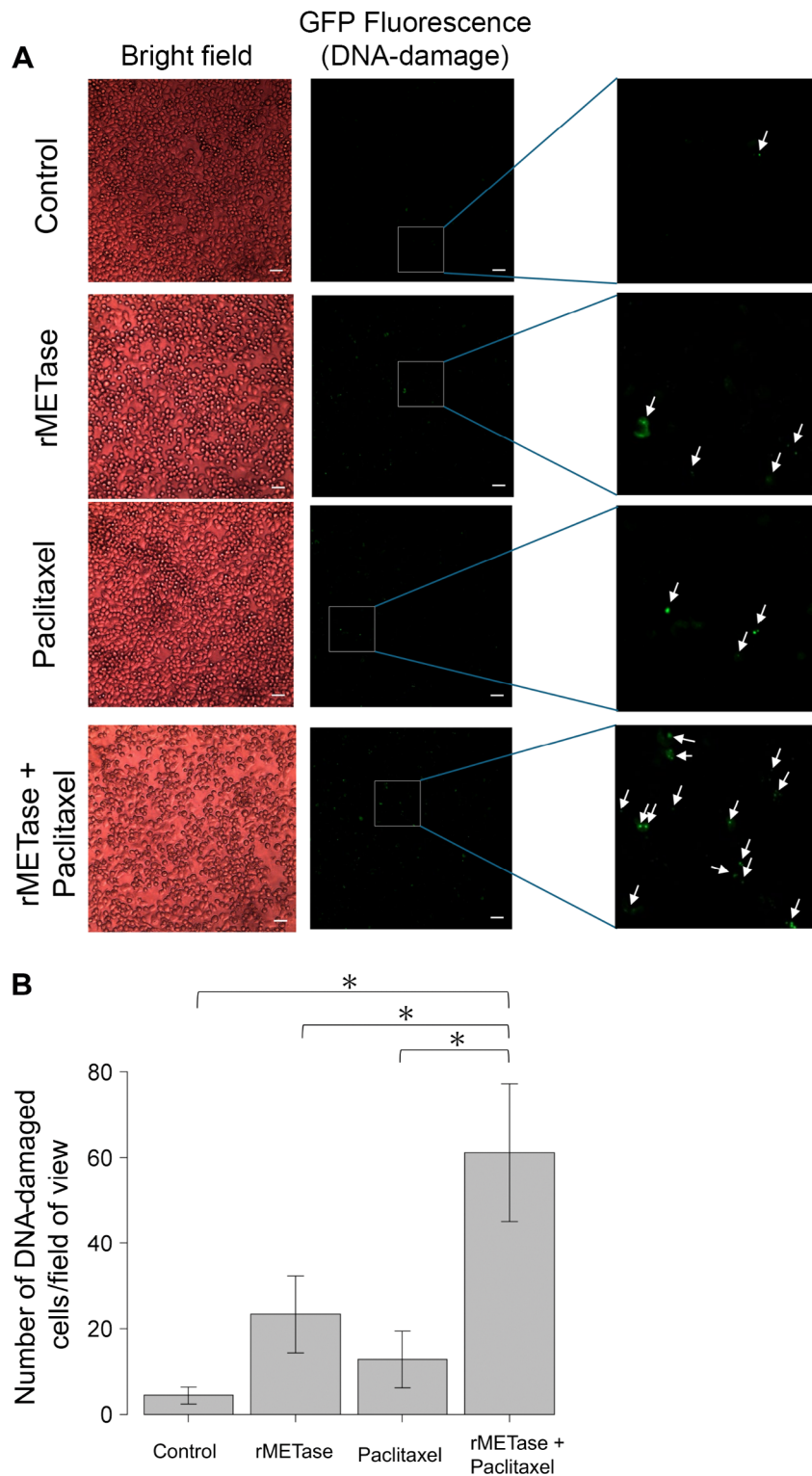


Figure 3. Effect of paclitaxel alone, recombinant methioninase (rMETase) alone and their combination on DNA damage in MiaPaCa-2^{Tet-On} 53BP1-GFP cells. Cells were untreated (Control) or treated with 5.1 nM (IC₅₀) paclitaxel alone, 2.3 U/ml (IC₅₀) rMETase alone or their combination. (A) Images of DNA damage and repair in MiaPaCa-2^{Tet-On} 53BP1-GFP cells expressing green fluorescent protein (GFP). Scale bars: 100 μ m. (B) Quantification of DNA damage in treated cells. GFP fluorescence was observed with an IX71 fluorescence microscope (Olympus, Tokyo, Japan). * $p < 0.05$ (n=6) using Tukey-Kramer analysis.

We recently showed that methionine restriction induced an alternate DNA repair enzyme in BRCA1-mutant breast cancer cells (21). 53BPI mediates and effects the response to double-strand breaks (DSB) of DNA (10, 11) suggesting DSBs occur during S-phase-induced cell-cycle arrest of cancer cells by methionine restriction, enhanced by chemotherapy. Further studies are warranted to determine the role of DNA damage in the synergistic efficacy of rMETase and chemotherapy.

rMETase is effective because it targets the fundamental hallmark of cancer, methionine addiction, termed the Hoffman effect (1-3, 5, 6, 22-43).

Conflicts of Interest

The Authors declare that there are no competing interests.

Authors' Contributions

SM, RMH and QH designed the study. QH provided rMETase. SM performed experiments. SM was the major contributor to writing the article and RMH revised the article. KM, BMK, MS, MB, NY, KH, HK, SM, KI, TH, HT and SD critically read the manuscript.

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