

Simultaneous Targeting of Multiple Hallmarks of Cancer With Recombinant Methioninase, Rapamycin and Chloroquine Is Specific and Synergistic to MiaPaCa-2 Pancreatic-Cancer Cells in Contrast to Hs-27 Normal Fibroblasts

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Abstract

Background/Aim: Pancreatic ductal adenocarcinoma (PDAC) is recalcitrant to conventional therapies. Recombinant methioninase (rMETase), rapamycin, and chloroquine target fundamental hallmarks of cancer. The present study examined the efficacy of each agent alone and in all combinations against PDAC cells compared to normal fibroblasts. **Materials and Methods:** The 30% inhibitory concentration (IC₃₀) of rMETase, rapamycin, and chloroquine on MiaPaCa-2 PDAC cells and Hs-27 normal human fibroblasts was determined with *in vitro* cell-viability assays using the WST-8 reagent. Combination treatment was performed at IC₃₀ concentrations for MiaPaCa-2 cells to evaluate synergistic efficacy of all combinations of rMETase, rapamycin, and chloroquine on each cell type.

Results: rMETase alone led to significantly higher cytotoxicity against MiaPaCa-2 cells than Hs-27 fibroblasts ($p < 0.05$). The triple combination of rMETase, rapamycin and chloroquine synergistically eradicated MiaPaCa-2 cells, reducing viability to 3.8% ($p < 0.05$). In contrast, normal Hs-27 fibroblasts were not synergistically inhibited by the triple combination.

Conclusion: The combination of rMETase, rapamycin and chloroquine had highly synergistic and selective efficacy against PDAC cells *in vitro* compared to normal cells, supporting its potential as a precision-targeted metabolic clinical therapy for pancreatic cancer.

Keywords: Pancreatic cancer, methionine addiction, Hoffman effect, recombinant methioninase, rMETase, rapamycin, RAPA, chloroquine, CQ, combination therapy, synergy.



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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a recalcitrant disease with limited therapeutic options. The overall 5-year survival rate for patients with PDAC is approximately 12%, which is largely due to late-stage diagnosis and aggressive tumor behavior, and has not significantly improved for many decades (1, 2).

Methionine addiction is a fundamental and general hallmark of cancer, commonly referred to as the Hoffman effect (3-5). Methionine addiction has been targeted because cancers show specific vulnerability to methionine restriction (6-13). An effective approach of methionine restriction of cancer cells involves the use of recombinant methioninase (rMETase), an enzyme which degrades methionine (6). Our previous studies have demonstrated synergistic efficacy of rMETase with numerous anti-cancer drugs in all major cancer types (14).

Previous studies demonstrated that rapamycin, a mechanistic target of rapamycin kinase (mTOR) inhibitor, has synergistic efficacy with rMETase in breast osteosarcoma *in vivo* (15) and colon cancer cells (16) *in vitro*. Chloroquine, an autophagy inhibitor, was shown to have synergistic efficacy with rapamycin in a patient-derived orthotopic xenograft mouse model and in *in vitro* experiments on differentiated liposarcoma (15, 17). Based on these results, we previously conducted *in vitro* experiments on the triple combination of rMETase, rapamycin, and chloroquine. We have shown the triple combination has synergy against osteosarcoma cells (18), colon cancer cells (19), and triple-negative breast cancer cells (20). The aim of the present study was to evaluate the synergistic efficacy of the triple combination of rMETase, rapamycin, and chloroquine on PDAC *in vitro* compared to normal fibroblasts.

Materials and Methods

Cell culture. MiaPaCa-2 human PDAC cells and Hs-27 normal human fibroblasts were obtained from the American Type Culture Collection (Manassas, VA, USA).

The cells were grown in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mixture F-12 with GlutaMAX™ supplement (DMEM/F-12), with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified incubator containing 5% CO₂.

Recombinant methioninase production. rMETase was produced by AntiCancer Inc. (San Diego, CA, USA) *via* fermentation of recombinant *Escherichia coli* transformed with the *methioninase* gene from *Pseudomonas putida*. The purification process involved heat treatment at 60°C, polyethylene glycol precipitation, and diethylaminoethyl sepharose fast-flow ion-exchange column chromatography, as previously described elsewhere (21).

Reagents. Rapamycin was obtained from MedChemExpress (Monmouth Junction, NJ, USA) and dissolved in dimethyl sulfoxide at a stock concentration of 10 mM. Chloroquine diphosphate was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS) at a stock concentration of 10 mM.

Cell viability assay. MiaPaCa-2 and Hs-27 cells were seeded at 2.0×10^3 cells per well in 96-well plates and incubated overnight. The next day, cells were treated for 72 h with different concentrations of rMETase (0.125-4 U/ml), rapamycin (1-128 μM), and chloroquine (1-128 μM). Cell viability was assessed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) with the WST-8 reagent. Ten microliters of WST-8 solution were added to each well, followed by a 1 h incubation at 37°C. Absorbance was then measured at 450 nm using a microplate reader (Sunrise; Tecan, Männedorf, Switzerland). Cell viability was calculated as a percentage relative to that of untreated control cells. Drug-sensitivity curves, half-maximal inhibitory concentration (IC₅₀), and 30% inhibitory concentration (IC₃₀) values were generated using Microsoft 365 Excel for MacOS (Microsoft, Redmond, WA, USA), ImageJ version 1.54g (National Institutes of Health, Bethesda, MD, USA), and GraphPad Prism version 10.4.2 (GraphPad Software, Inc., San Diego, CA, USA).

Combination treatment. MiaPaCa-2 and Hs-27 cells were seeded in 96-well plates at a density of 2.0×10^3 cells/well in DMEM/F-12. After 24 h, the cells were treated with all combinations of rMETase, rapamycin and chloroquine at the IC_{30} values on MiaPaCa-2 cells for each agent for 72 h. Cell viability was then determined. All experiments were performed in triplicate.

Statistical analysis. Dose–response curves were fitted using a four-parameter logistic model with nonlinear regression. To statistically compare the IC_{50} values between groups, we performed an extra sum-of-squares F-test to determine whether the data sets were better represented by a single shared curve or by separate curves. A value of $p \leq 0.05$ was considered statistically significant. Data are presented as the mean \pm standard deviation. Comparisons between groups were performed using one-way analysis of variance. Statistical significance was determined using Dunnett’s multiple comparison test, with values of $p \leq 0.05$ considered significant.

Results

Determination of IC_{50} and IC_{30} values of rMETase, rapamycin and chloroquine against MiaPaCa-2 PDAC cells and Hs-27 normal fibroblasts. The IC_{50} value of rMETase was 0.23 U/ml; for rapamycin was 10.32 μ M; and for chloroquine was 21.31 μ M, for MiaPaCa-2 cells. The IC_{50} value for rMETase was 1.14 U/ml; for rapamycin was 10.53 μ M; for chloroquine was 18.61 μ M, for Hs-27 cells. The IC_{30} value of rMETase was 0.14 U/ml; 6.76 μ M for rapamycin; and, 15.31 μ M for chloroquine, for MiaPaCa-2 cells. For Hs-27 cells the IC_{30} of rMETase was 0.83 U/ml; 5.99 μ M for rapamycin; 12.98 μ M for chloroquine (Table I). The IC_{50} of rMETase was significantly lower in MiaPaCa cells than in Hs-27 cells ($p < 0.05$; Figure 1A; Table I). The difference in IC_{50} values of rapamycin for MiaPaCa-2 and Hs-27 cells was not significant ($p = 0.212$); The difference in IC_{50} values for chloroquine on MiaPaCa-2 cells and Hs27 cells was significant ($p = 0.025$; Figure 1B and C; Table I).

Table I. Sensitivity profiles of MiaPaCa-2 and Hs-27 cells *in vitro*. Values were calculated from the best fit of the drug-sensitivity curve.

Treatment	IC_{50}		IC_{30}		<i>p</i> -Value*
	MiaPaCa-2	Hs-27	MiaPaCa-2	Hs-27	
rMETase (U/ml)	0.23	1.14	0.14	0.83	<0.05
Rapamycin (μ M)	10.32	10.53	6.76	5.99	0.212
Chloroquine (μ M)	21.31	18.61	15.31	12.98	0.025

*Determined by sum-of-squares F-test.

Efficacy of combinations of rMETase, rapamycin, and chloroquine against MiaPaCa-2 and Hs-27 cells. At the IC_{30} concentrations for MiaPaCa-2 cells, treatment with rMETase, rapamycin, or chloroquine alone induced moderate inhibition of the MiaPaCa-2 cells, whereas combination treatments exhibited enhanced efficacy. The triple combination of rMETase, rapamycin, and chloroquine achieved the greatest inhibition (96.24%), essentially eradicating the MiaPaCa-2 cells. For Hs-27 normal fibroblasts, rMETase, rapamycin, or chloroquine alone produced moderate inhibition, while the triple combination treatment resulted in much less inhibition than observed on MiaPaCa-2 cells. The details are shown in Figure 2 and Table II. All comparisons involving the triple combination *versus* any other treatment of the MiaPaCa-2 cells were statistically significant ($p < 0.05$), indicating highly synergistic efficacy against MiaPaCa-2 cells. In contrast, multiple comparisons for Hs-27 cells showed no statistically-significant differences between the combinations, suggesting no synergistic cytotoxicity on normal fibroblasts (Figure 2).

Discussion

The present study demonstrates that the combination of rMETase, rapamycin and chloroquine showed highly synergistic efficacy against MiaPaCa-2 PDAC cells but not normal human fibroblasts, highlighting its therapeutic potential, with reduced toxicity. Out of the three agents tested alone, rMETase greatly distinguished pancreatic cancer and normal cells. The triple combination was significantly more effective on cancer than normal cells.

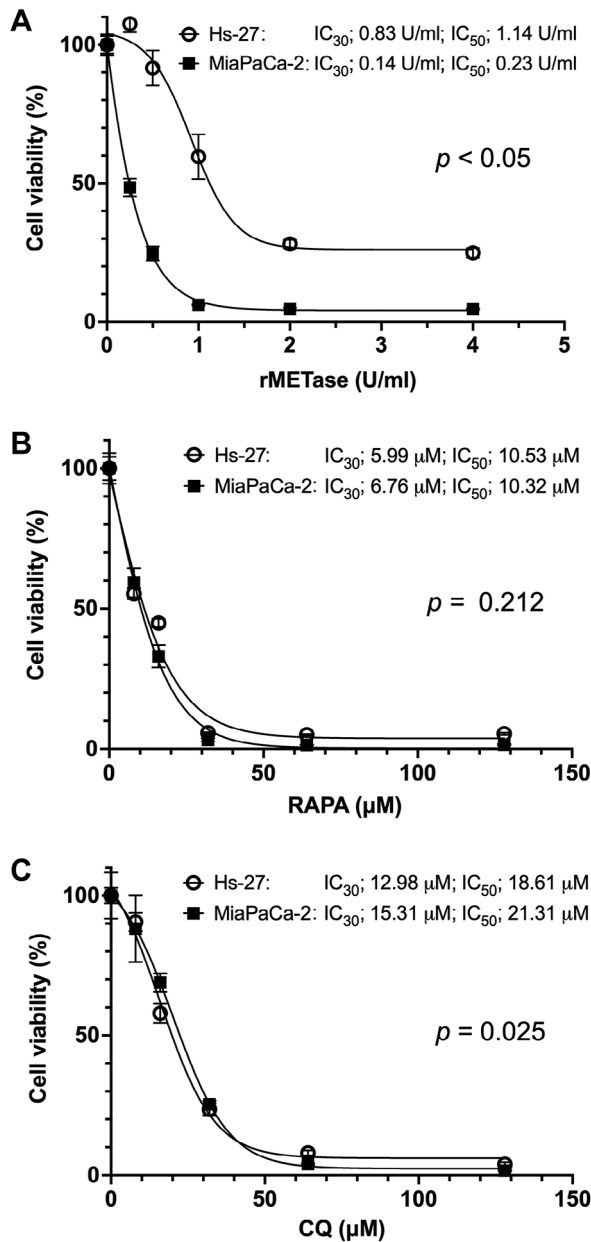


Figure 1. Determination of half-maximal (IC_{50}) and 30% inhibitory (IC_{30}) concentrations of recombinant methioninase (rMETase) (A); rapamycin (RAPA) (B); and chloroquine (CQ) (C); against MiaPaCa-2 and Hs-27 cells *in vitro*. IC_{50} and IC_{30} values were calculated by nonlinear regression using a four-parameter logistic model. Data are shown as the mean±standard deviation. Please see Materials and Methods for details.

Methionine restriction of cancer cells leads to a reduction in intracellular S-adenosylmethionine (SAM) levels (4), which subsequently inhibits mTOR *via*

Table II. Percentage inhibition of MiaPaCa-2 pancreatic ductal adenocarcinoma cells and normal Hs-27 fibroblasts relative to untreated control cells induced by recombinant methioninase (rMETase), rapamycin, and chloroquine and their combination at the 30% inhibitory concentration (IC_{30}) for MiaPaCa-2 cells. Data are shown as the mean±standard deviation.

Treatment	MiaPaCa-2 percent inhibition	Hs-27 percent inhibition
Control		
rMETase	33.18±5.39	10.15±4.09
Rapamycin	36.01±5.41	51.69±2.3
Chloroquine	50.63±2.83	53.18±1.58
rMETase+rapamycin	61.01±3.24	64.72±2.98
rMETase+chloroquine	68.49±2.82	65.54±2.87
Rapamycin+chloroquine	87.04±0.93	66.15±0.57
rMETase+rapamycin+chloroquine	96.24±1.31	66.31±2.33

disruption of SAM-TOR (16, 22). Methionine restriction also inhibits autophagy, which may explain the synergy of rMETase and chloroquine (23, 24). We recently reported synergy of rMETase, rapamycin and chloroquine against osteosarcoma, colon cancer, and triple-negative breast cancer cell lines (18-20). The present study showed synergy of this triple combination against the MiaPaCa-2 PDAC cell line in contrast to normal fibroblasts.

However, the lack of significant cytotoxicity of the triple combination of rMETase, rapamycin and chloroquine on normal Hs-27 fibroblasts, suggests that the synergistic efficacy of these agents is specific to methionine-addicted cancer cells (6-13). This selectivity may be able to minimize off-target toxicity, an essential feature of effective combination therapies.

In conclusion, the present findings support the hypothesis that targeting fundamental hallmarks of cancer, including methionine restriction (3-14), mTOR signaling (15-20, 22), and autophagy (23, 24) is a promising strategy for clinical pancreatic cancer treatment. Further *in vivo* studies and clinical investigations are warranted to validate the translational relevance of this combination.

Conflicts of Interest

All Authors have no conflicts of interest or financial ties to disclose related to this study.

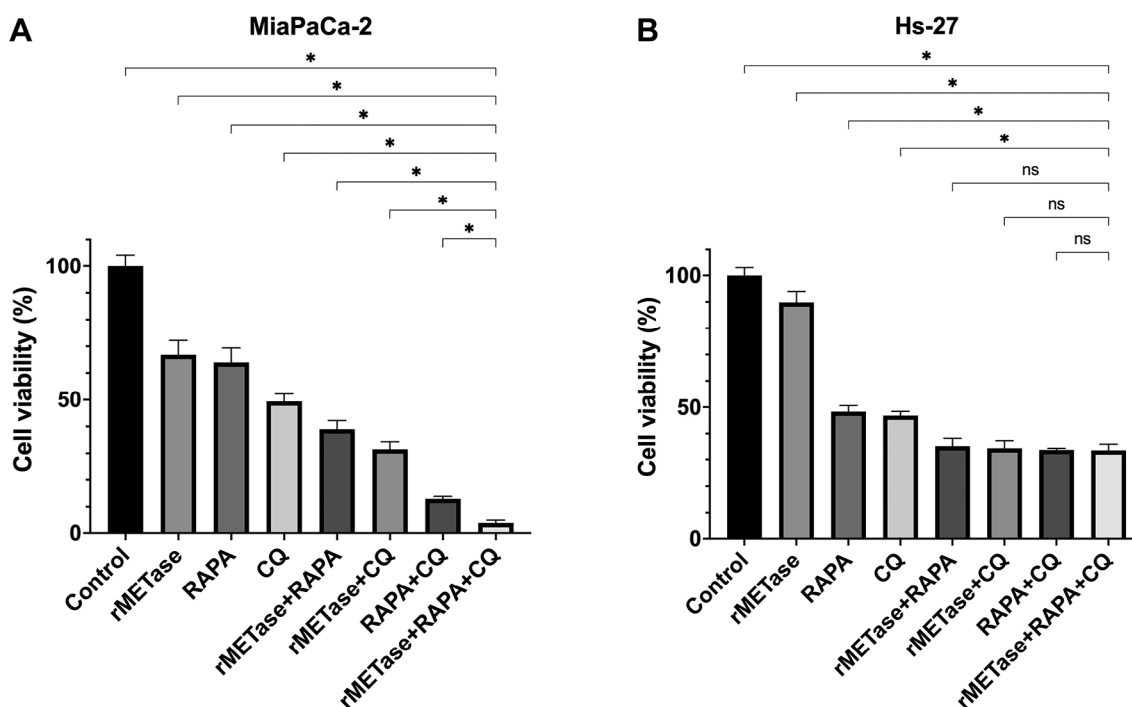


Figure 2. Cytotoxicity of recombinant methioninase (rMETase); rapamycin (RAPA); and chloroquine (CQ); tested alone and in combination at 30% inhibitory concentrations (IC_{30}) on MiaPaCa-2 cells. (A) MiaPaCa-2 pancreatic cancer cells; and (B) Hs-27 normal fibroblasts. *Significantly different at $p < 0.05$; ns: Not significantly different. Please see Materials and Methods for details.

Authors' Contributions

JK and RMH designed the study. QH and SL produced rMETase. JK conducted all experiments and wrote the article. RMH revised the article. BMK, KM, YA, YM and MB critically read the article.

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Artificial Intelligence (AI) Disclosure

No artificial intelligence (AI) tools, including large language models or machine learning software, were used in the preparation, analysis, or presentation of this article.

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