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Synergy of Rapamycin and Methioninase on Colorectal Cancer Cells Requires Simultaneous and Not Sequential Administration: Implications for mTOR Inhibition

DANIEL ARDJMAND[1], MOTOKAZU SATO[1,2], QINGHONG HAN[1], YUTARO KUBOTA[1,2], KOHEI MIZUTA[1,2], SEI MORINAGA[1,2] and ROBERT M. HOFFMAN[1,2]

[1]*AntiCancer Inc., San Diego, CA, U.S.A.;*
[2]*Department of Surgery, University of California San Diego, San Diego, CA, U.S.A.*

Abstract. *Background/Aim: Rapamycin inhibits the mTOR protein kinase. Methioninase (rMETase), by degrading methionine, targets the methionine addiction of cancer cells and has been shown to improve the efficacy of chemotherapy drugs, reducing their effective doses. Our previous study demonstrated that rapamycin and rMETase work synergistically against colorectal-cancer cells, but not on normal cells, when administered simultaneously in vitro. In the present study, we aimed to further our previous findings by exploring whether synergy exists between rapamycin and rMETase when used sequentially against HCT-116 colorectal-carcinoma cells, compared to simultaneous administration, in vitro. Materials and Methods: The half-maximal inhibitory concentrations (IC_{50}) of rapamycin alone and rMETase alone against the HCT-116 human colorectal-cancer cell line were previously determined using the CCK-8 cell viability assay (11). We then examined the efficacy of rapamycin and rMETase, both at their IC_{50}, administered simultaneously or sequentially on the HCT-116 cell line, with rapamycin administered before rMETase and vice versa. Results: The IC_{50} for rapamycin and rMETase, determined from previous experiments (11), was 1.38 nM and 0.39 U/ml, respectively, of HCT-116 cells. When rMETase was administered four days before rapamycin, both at the IC_{50}, there was a 30.46% inhibition of HCT-116 cells. When rapamycin was administered four days before rMETase, both at the IC_{50}, there was an inhibition of 41.13%. When both rapamycin and rMETase were simultaneously administered, both at the IC_{50}, there was a 71.03% inhibition. Conclusion: Rapamycin and rMETase have synergistic efficacy against colorectal-cancer cells in vitro when administered simultaneously, but not sequentially.*

Correspondence to: Robert M. Hoffman, Ph.D., AntiCancer Inc, 7917 Ostrow St, San Diego, CA, 92111, U.S.A. Tel: +1 6198852284, e-mail: all@anticancer.com

Key Words: Methioninase, rMETase, rapamycin, mTOR, SAMTOR, SAM, combination, simultaneous, sequential, synergy, cancer cells, IC_{50}, HCT-116, methionine addiction, Hoffman Effect.

Mammalian target of rapamycin (mTOR) is a serine-threonine protein kinase that regulates critical aspects of cellular metabolism. Rapamycin (sirolimus) and its analogs, such as temsirolimus and everolimus, are inhibitors of mTOR and have shown modest efficacy against various cancers (1).

mTOR (mTORC1) has been shown to regulate glycolysis, glutamine metabolism, autophagy, and its activity partially depends on sensing methionine. The methionine metabolite S-adenosylmethionine (SAM) activates mTOR by binding SAMTOR (2). The cellular concentration of SAM is rapidly reduced by methionine restriction of cancer cells (3), which are methionine addicted, due to overuse of methionine and SAM for abnormally-elevated transmethylation reactions (4-8).

To target the methionine addiction of cancer cells, recombinant methioninase (rMETase), cloned from *Pseudomonas putida* into *E. Coli*, is used to degrade methionine (9). We have previously shown that rMETase combined with rapamycin synergistically eradicated an osteosarcoma of the breast, in a patient-derived orthotopic xenograft (PDOX) mouse model without toxicity (10). The combination of rMETase and rapamycin administered simultaneously also demonstrated great synergy on HCT-116 colorectal-carcinoma cells, but not normal fibroblasts, *in vitro* (11). These results suggest that an acute deficiency of SAM, effected by methionine restriction, in cancer cells, in combination with rapamycin, greatly inhibits mTOR's protein kinase activity, preventing cancer-cell proliferation (3, 12).

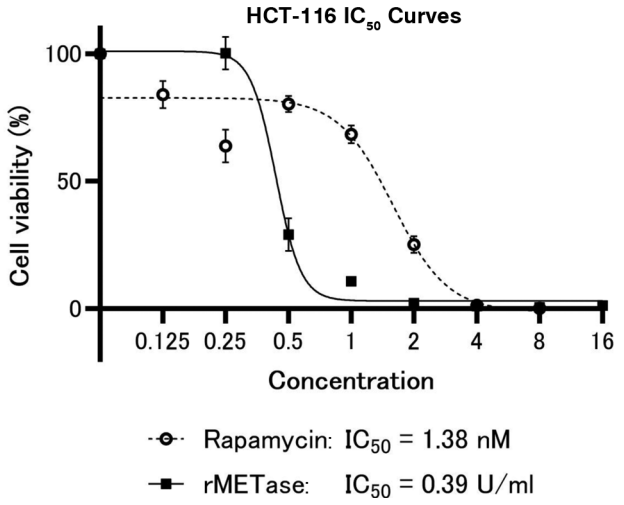

Figure 1. *The efficacy of recombinant methioninase (rMETase) and rapamycin on HCT-116 cells in vitro. Cell viability was measured using the WST-8 reagent. The concentration axis uses a log2 scale. IC_{50}: Half-maximal inhibitory concentration. Data are from (11).*

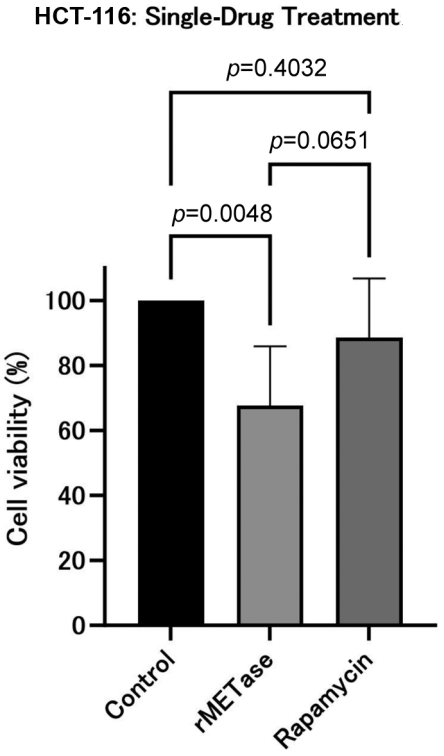

Figure 2. *Single-drug treatment of HCT-116 cells in vitro with recombinant methioninase (rMETase) or rapamycin at their half-maximal inhibitory concentrations (IC_{50}) (0.39 U/ml and 1.38 nM, respectively [11]). rMETase treatment significantly reduced the viability of the cancer cells. Cell viability was measured using the WST-8 reagent.*

In the present study, we administered rapamycin and rMETase both simultaneously and sequentially to human colorectal carcinoma cells (HCT-116) *in vitro* to determine whether the synergy of these two agents depends on the timing of their administration. The results suggest a possible novel mechanism of mTOR inhibition.

Materials and Methods

Cell culture. The HCT-116 human colon cancer cell line (American Type Culture Collection Manassas, VA, USA) was grown in Dulbecco's modified Eagles' medium (DMEM) with 10% fetal bovine serum and 100 IU/ml of penicillin/streptomycin.

rMETase production and formulation. rMETase was produced at AntiCancer Inc. (San Diego, CA, USA). *Escherichia coli* was previously transformed with the *methioninase* gene from *Pseudomonas putida* and fermented (9). rMETase was purified from recombinant *E. Coli* with a 60˚C heat step, precipitation with polyethylene-glycol, and final purification with diethylaminoethyl-sepharose fast-flow ion-exchange column chromatography (9).

Cell viability testing. HCT-116 cells were cultured at subconfluence overnight in DMEM in 96-well plates (1.0×10^3 cells per well). The following day, HCT-116 cells were treated with IC_{50} concentrations of rapamycin (IC_{50}=1.38 nM [11]) or rMETase (IC_{50}=0.39 U/ml [11]), either simultaneously or sequentially. HCT-116 cells were treated for eight days with rMETase or rapamycin alone. The HCT-116 cells were treated for 8 days with the simultaneous combination of rMETase and rapamycin. For sequential treatment, HCT-116 cells were treated for four days with rMETase first, followed by a wash with phosphate-buffered saline (PBS), and then treated with rapamycin for another four days, or *vice versa*. Cell viability was determined with the Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) using the WST-8 reagent.

Table I. *p-Values obtained upon comparison of HCT-116 cell viability for each experimental condition.*

Treatment comparison	*p*-Value
Control *vs*. rMETase (M)	0.0037
Control *vs*. Rapamycin (R)	0.7066
Control *vs*. Simultaneous	<0.0001
Control *vs*. M–>R	0.0071
Control *vs*. R–>M	<0.0001
rMETase *vs*. R–>M	0.8785
rMETase *vs*. M–>R	0.9999
Rapamycin *vs*. R–>M	0.0088
Rapamycin *vs*. M–>R	0.1857
Simultaneous *vs*. R–>M	0.0006
Simultaneous *vs*. M–>R	0.0002

M→R: Methioninase administered before rapamycin. R→M: Rapamycin administered before methioninase. Simultaneous: Rapamycin and methioninase administered simultaneously.

ImageJ version 1.53 (National Institutes of Health, Bethesda, MD, USA) was applied to produce IC_{50} and sensitivity curves. IC_{50} values were calculated from the raw data. Each experiment was carried out in triplicate.

Table II. *Percent inhibition of HCT-116 cells for each experimental condition.*

Condition	rMETase (M)	Rapamycin (R)	Simultaneous	M–>R	R–>M
Average inhibition %	32.4	11.4	71.0	30.5	41.1

rMETase was administered at its IC_{50} (0.39 U/ml [11] and/or rapamycin at its IC_{50} (1.38 nM [11]). Percent inhibition was calculated using the following formula: [% cell viability of control (no inhibitor)] – (% cell viability with inhibitor present)=% inhibition. M→R: Methioninase administered before rapamycin. R→M: Rapamycin administered before methioninase.

Statistics. GraphPad Prism 9.4.0 (GraphPad Software, Inc., San Diego, CA, USA) was used to conduct all statistical analyses. Tukey's multiple comparison test was performed for the parametric test of comparison between groups. All data are presented as the mean and standard deviation. The significance level was set at $p \leq 0.05$.

Results

The IC_{50} of HCT-116 cells for rapamycin alone and rMETase alone was 1.38 nM (11) and 0.39 U/ml (11), respectively (Figure 1). The IC_{50} of rMETase alone significantly inhibited the HCT-116 cells (p=0.0048) but the IC_{50} of rapamycin alone did not significantly inhibit the HCT-116 cells (p=0.4032) (Figure 2). Figures 1 and 2 are from independent experiments. When both rapamycin and rMETase were simultaneously administered, both at the IC_{50}, there was a 71.03% inhibition (Table I, Table II, Figure 3, Figure 4). When rMETase was administered four days before rapamycin, both at the IC_{50}, there was a 30.5% inhibition (Table I, Table II, Figure 3, Figure 4). When rapamycin was administered for four days before rMETase, both at the IC_{50}, there was an inhibition of 41.1% (Table I, Table II, Figure 3, Figure 4).

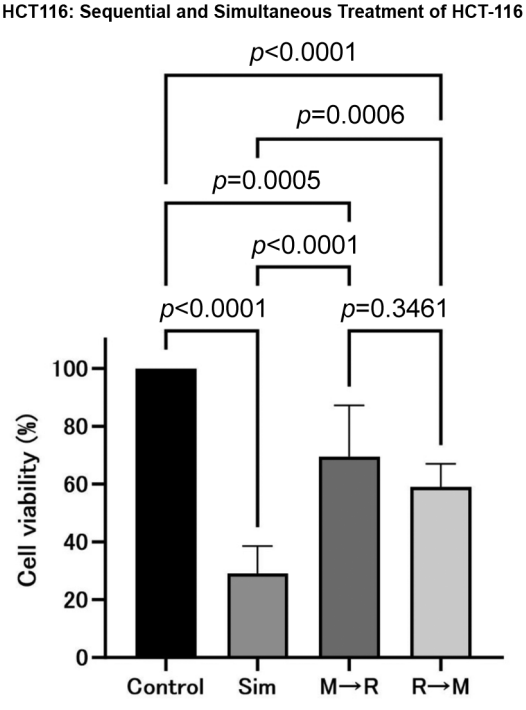

Figure 3. *Simultaneous and sequential treatment of HCT-116 cells in vitro with recombinant methioninase (rMETase) and rapamycin at their half-maximal inhibitory concentrations (0.39 U/ml and 1.38 nM, respectively [11]). The simultaneous treatment synergistically reduced the viability of the cancer cells. Cell viability was measured using the WST-8 reagent. Sim: Simultaneous treatment. M→R: Methioninase administered before rapamycin. R→M: Rapamycin administered before methioninase.*

Discussion

Methionine addiction is termed the Hoffman Effect and is a fundamental hallmark of cancer (4, 12-19). Due to methionine addiction, cancer cells are inhibited by methionine restriction, which severely depletes methionine and SAM in the cancer cells (3, 4, 12). rMETase indirectly inhibits mTOR activity by acute depletion of methionine (MET) which depletes SAM in cancer cells resulting in SAMTOR binding to GATOR instead of SAM, thereby inhibiting mTOR (3) (Figure 4B, Figure 4C). Rapamycin forms an inhibitory complex with FKBP12 to block the kinase activity of mTOR (Figure 4A, Figure 4C). Rapamycin and rMETase when used simultaneously have synergistic efficacy against HCT-116 human colorectal-cancer cells *in vitro* (11) as well as against osteosarcoma of the breast *in vivo* (10). As shown in the present study, synergy was not observed when rapamycin and methioninase were administered sequentially (Table I, Table II, Figure 3, Figure 4). These results suggest that the synergistic inhibition of mTOR requires rapamycin and rMETase to be simultaneously present (Table I, Table II, Figure 3, Figure 4). Further studies are needed to describe the mechanism in detail. The present *in vitro* result and our previous *in vitro* (11) and *in vivo* results (10) showing synergy of rapamycin and methioninase against cancer cells and not normal cells, suggest that this combination has the potential for future clinical use when administered simultaneously as it targets a fundamental hallmark of cancer (1-6, 11-19, 20-31), methionine addiction, known as the Hoffman effect (16, 18, 19).

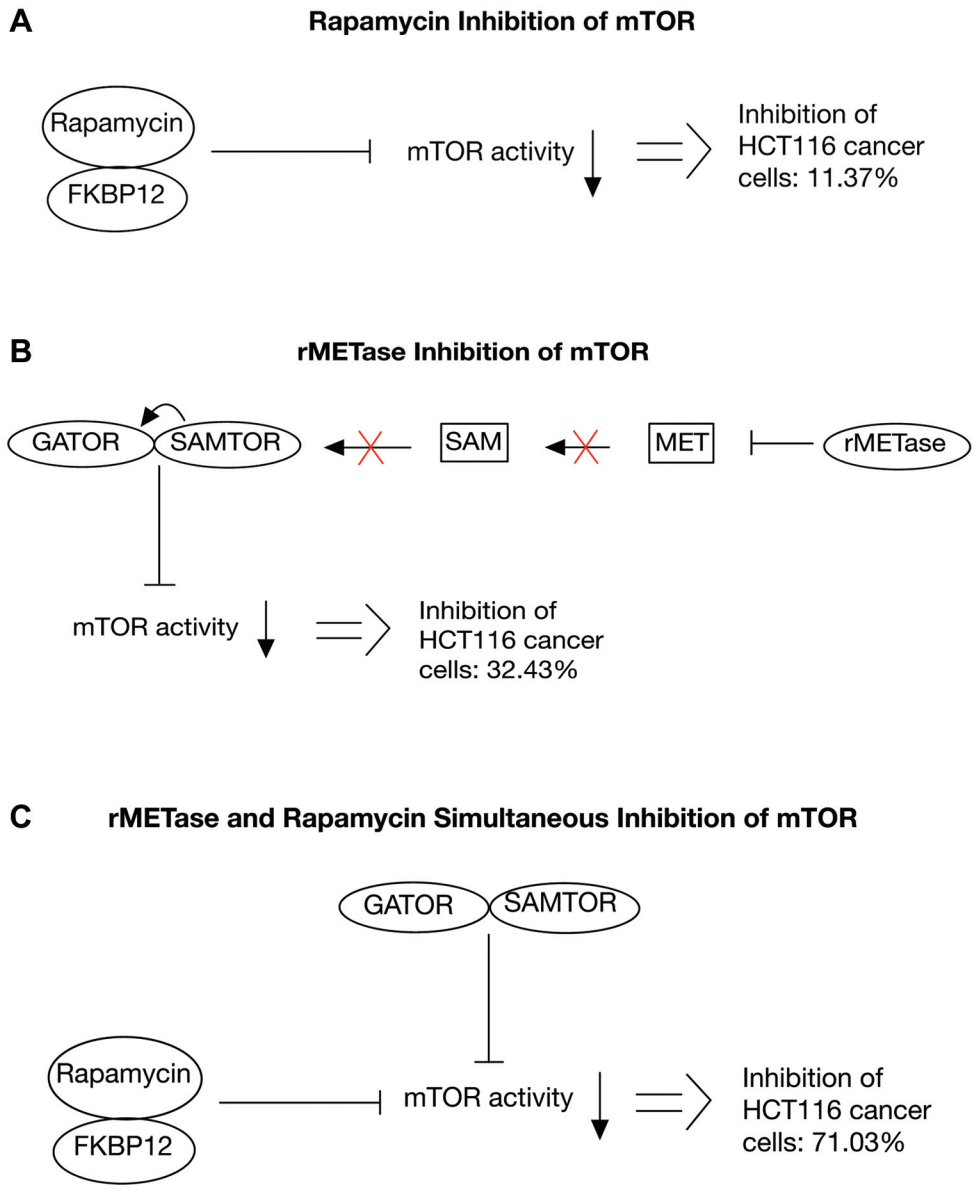

Figure 4. *Models of mTOR inhibition are shown. (A) Rapamycin binds the FKBP12 protein and inhibits mTOR activity; HCT-116 cancer-cell viability decreased by 11.37%. (B) rMETase depletes methionine (MET) levels thereby decreasing SAM, which no longer binds an S-adenosylmethionine sensor (SAMTOR), allowing SAMTOR to bind a GTPase-activating protein (GATOR) and ultimately to inhibit mTOR; HCT-116 cancer-cell viability decreased by 32.43%. (C) The simultaneous inhibition of mTOR by rapamycin and rMETase; HCT-116 cancer cell viability decreased by 71.03%. Percent inhibition was calculated using the following formula: [% cell viability of control (no inhibitor)] – (% cell viability with inhibitor present)=% inhibition.*

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

DA performed experiments. QH supplied methioninase. DA and RMH contributed the concept of the study and wrote the manuscript. DA and RMH revised the manuscript. DA, YK, MS, QH, KM, SM, and RMH critically read the manuscript.

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