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# The Combination of Methionine Adenosyltransferase 2A (MAT2A) Inhibitor AG-270 and Recombinant Methioninase Is Not Cancer-selective in a Co-culture Model of Colon Cancer Cells and Normal Fibroblasts

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## Abstract

*Background/Aim*: Methionine addiction is a fundamental and general hallmark of cancer cells. Recombinant *methioninase* (rMETase) degrades extracellular methionine. rMETase, or other means of restricting methionine, in combination with numerous types of chemotherapy have shown synergistic cancer-selective efficacy. AG-270, a methionine adenosyltransferase 2A (MAT2A) inhibitor, blocks intracellular conversion of methionine to S-adenosylmethionine (SAM), the central reaction of the methionine cycle. The present study aimed to evaluate the synergistic and cancer-selective efficacy of the combination of AG-270 and rMETase in a co-culture model of cancer and normal cells.

*Materials and Methods:* HCT116 human colon-cancer cells expressing green fluorescent protein (GFP) and human Hs-27 normal fibroblasts were co-cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum in 12-well plates. Co-cultures were treated with AG-270 (6 μM and 10 μM) and rMETase (0.3 U/ml and 0.5 U/ml) alone or in combination. Cell growth and viability were assessed by phase-contrast microscopy and fluorescence imaging over 6 days.

*Results:* Treatment with AG-270 or rMETase alone inhibited HCT116 colon-cancer cell viability in a dose-dependent manner, whereas Hs-27 normal fibroblasts remained viable on day 6 in co-culture. In contrast, the combination of AG-270 and rMETase produced a strong, synergistic reduction of the viability of both HCT116 and Hs-27 cells, accompanied by extensive morphological damage, in co-culture. GFP-expressing HCT116 colon-cancer cells were

*continued*

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nearly eradicated by the combination treatment, as visualized by fluorescence imaging on day 6 in co-culture with Hs-27 fibroblasts.

*Conclusion:* Dual inhibition of methionine metabolism by AG-270 and rMETase was toxic to both cancer cells and normal fibroblasts in a co-culture model which is internally controlled. In contrast, rMETase combined with numerous first-line chemotherapeutic drugs acted selectively and synergistically against cancer cells while sparing normal cells, including co-culture models. The present results suggest that AG-270 may have limited potential as an anticancer agent.

**Keywords:** Methionine addiction, Hoffman effect, MAT2A inhibitor, AG-270, recombinant methioninase, HCT116 colon cancer cells, Hs-27 normal fibroblasts, co-culture, combination treatment.

## Introduction

Methionine addiction is a fundamental and general metabolic hallmark cancer, commonly referred to as the Hoffman effect (1-3). Methionine addiction has been targeted for cancer because cancers show specific vulnerability to methionine restriction (4-8). Normal cells can proliferate under methionine-restriction by utilizing homocysteine in contrast to cancer cells which need large amount of exogenous methionine for elevated transmethylation reactions (8-10). Recombinant methioninase (rMETase) can degrade methionine to achieve strong methionine restriction (11, 12). rMETase has shown synergistic efficacy with numerous first-line chemotherapeutic agents in many different types of cancer cells, but not on normal cells, including co-cultures of cancer and normal cells (13-19).

Methionine adenosyltransferase 2A (MAT2A) catalyzes the conversion of methionine to S-adenosylmethionine (SAM), the universal methyl donor essential for cell proliferation (20). AG-270, a MAT2A inhibitor, suppresses intracellular methyl metabolism and epigenetic maintenance is being developed as a cancer drug (21-25).

In mono-culture models of cancer and normal cells we previously showed that AG-270 combined with rMETase did not have cancer specificity (26).

The present study used an internally-controlled co-culture model of HCT116 colon-cancer cells and Hs-27 normal fibroblasts to evaluate the selectivity and synergistic efficacy of combination therapy of AG-270 and rMETase on cancer and normal cells.

## Materials and Methods

*Co-culture.* HCT116 human colon-cancer cells and Hs-27 normal human fibroblasts were obtained from the American Type Culture Collection (Manassas, VA, USA). HCT116 colon-cancer cells were engineered to stably express green-fluorescent protein (GFP) as previously reported (27). The cells were co-cultured in 12-well plates in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 with GlutaMAX™ supplement (DMEM/F-12; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were allowed to adhere overnight at 37°C in a humidified incubator containing 5% $CO_2$.

*Recombinant methioninase production.* rMETase was produced by AntiCancer Inc. (San Diego, CA, USA) using *Escherichia coli* transformed with the *Pseudomonas putida methioninase* gene. The enzyme was purified by heat treatment at 60°C, polyethylene glycol precipitation, and diethylaminoethyl (DEAE) Sepharose ion-exchange chromatography as previously described (12).

*Reagents.* AG-270 was purchased from MedChemExpress (Monmouth Junction, NJ, USA) and dissolved in dimethyl sulfoxide at a stock concentration of 10 mM.

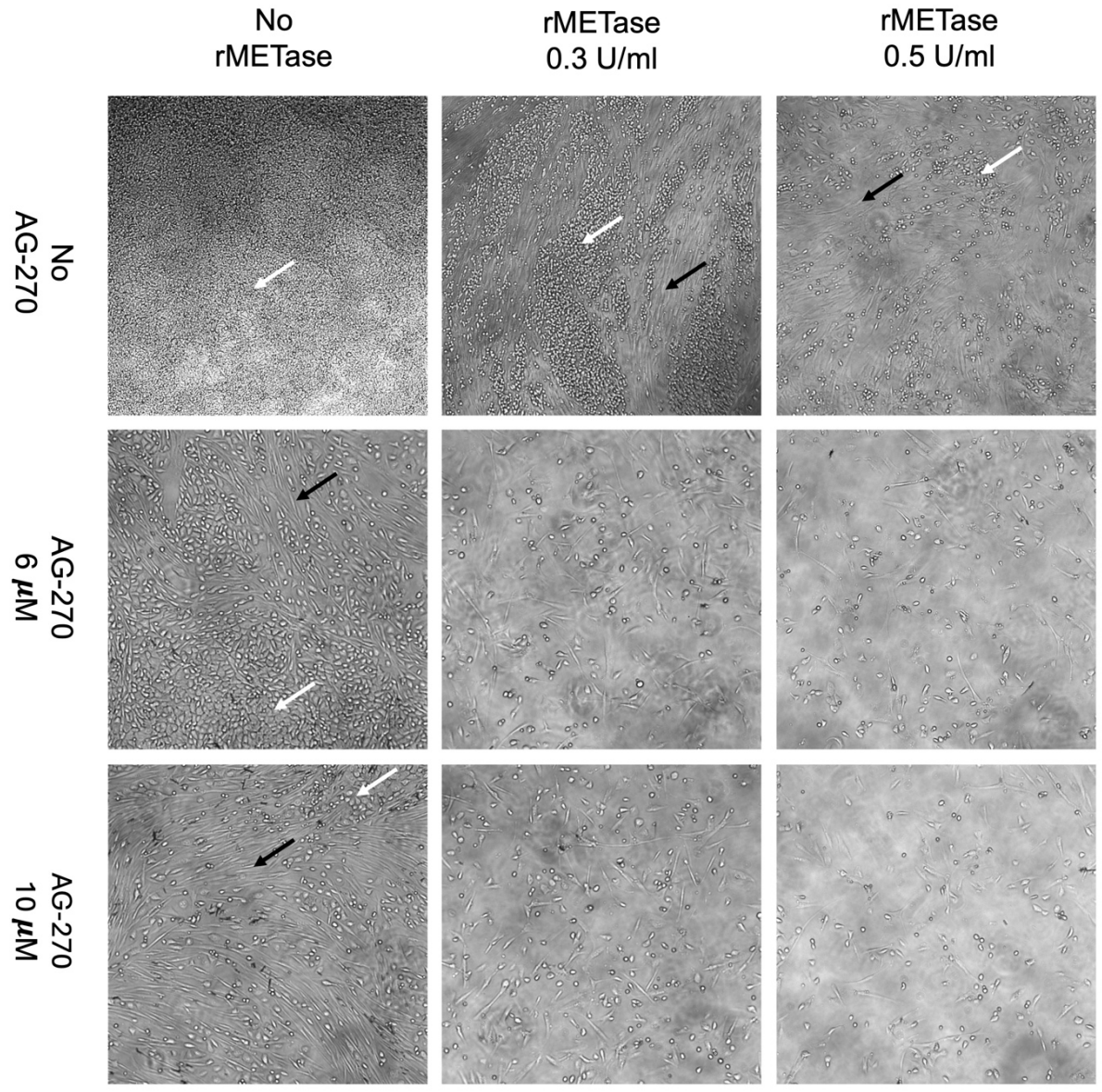


Figure 1. *Efficacy of AG-270 and rMETase in a co-culture system of HCT116 colon cancer cells and Hs-27 normal fibroblasts. HCT116 colon-cancer cells were co-cultured with Hs-27 normal fibroblasts and treated for 6 days with the MAT2A inhibitor AG-270 (0, 6, or 10 μM) and/or recombinant methioninase (rMETase; 0, 0.3, or 0.5 U/ml), as indicated. Phase-contrast images show extensive overgrowth of HCT116 cells in untreated co-cultures, whereas single-agent treatment with AG-270 or rMETase reduced the number of cancer cells while largely preserving the spindle-shaped Hs-27 fibroblasts. Combined treatment at higher doses resulted in a pronounced reduction in cancer cells as well as normal fibroblasts, indicating that the cytotoxicity of the combination of AG-270 and rMETase is not cancer-specific in this model. Black arrows indicate normal fibroblasts; white arrows indicate HCT116 cancer cells. Representative images; original magnification ×100.*

*Dose selection and treatment of HCT116 and Hs-27 cells with AG-270 and rMETase alone and in combination in co-culture.* To determine appropriate concentrations of AG-270 and rMETase, preliminary dose–response experiments were performed. Co-cultures were treated with AG-270 at 2, 6, 10, and 14 μM, or rMETase at 0.1, 0.3,

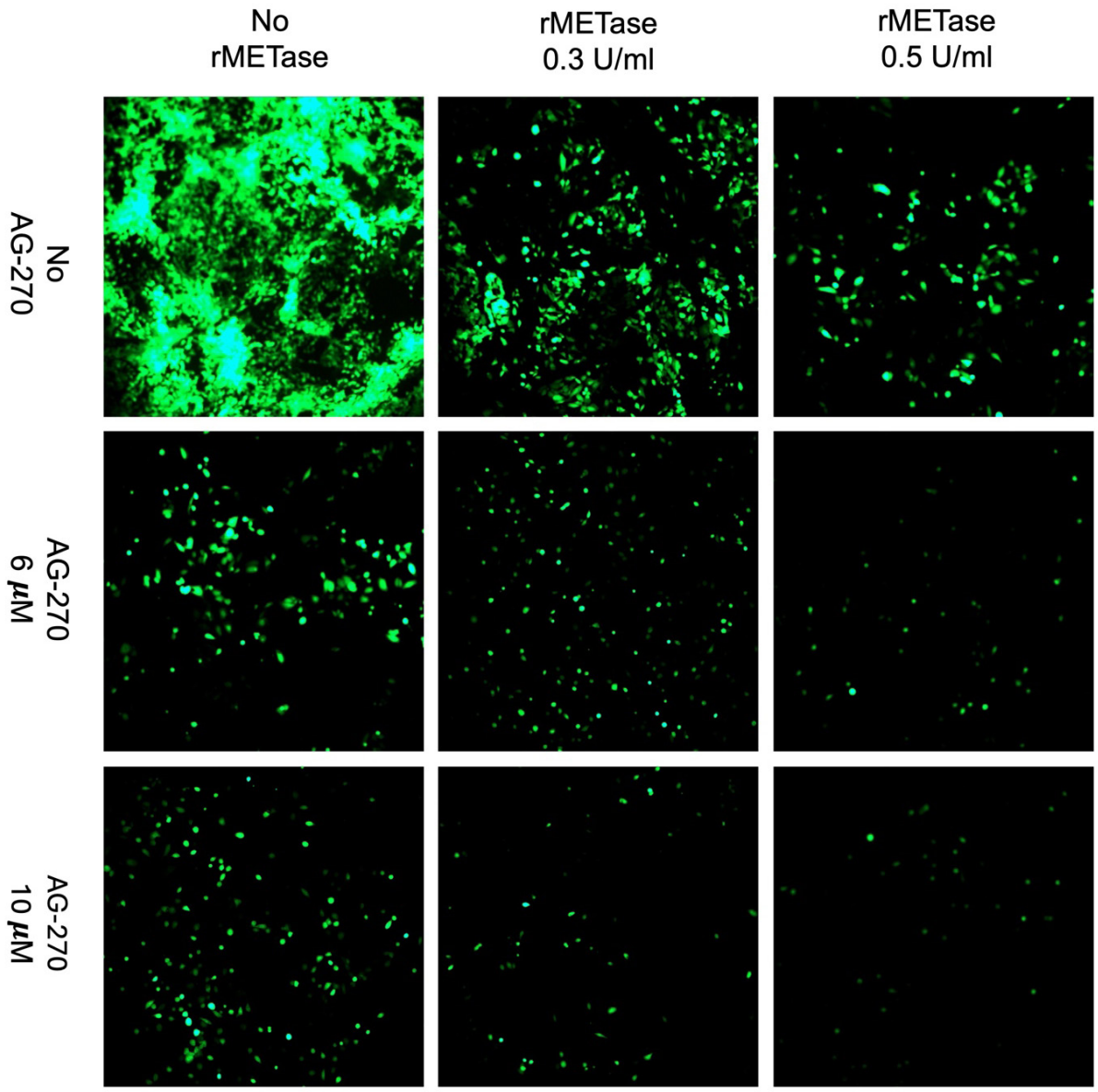


Figure 2. *GFP fluorescence images of HCT116 colon-cancer cells in co-culture with Hs-27 normal fibroblasts after treatment with AG-270 and rMETase. HCT116 cells stably expressing GFP were co-cultured with Hs-27 fibroblasts and treated for 6 days with AG-270 (0, 6, or 10 μM) and/or rMETase (0, 0.3, or 0.5 U/ml), as indicated. In untreated co-cultures, intense and confluent green fluorescence reflects massive overgrowth of GFP-expressing HCT116 cells. Treatment with either AG-270 or rMETase alone results in a concentration-dependent decrease in the number of GFP-expressing cells. Combined treatment with AG-270 and rMETase further diminishes, and at higher doses almost abolishes, GFP fluorescence, indicating elimination of viable cancer cells in the co-culture system. Representative fluorescence images; original magnification ×100.*

0.5, and 0.8 U/ml for 6 days. Among these concentrations, rMETase alone at 0.3 U/ml and 0.5 U/ml and AG-270 alone at 6 μM and 10 μM produced a clear reduction in cancer-cell density while the spindle-shaped normal fibroblasts were largely preserved (data not shown). Therefore, these concentrations were selected for

subsequent co-culture experiments. For the main experiments, co-cultures were treated with rMETase (0.3 U/ml or 0.5 U/ml) and/or AG-270 (6 μM or 10 μM). A control group without either drug was included in each assay. Treatments were maintained for 6 days when the control group reached full confluence. The culture medium containing the corresponding drugs was refreshed at regular intervals.

After 6 days of treatment, representative fields were imaged using an inverted phase-contrast microscope (IX71, Olympus Corporation, Tokyo, Japan) equipped with digital camera, at 100-fold magnification. Fluorescence-imaging of GFP of HCT116 was obtained on day 6 with the IX71 microscope.

## Results

*Lack of cancer selectivity by the combination of AG-270 and rMETase on the co-culture of cancer and normal cells.* In the untreated co-culture, HCT116 colon-cancer cells overgrew and eventually covered the normal Hs-27 fibroblasts, obscuring the fibroblast layer and almost completely filling the well surface. When treated with either AG-270 (6 μM or 10 μM) or rMETase (0.3 U/ml or 0.5 U/ml) alone, the structural integrity and spindle-shaped morphology of Hs-27 fibroblasts were well maintained, whereas the number of HCT116 cells greatly decreased in a concentration-dependent manner. The combination of AG-270 and rMETase at the above-described concentrations greatly reduced the density of HCT116 cancer cells in the co-culture. The normal fibroblasts also became sparse and disrupted (Figure 1). These results demonstrated the lack of cancer selectivity of the combination of AG-270 and rMETase on the co-culture of cancer and normal cells.

In the HCT116 and Hs-27 co-culture the GFP-labeled HCT116 cells' intense and confluent green fluorescence was observed in the untreated group, reflecting overgrowth of cancer cells. Treatment with rMETase or AG-270 alone visibly reduced the number of GFP-expressing cells in a dose-dependent manner, and their combination almost abolished GFP fluorescence, indicating a strong reduction of viable cancer cells in the co-culture system (Figure 2).

Figure 1 of the present study demonstrates that phase-contrast microscopy readily distinguishes cancer cells and normal fibroblasts by their very different morphology showing their relative proportion under each condition. Figure 1 shows also the combination of AG-270 and rMETase is toxic to both cancer and normal cells. Figure 2 demonstrates by fluorescence microscopy similar behavior of the GFP-expressing cancer cells as that shown by phase-contrast microscopy in Figure 1, indicating that the normal fibroblasts did not affect the interpretation of the GFP signal in Figure 2.

## Discussion

We demonstrated 40 years ago in co-culture models that cancer cells are selectively eliminated when methionine restriction is combined with chemotherapy (13). We also recently showed that methionine restriction *via* rMETase alone selectively eliminates cancer cells co-cultured with normal cells (28). Our previous studies have shown that the combination of rMETase with numerous chemotherapeutic agents, including 5-fluoruracil cisplatinum, eribulin, docetaxel, gemcitabine and rapamycin. exhibits selective synergy against cancer cells but not normal fibroblasts including in co-culture of cancer and normal cells (14-19, 29).

In contrast, in the present study, AG-270 combined with rMETase, exhibits different results compared to first-line chemotherapy drugs listed above. Both HCT116 colon-cancer cells and Hs-27 normal fibroblasts had similar sensitivity to the combination treatment of rMETase and AG-270 in the co-culture model. MAT2A is a crucial enzyme because it catalyzes the activation of methionine to S-adenosylmethionine (SAM) which is the universal methyl donor in all cells. MAT2A has been chosen as an anti-cancer target, and AG-270 was developed as a novel anti-cancer agent to target MAT2A (22-25). The present study, and our previous study (26) demonstrate that a MAT2A inhibitor in

combination with rMETase is toxic to both cancer and normal cells, suggesting AG-270 is not a promising anti-cancer agent.

rMETase and other means of methionine restriction are effective because they target methionine addiction, the fundamental and general hallmark of cancer (1-19, 29-74). rMETase is showing clinical promise (75).

## Conflicts of Interest

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

## 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 manuscript.

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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 manuscript.

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