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Recombinant Methioninase Lowers the Effective Dose of Regorafenib Against Colon-Cancer Cells: A Strategy for Widespread Clinical Use of a Toxic Drug

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Abstract. *Background/Aim: Regorafenib is a multi-kinase inhibitor, targeting vascular endothelial growth factor receptor 2, fibroblast growth factor receptor 1 and other oncogenic kinases. Regorafenib has efficacy in metastatic colon cancer, but has severe dose-limiting toxicities which cause patients to stop taking the drug. The aim of the present study was to determine if recombinant methioninase (rMETase) could lower the effective concentration of regorafenib in vitro against a colorectal-cancer cell line. Materials and Methods: Firstly, we examined the half-maximal inhibitory concentration (IC_{50}) of regorafenib alone and rMETase alone for the HCT-116 human colorectal-cancer cell line. After that, using the IC_{50} concentration of each drug, we investigated the efficacy of the combination of regorafenib and rMETase. Results: While both methioninase alone (IC_{50}=0.61 U/ml) and regorafenib alone (IC_{50}=2.26 U/ml) inhibited the viability of HCT-116 cells, the combination of the two agents was more than twice as effective as either alone. Addition of rMETase at 0.61 U/ml lowered the IC_{50} of regorafenib from 2.26 μM to 1.46 μM. Conclusion: rMETase and regorafenib are synergistic, giving rise to the possibility of lowering the effective dose of regorafenib in patients, thereby reducing its severe toxicity, allowing more cancer patients to be treated with regorafenib.*

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Methionine addiction is a fundamental and general hallmark of cancer, termed the Hoffman effect (1-5). Methionine restriction was shown to increase the efficacy of chemotherapy drugs on cancer cells almost 40 years ago (2, 3). Since then, methionine restriction using recombinant methioninase (rMETase), cloned from *Pseudomonas putida*, has been shown to increase the efficacy of all cytotoxic chemotherapy drugs tested (2). rMETase targets the methionine addiction of cancer (1-5), and selectively arrests cancer cells in late-S/G_2 phase at which many cytotoxic chemotherapy drugs are active (6-9).

However, rMETase has not been tested in combination with a multiple kinase inhibitor, such as regorafenib. Regorafenib has increased the overall survival of patients with metastatic colorectal cancer (10) and gastro-intestinal stromal tumor (11) with whom first- or second-line therapies have failed. However, the toxicity of regorafenib appears within 3-4 days of the start of treatment and includes severe skin rash, diarrhea, hypertension and fatigue (12). These severe side-effects make it difficult to start regorafenib at the recommended dose of 160 mg/day. We previously reported that 160 mg/day regorafenib increased blood levels of M2 and M5, which are active metabolites of regorafenib, responsible for the severe side-effects. Thus the dose intensity of regorafenib was then reduced, thereby reducing its efficacy (13). Recently, clinical trials showed that starting at a low dose of regorafenib (80 or 120 mg/day) can reduce side-effects and increase dose intensity (14, 15). According to these results, the NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) recommends a starting dose of 80 mg/day rather than the usual 160 mg/day dose, with dose escalation depending on side-effects (16). However, low-dose regorafenib can also reduce efficacy for some patients.

In the present study, we tested regorafenib in combination with rMETase on a human colon cancer cell line *in vitro* to determine whether rMETase can reduce the effective dosage of regorafenib.

Materials and Methods

Cell culture. The HCT-116 human colon cancer cell line was acquired from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco's minimum essential 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) by fermentation of recombinant *Escherichia coli* transformed with the *methioninase* gene from *Pseudomonas putida*. rMETase was purified using a high-yield method, including a 60˚C thermal step, polyethyleneglycol precipitation, and diethylaminoethyl-sepharose fast-flow column chromatography (17).

Cell viability testing. HCT-116 cells were cultivated at subconfluence overnight in Dulbecco's modified Eagle's medium in 96-well plates (1.0×10^3 cells per well). The following day, HCT-116 cells were treated with concentrations of rMETase ranging from 0.125 to 8 U/ml or regorafenib ranging from 0.25 μM to 16 μM. Regorafenib was purchased from MedChemExpress (Monmouth Junction, NJ, USA). After 72 h of treatment, cell viability was assessed using the Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) with the WST-8 reagent.

ImageJ version 1.53 (National Institutes of Health, Bethesda, MD, USA) was applied to calculate IC_{50} values and sensitivity curves. After calculating the half-maximal inhibitory concentration (IC_{50}) for rMETase and regorafenib, the IC_{50} concentrations of both drugs were used to determine the synergistic efficacy of the combination of the drugs. Finally, we repeated the IC_{50} study for regorafenib on HCT-116 cells treated in combination with the IC_{50} concentration of rMETase to examine whether methionine restriction increased the efficacy of regorafenib. Each experiment was carried out in triplicate.

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 $p\leq0.05$.

Results

Determination of the IC_{50} of rMETase alone and regorafenib alone and efficacy of their combination on HCT-116 cells in vitro. We first evaluated the sensitivity to rMETase alone and regorafenib alone of HCT-116 cells *in vitro*, and IC_{50} values were calculated. The IC_{50} of rMETase on HCT-116 cells was 0.61 U/ml and the IC_{50} of regorafenib was 2.26 μM (Figure 1 and Figure 2). The combination of rMETase and regorafenib at their IC_{50} highly reduced the viability of HCT-116 cells in comparison to either agent alone at its IC_{50} (Figure 3).

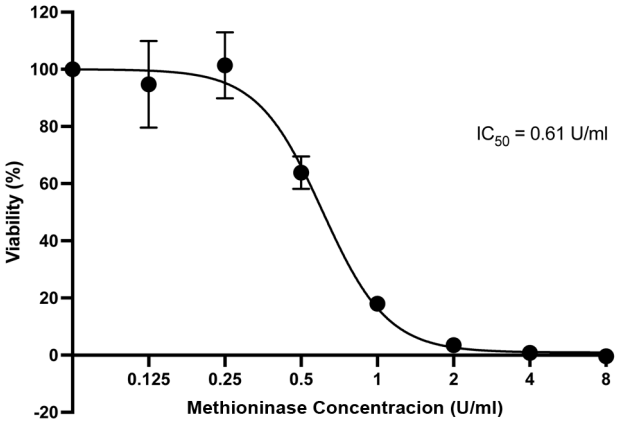

Figure 1. *Recombinant methioninase (rMETase) efficacy on HCT-116 cells in vitro. Cell viability was measured with the WST-8 reagent. The concentration axis uses a log_2 scale. IC_{50}: Half-maximal inhibitory concentration.*

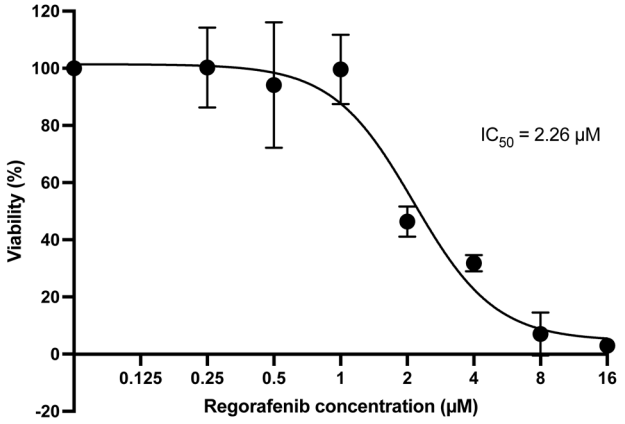

Figure 2. *Regorafenib efficacy on HCT-116 cells in vitro. Cell viability was measured with the WST-8 reagent. The concentration axis uses a log_2 scale. IC_{50}: Half-maximal inhibitory concentration.*

Reduction of the IC_{50} of regorafenib in combination with the IC_{50} of rMETase. The IC_{50} of regorafenib against HCT-116 was reduced from 2.26 μM to 1.46 μM when cells were treated in the presence of rMETase at its IC_{50} (Figure 4).

Discussion

Regorafenib is a third-line chemotherapy drug for metastatic colorectal cancer and gastro-intestinal stromal tumor, used after the failure of first- or second-line therapy. The problem with regorafenib is its dose-limiting toxicity. The results of the present study suggest that rMETase, when combined with regorafenib, can reduce the effective dose of

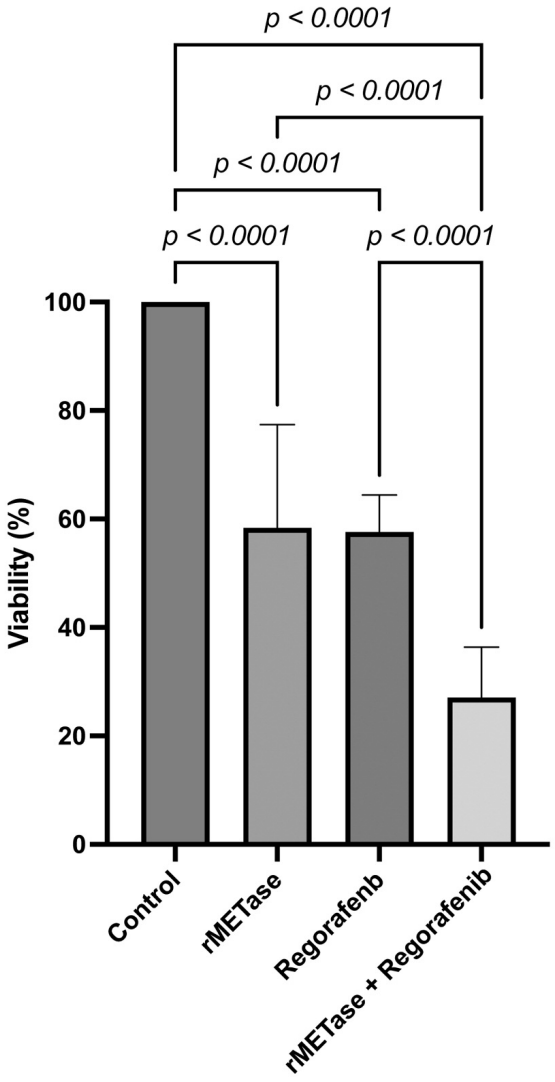

Figure 3. *Synergy of the combination of recombinant methioninase (rMETase) and regorafenib at their half-maximal inhibitory concentrations (0.61 U/ml and 2.2 μM, respectively) against HCT-116 cells in vitro. Cell viability was measured with the WST-8 reagent.*

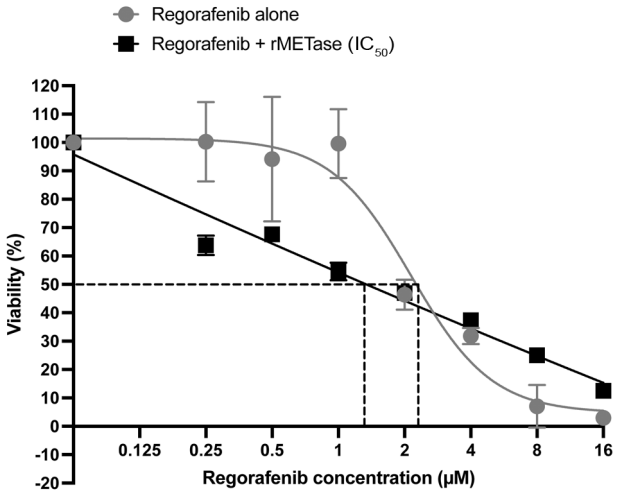

Figure 4. *Recombinant methioninase (rMETase) lowered the half-maximal inhibitory concentration (IC_{50}) of regorafenib against HCT-116 cells in vitro from 2.26 μM to 1.46 μM. Cell viability was measured with the WST-8 reagent. The concentration axis uses a log_2 scale. IC_{50}: Half-maximal inhibitory concentration.*

regorafenib and thereby also reduce its toxicity. Previously, we showed in an orthotopic mouse model of osteosarcoma that combining rMETase with cisplatinum reduced its effective dose by 50% (18). A similar effect is predicted for the combination of regorafenib and rMETase in future *in vivo* experiments.

Regorafenib is a multikinase inhibitor of angiogenic, stromal, and oncogenic-receptor tyrosine kinases. In an *in vitro* study using the human colorectal cancer cell line HT-29, regorafenib increased the intra-cellular *S*-adenosyl-L-methionine (SAM) level by 34.5% compared to the control (19). SAM is the only provider of methyl groups to DNA, RNA, histones and other proteins. This suggests that regorafenib inhibits transmethylation reactions which use SAM. Methionine depletion reduces SAM levels in cells, further reducing transmethylation reactions which are elevated in cancer cells (4, 20-29). Therefore, the combination of rMETase and regorafenib had synergistic efficacy in the present study, giving rise to the possibility of lowering the effective dose of regorafenib in patients, thereby reducing its severe toxicity, and increasing the use of regorafenib in the clinic. rMETase is effective because it targets methionine addiction, the fundamental hallmark of cancer (1-5, 20-33).

Conflicts of Interest

The Authors declare no competing interests regarding this work.

Authors' Contributions

BBC and YK performed experiments. BBC, YK, and RMH wrote the article. QH provided methioninase. DA, SM, KM, MB, and TT critically reviewed the article.

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