ANTICANCER RESEARCH *44*: 929-933 (2024)
doi:10.21873/anticanres.16887

Selective Synergy of Rapamycin Combined With Methioninase on Cancer Cells Compared to Normal Cells

DANIEL ARDJMAND[1], YUTARO KUBOTA[1,2], MOTOKAZU SATO[1,2], QINGHONG HAN[1], 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 and recombinant methioninase (rMETase) have both shown efficacy to target cancer cells. Rapamycin prevents cancer-cell growth by inhibition of the mTOR protein kinase. rMETase, by degrading methionine, targets the methionine addiction of cancer and has been shown to improve the efficacy of chemotherapy drugs. In the present study, we aimed to determine if a synergy exists between rapamycin and rMETase when used in combination against a colorectal-carcinoma cell line, compared to normal fibroblasts, 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 and Hs-27 human fibroblasts were determined using the CCK-8 Cell Viability Assay. After calculating the IC_{50} of each drug, we determined the efficacy of rapamycin and rMETase combined on both HCT-116 and Hs-27. Results: Hs-27 normal fibroblasts were more sensitive to rapamycin than HCT-116 colon-cancer cells (IC_{50}=0.37 nM and IC_{50}=1.38 nM, respectively). HCT-116 cells were more sensitive to rMETase than Hs-27 cells (IC_{50} 0.39 U/ml and IC_{50} 0.96 U/ml, respectively). The treatment of Hs-27 cells with the combination of rapamycin (IC_{50}=0.37 nM) and rMETase (IC_{50}=0.96 U/ml) showed no significant difference in their effect on Hs-27 cell viability compared to the two drugs being used separately. However, the treatment of HCT-116 cells with the combination of rapamycin (IC_{50}=1.38 nM) and rMETase (IC_{50}=0.39 U/ml) was able to decrease cancer-cell viability significantly more than either single-drug treatment. Conclusion: Rapamycin and rMETase, when used in combination against colorectal-cancer cells, but not normal fibroblasts, in vitro, have a cancer-specific synergistic effect, suggesting that the combination of these drugs can be used as an effective, targeted cancer therapy.*

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, synergy, cancer cells, normal cells, IC_{50}, HCT-116, Hs-27, methionine addiction, Hoffman effect.

Rapamycin is an inhibitor of mammalian target of rapamycin (mTOR). mTOR is a serine-threonine protein kinase. Rapamycin is produced by *Streptomyces hygroscopicus* and has been used as an anti-fungal drug, an immunosuppressant to prevent transplanted-organ rejection, and as an anti-cancer agent (1). As an anti-cancer agent, rapamycin (sirolimus) has shown tolerability in the clinic with limited efficacy on a variety of cancers (1). Rapamycin analogs such as temsirolimus and everolimus have also shown limited efficacy against various cancers (1).

mTOR (mTORC1) is activated in many cancers and is involved in metabolic reprogramming that increases glycolysis, glutamine metabolism, and other cellular functions. Methionine also activates mTOR (2) through its metabolite S-adenosylmethionine (SAM) which binds SAMTOR, which in turn activates mTOR. Therefore, at low concentrations of cellular methionine, SAM levels are reduced and do not efficiently bind SAMTOR, deactivating mTOR (2). SAM levels become acutely reduced by methionine restriction of cancer cells (3) due to the methionine addiction of cancer cells that overuse methionine and SAM for elevated transmethylation reactions (4-8). Thus, methionine restriction may have a far greater effect on mTOR in cancer cells than normal cells, which are not methionine addicted.

Methionine addiction, termed the Hoffman Effect, is the fundamental and general hallmark of cancer (4, 9-13). Therefore, cancer cells are much more sensitive to methionine restriction than normal cells (14-19). Methionine restriction, uses a recombinant methioninase (rMETase), which was cloned from *Pseudomonas putida* into *E. Coli,* to degrade methionine (20-22).

rMETase, in combination with rapamycin, has previously been shown to synergistically eradicate an osteosarcoma of the

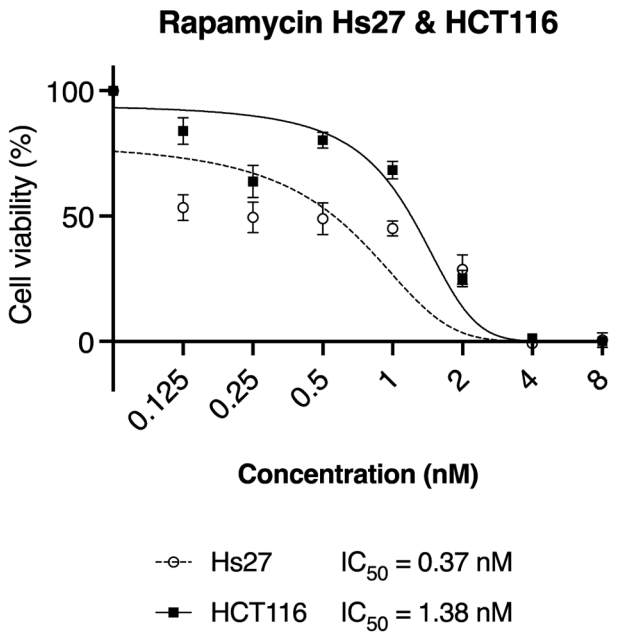

Figure 1. *Rapamycin efficacy on HCT-116 cells and Hs-27 cells in vitro. Cell viability was measured with the WST-8 reagent. The concentration axis uses a log_2 scale. IC_{50}: Half-maximal inhibitory concentration.*

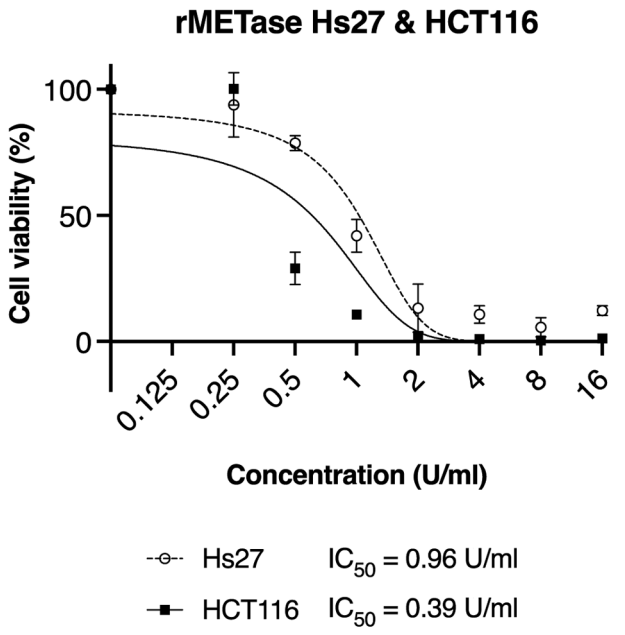

Figure 2. *Recombinant methioninase (rMETase) efficacy on HCT-116 cells and Hs-27 cells in vitro. Cell viability was measured with the WST-8 reagent. The concentration axis uses a log_2 scale. IC_{50}: Half-maximal inhibitory concentration.*

breast in a patient-derived orthotopic xenograft (PDOX) mouse model without toxicity (23). This result suggests the possibility that mTOR *via* SAMTOR and SAM may have a very different effect in cancer cells due to the acute deficiency of SAM under methionine restriction which may greatly inhibit mTOR's protein kinase activity, in contrast to normal cells where methionine restriction does not cause an acute deficiency of SAM (3, 4).

In the present study, we tested rapamycin and rMETase alone and in combination on both human colorectal carcinoma cells (HCT-116) and normal human fibroblast cells (Hs-27) *in vitro* to determine if there is a differential effect of the two agents alone and in combination on the survival of cancer cells and normal fibroblasts.

Materials and Methods

Cell culture. The HCT-116 human colon cancer cell line and Hs-27 human fibroblasts were acquired from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's 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, polyethylene glycol precipitation, and diethylaminoethyl-sepharose fast-flow ion-exchange column chromatography (20-22).

Cell viability testing. HCT-116 cells and Hs-27 cells were cultivated at subconfluence overnight in DMEM in 96-well plates (1.0×10^3 cells per well). The following day, HCT-116 cells and Hs-27 cells were treated with concentrations of rapamycin ranging from 0.125 nM to 8 nM or rMETase ranging from 0.125 U/ml to 16 U/ml. After 96 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 used to calculate IC_{50} values and sensitivity curves. After calculating the half-maximal inhibitory concentration (IC_{50}) for rMETase and rapamycin, the IC_{50} concentrations of both drugs were used to determine the synergistic efficacy of the combination of the drugs. Finally, we treated both HCT-116 cells and Hs-27 cells with a combination of rMETase and rapamycin using the IC_{50} values from the cell viability testing to determine whether the combination of methionine restriction and rapamycin produced a synergistic effect. 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 rapamycin alone and rMETase alone on HCT-116 and Hs-27 cells in vitro. We determined the sensitivity to rapamycin alone and rMETase alone of HCT-116

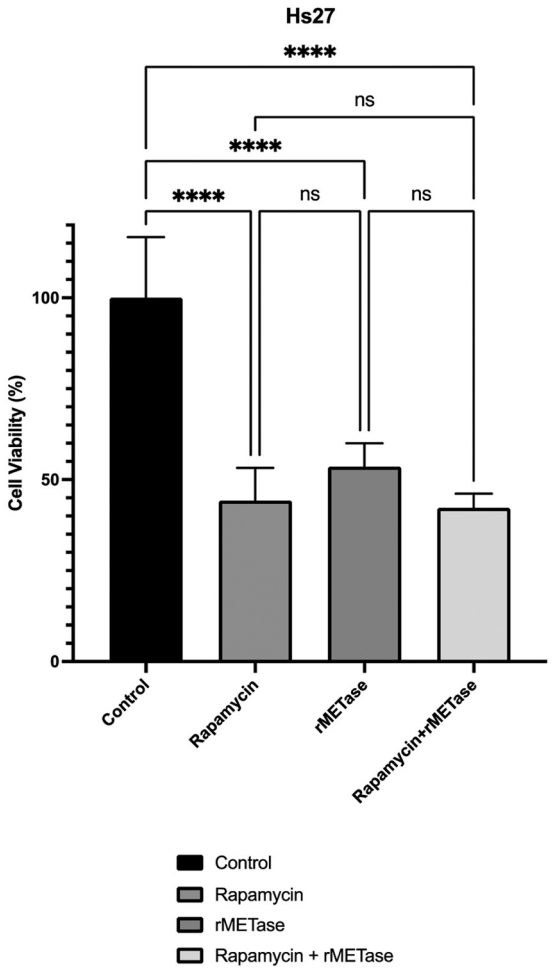

Figure 3. *Treatment of Hs-27 cells in vitro with recombinant methioninase (rMETase) and rapamycin at their half-maximal inhibitory concentrations (0.96 U/ml and 0.37 nM, respectively) alone and in combination. The combination treatment did not synergistically affect the viability of Hs-27 cells more than either drug alone. Cell viability was measured with the WST-8 reagent. ****p<0.0001.*

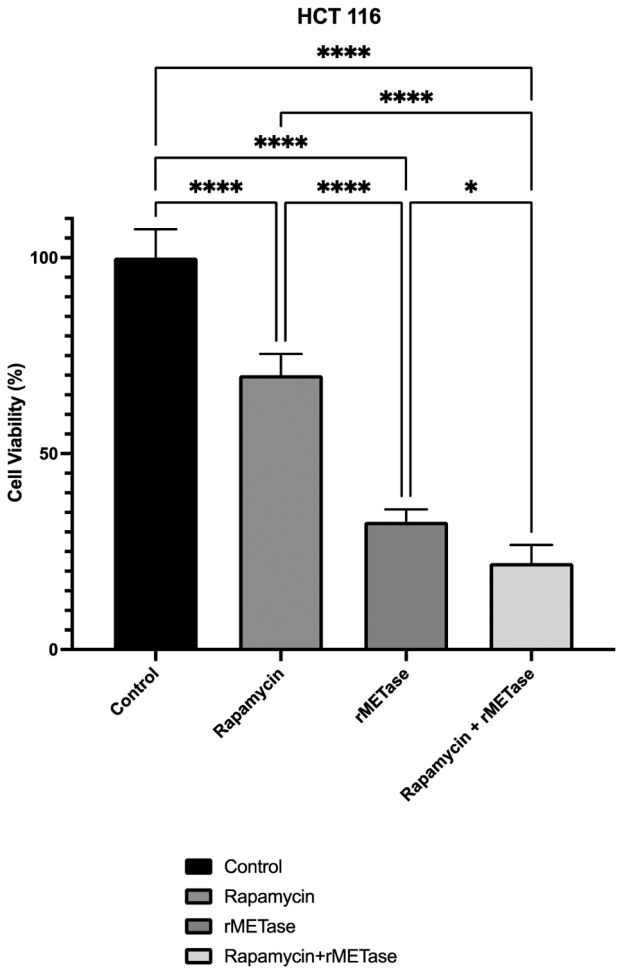

Figure 4. *Treatment of HCT-116 cells in vitro with recombinant methioninase (rMETase) and rapamycin at their half-maximal inhibitory concentrations (0.39 U/ml and 1.38 nM, respectively) alone and in combination. The combination treatment significantly reduced the viability of cancer cells. Cell viability was measured with the WST-8 reagent. ****p<0.0001, *p=0.0131.*

cells and Hs-27 cells and IC_{50} values were calculated. The IC_{50} of rapamycin alone on HCT-116 cells was 1.38 nM and the IC_{50} of rapamycin alone on Hs-27 cells was 0.37 nM (Figure 1). Thus, normal fibroblasts were more sensitive to rapamycin than cancer cells. The IC_{50} of rMETase on HCT-116 cells was 0.39 U/ml and the IC_{50} of rMETase on Hs-27 cells was 0.96 U/ml (Figure 2). Thus, cancer cells were more sensitive to rMETase than normal fibroblasts.

Combination of rapamycin and rMETase showed synergy only in the cancer cells not normal cells, despite greater sensitivity of normal cells to rapamycin alone. The combination of rMETase and rapamycin at their IC_{50} values (Hs-27: rMETase IC_{50}=0.96 U/ml; rapamycin IC_{50}=0.37 nM; HCT-116: rMETase IC_{50}=0.39 U/ml; rapamycin IC_{50}=1.38 nM) greatly reduced the viability of cancer cells but did not affect normal fibroblasts more than either drug alone (Figure 3 and Figure 4).

Discussion

The present results suggest that rapamycin as well as methioninase have different effects on normal and cancer cells. This difference is greater when rapamycin and methioninase are combined which can selectively reduce the survival of cancer cells with respect to rapamycin alone and methionine alone, but not normal cells where methioninase did not reduce the viability of normal cells when combined with rapamycin compared to rapamycin alone.

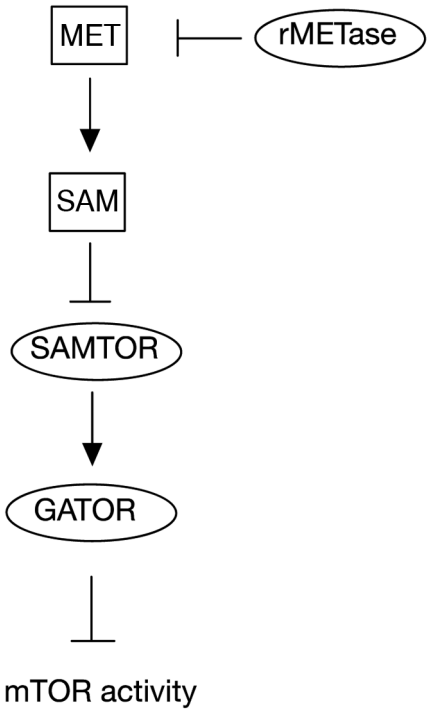

Figure 5. *Model showing how rMETase indirectly inhibits mTOR activity by acute depletion of methionine (MET) and SAM in cancer cells resulting in SAMTOR binding to GATOR, thereby inihibiting mTOR.*

The results suggest that mTOR through SAMTOR and SAM may react differently in normal and cancer cells due to the acute depletion of SAM in cancer cells under methionine restriction (3, 4), but not normal cells under methionine restriction, causing an increase in efficacy of rapamycin against the cancer cells when combined with methioninase (Figure 5).

Methioninase causes a greater drop of SAM in cancer cells compared to normal cells, due to the cancer cells addiction to methionine which causes depleted SAM levels under methionine restriction (3, 4), which may result in mTOR inhibition *via* SAMTOR and cell death (Figure 5).

The present *in vitro* results and our previous in vivo results (23) showing synergy of rapamycin and methioninase against cancer cells, suggest future clinical promise of this combination. Future experiments will investigate this possibility.

Conclusion

Rapamycin is an inhibitor of mTOR. mTOR is also inhibited by methionine restriction of cancer cells due to reduced SAM which binds SAMTOR and is necessary for mTOR activity (3). Therefore, there is synergy between rapamycin and rMETase on cancer cells and not normal cells due to the methionine addiction of cancer cells in which methionine restriction acutely depletes methionine and SAM, but not normal cells (3-6, 9-19, 23-28).

Conflicts of Interest

QH is an employee of AntiCancer Inc. DA, YK, MS, KM, SM, and RMH are or were unsalaried associates of AntiCancer Inc.

Authors' Contributions

DA and YK performed experiments. QH supplied methioninase. DA, YK, 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.

Acknowledgements

This paper is dedicated to the memory of A. R. Moossa, MD, Sun Lee, MD, Gordon H. Sato, PhD, Professor Li Jiaxi, Masaki Kitajima, MD, Shigeo Yagi, PhD, Jack Geller, MD, Joseph R Bertino, MD, and J.A.R. Mead, PhD. The Robert M Hoffman Foundation for Cancer Research provided funds for this study.

References

1 Li J, Kim SG, Blenis J: Rapamycin: one drug, many effects. Cell Metab 19(3): 373-379, 2014. DOI: 10.1016/j.cmet.2014.01.001

2 Gu X, Orozco JM, Saxton RA, Condon KJ, Liu GY, Krawczyk PA, Scaria SM, Harper JW, Gygi SP, Sabatini DM: SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. Science 358(6364): 813-818, 2017. DOI: 10.1126/science.aao3265

3 Coalson DW, Mecham JO, Stern PH, Hoffman RM: Reduced availability of endogenously synthesized methionine for S-adenosylmethionine formation in methionine-dependent cancer cells. Proc Natl Acad Sci USA 79(14): 4248-4251, 1982. DOI: 10.1073/pnas.79.14.4248

4 Wang Z, Yip LY, Lee JHJ, Wu Z, Chew HY, Chong PKW, Teo CC, Ang HY, Peh KLE, Yuan J, Ma S, Choo LSK, Basri N, Jiang X, Yu Q, Hillmer AM, Lim WT, Lim TKH, Takano A, Tan EH, Tan DSW, Ho YS, Lim B, Tam WL: Methionine is a metabolic dependency of tumor-initiating cells. Nat Med 25(5): 825-837, 2019. DOI: 10.1038/s41591-019-0423-5

5 Stern PH, Hoffman RM: Elevated overall rates of transmethylation in cell lines from diverse human tumors. In Vitro 20(8): 663-670, 1984. DOI: 10.1007/BF02619617

6 Yamamoto J, Aoki Y, Inubushi S, Han Q, Hamada K, Tashiro Y, Miyake K, Matsuyama R, Bouvet M, Clarke SG, Endo I, Hoffman RM: Extent and instability of trimethylation of histone H3 lysine increases with degree of malignancy and methionine addiction. Cancer Genomics Proteomics 19(1): 12-18, 2022. DOI: 10.21873/cgp.20299

7 Ghergurovich JM, Xu X, Wang JZ, Yang L, Ryseck RP, Wang L, Rabinowitz JD: Methionine synthase supports tumour tetrahydrofolate pools. Nat Metab 3(11): 1512-1520, 2021. DOI: 10.1038/s42255-021-00465-w

8 Sullivan MR, Darnell AM, Reilly MF, Kunchok T, Joesch-Cohen L, Rosenberg D, Ali A, Rees MG, Roth JA, Lewis CA, Vander Heiden MG: Methionine synthase is essential for cancer cell

proliferation in physiological folate environments. Nat Metab 3(11): 1500-1511, 2021. DOI: 10.1038/s42255-021-00486-5

9 Sugimura T, Birnbaum SM, Winitz M, Greenstein JP: Quantitative nutritional studies with water-soluble, chemically defined diets. VIII. The forced feeding of diets each lacking in one essential amino acid. Arch Biochem Biophys 81(2): 448-455, 1959. DOI: 10.1016/0003-9861(59)90225-5

10 Yamamoto J, Han Q, Inubushi S, Sugisawa N, Hamada K, Nishino H, Miyake K, Kumamoto T, Matsuyama R, Bouvet M, Endo I, Hoffman RM: Histone methylation status of H3K4me3 and H3K9me3 under methionine restriction is unstable in methionine-addicted cancer cells, but stable in normal cells. Biochem Biophys Res Commun 533(4): 1034-1038, 2020. DOI: 10.1016/j.bbrc.2020.09.108

11 Kaiser P: Methionine dependence of cancer. Biomolecules 10(4): 568, 2020. DOI: 10.3390/biom10040568

12 Mecham JO, Rowitch D, Wallace C, Stern PH, Hoffman RM: The metabolic defect of methionine dependence occurs frequently in human tumor cell lines. Biochem Biophys Res Commun 117(2): 429-434, 1983. DOI: 10.1016/0006-291x(83)91218-4

13 Guo R, Liang JH, Zhang Y, Lutchenkov M, Li Z, Wang Y, Trujillo-Alonso V, Puri R, Giulino-Roth L, Gewurz BE: Methionine metabolism controls the B cell EBV epigenome and viral latency. Cell Metab 34(9): 1280-1297.e9, 2022. DOI: 10.1016/j.cmet.2022.08.008

14 Hoffman RM, Erbe RW: High in vivo rates of methionine biosynthesis in transformed human and malignant rat cells auxotrophic for methionine. Proc Natl Acad Sci USA 73(5): 1523-1527, 1976. DOI: 10.1073/pnas.73.5.1523

15 Yamamoto J, Aoki Y, Han Q, Sugisawa N, Sun YU, Hamada K, Nishino H, Inubushi S, Miyake K, Matsuyama R, Bouvet M, Endo I, Hoffman RM: Reversion from methionine addiction to methionine independence results in loss of tumorigenic potential of highly-malignant lung-cancer cells. Anticancer Res 41(2): 641-643, 2021. DOI: 10.21873/anticanres.14815

16 Hoffman RM, Jacobsen SJ, Erbe RW: Reversion to methionine independence in simian virus 40-transformed human and malignant rat fibroblasts is associated with altered ploidy and altered properties of transformation. Proc Natl Acad Sci USA 76(3): 1313-1317, 1979. DOI: 10.1073/pnas.76.3.1313

17 Kubota Y, Sato T, Hozumi C, Han Q, Aoki Y, Masaki N, Obara K, Tsunoda T, Hoffman RM: Superiority of [(11)C]methionine over [(18)F]deoxyglucose for PET imaging of multiple cancer types due to the methionine addiction of cancer. Int J Mol Sci 24(3): 1935, 2023. DOI: 10.3390/ijms24031935

18 Stern PH, Hoffman RM: Enhanced *in vitro* selective toxicity of chemotherapeutic agents for human cancer cells based on a metabolic defect. J Natl Cancer Inst 76(4): 629-639, 1986. DOI: 10.1093/jnci/76.4.629

19 Hoffman RM, Coalson DW, Jacobsen SJ, Erbe RW: Folate polyglutamate and monoglutamate accumulation in normal and SV40-transformed human fibroblasts. J Cell Physiol 109(3): 497-505, 1981. DOI: 10.1002/jcp.1041090316

20 Takakura T, Ito T, Yagi S, Notsu Y, Itakura T, Nakamura T, Inagaki K, Esaki N, Hoffman RM, Takimoto A: High-level expression and bulk crystallization of recombinant l-methionine γ-lyase, an anticancer agent. Appl Microbiol Biotechnol 70(2): 183-192, 2006. DOI: 10.1007/s00253-005-0038-2

21 Takakura T, Mitsushima K, Yagi S, Inagaki K, Tanaka H, Esaki N, Soda K, Takimoto A: Assay method for antitumor l-methionine γ-lyase: comprehensive kinetic analysis of the complex reaction with l-methionine. Anal Biochem 327(2): 233-240, 2004. DOI: 10.1016/j.ab.2004.01.024

22 Tan Y, Xu M, Tan X, Tan X, Wang X, Saikawa Y, Nagahama T, Sun X, Lenz M, Hoffman RM: Overexpression and large-scale production of recombinantl-methionine-α-deamino-γ-mercapto methane-lyase for novel anticancer therapy. Protein Expr Purif 9(2): 233-245, 1997. DOI: 10.1006/prep.1996.0700

23 Masaki N, Han Q, Samonte C, Wu NF, Hozumi C, Wu J, Obara K, Kubota Y, Aoki Y, Bouvet M, Hoffman RM: Oral-recombinant methioninase in combination with rapamycin eradicates osteosarcoma of the breast in a patient-derived orthotopic xenograft mouse model. Anticancer Res 42(11): 5217-5222, 2022. DOI: 10.21873/anticanres.16028

24 Stern PH, Mecham JO, Wallace CD, Hoffman RM: Reduced free-methionine in methionine-dependent SV40-transformed human fibroblasts synthesizing apparently normal amounts of methionine. J Cell Physiol 117(1): 9-14, 1983. DOI: 10.1002/jcp.1041170103

25 Aoki Y, Han Q, Tome Y, Yamamoto J, Kubota Y, Masaki N, Obara K, Hamada K, Wang JD, Inubushi S, Bouvet M, Clarke SG, Nishida K, Hoffman RM: Reversion of methionine addiction of osteosarcoma cells to methionine independence results in loss of malignancy, modulation of the epithelial-mesenchymal phenotype and alteration of histone-H3 lysine-methylation. Front Oncol 12: 1009548, 2022. DOI: 10.3389/fonc.2022.1009548

26 Yamamoto J, Inubushi S, Han Q, Tashiro Y, Sugisawa N, Hamada K, Aoki Y, Miyake K, Matsuyama R, Bouvet M, Clarke SG, Endo I, Hoffman RM: Linkage of methionine addiction, histone lysine hypermethylation, and malignancy. iScience 25(4): 104162, 2022. DOI: 10.1016/j.isci.2022.104162

27 Hoffman RM, Jacobsen SJ, Erbe RW: Reversion to methionine independence by malignant rat and SV40-transformed human fibroblasts. Biochem Biophys Res Commun 82(1): 228-234, 1978. DOI: 10.1016/0006-291x(78)90600-9

28 Tan Y, Xu M, Hoffman RM: Broad selective efficacy of recombinant methioninase and polyethylene glycol-modified recombinant methioninase on cancer cells In Vitro. Anticancer Res 30(4): 1041-6, 2010

Received November 21, 2023
Revised December 21, 2023
Accepted January 10, 2024