Methionine depletion with recombinant methioninase: *In vitro* and *in vivo* efficacy against neuroblastoma and its synergism with chemotherapeutic drugs

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Methionine starvation can modulate gene methylation, cell cycle transition and pathways related to survival following DNA damage. Methionine depletion by recombinant methioninase (rMETase) may have *in vitro* and *in vivo* efficacy against neuroblastoma (NB), especially when combined with chemotherapeutic drugs. rMETase from *Pseudomonas putida* was produced in *Escherichia coli* and purified by ion-exchange chromatography. rMETase alone inhibited the proliferation of 15/15 NB cell lines *in vitro*. Among these 15 cell lines, only 66N demonstrated rMETase-induced apoptosis. rMETase alone suppressed LAN-1 and NMB-7 xenografts ($p < 0.01$) and no toxicities were noted other than reversible weight loss. *In vitro* efficacy experiments combining rMETase and chemotherapeutic agents were carried out using SK-N-LD and SK-N-BE (1)N established at diagnosis, as well as LAN-1, SK-N-BE (2)C and NMB-7 established at relapse. Microtubule depolymerization agents including vincristine, vinorelbine, vinblastine and mebendazole showed synergism when tested in combination with rMETase in all 5 cell lines. Among DNA damaging agents, synergy with rMETase was observed only in cell lines established at diagnosis and not at relapse. Cell cycle analysis showed that rMETase arrested G2 phase and not M phase. *In vivo* efficacy experiments using LAN-1 and NMB-7 xenografts showed that rMETase rendered vincristine more effective than vincristine alone in tumor growth suppression ($p < 0.001$). In conclusion, methionine depletion inhibited NB proliferation and arrested tumor cells at G2 phase. rMETase synergized with microtubule depolymerization agents. Moreover, synergism between rMETase and DNA damaging agents was dependent on whether cell lines were established at diagnosis or at relapse.
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Methionine deprivation affects tumor cells with the propensity to divide and causes them to arrest predominantly in the G2 phase of the cell cycle and to eventually undergo apoptosis.[1–5] Reduction of plasma methionine below 5 μmol/L arrests human xenograft growth in athymic mice.[6] Methionine depletion can be achieved using parenteral L-methionine-deamino-mercaptomethane-lyase (methioninase, a methionine-cleaving enzyme derived from *Pseudomonas putida*)[7,8] which is cloned and expressed in *Escherichia coli*.[9,10] A variety of tumors including Lewis lung carcinoma,[11] several human colon cancer lines[12] and glioblastoma[13,14] have shown arrested growth by rMETase. To eradicate tumors, treatment has to be prolonged. However, prolonged deprivation of methionine is impractical because it may result in permanent liver damage[15] and tumor will rapidly regrow after rMETase is withdrawn. Since methionine starvation can broadly affect protein synthesis and modulate gene methylation, cell cycle transition and pathways related to survival following DNA damage in tumor,[3,6,13,14,16,17] but not in normal tissues, they may enhance the effects of chemotherapeutic drugs and decrease systemic toxicity. Indeed, there is growing evidence that certain classes of chemotherapeutic agents may be synergistically enhanced by methionine depletion; these include BCNU,[3] doxorubicin,[4] 5-fluorouracil, mitomycin C,[18] cisplatin[12] and nitrosoureas.[13,14]

Neuroblastoma (NB) is an embryonic neoplasm of the sympathetic nervous system.[19,20] Metastatic NB is a highly proliferative cancer in which gene methylation[21,22] and alkylator resistance[23] are strongly correlated with poor prognosis. Drawing from the successful experience of bacteria-derived asparaginase in the current treatment of childhood acute lymphoblastic leukemia,[24] we hypothesize that rMETase may have clinical utility against NB, which is methionine-dependent. In this report, we describe the production and purification of *P. putida* rMETase, and its *in vitro* and *in vivo* cytotoxicity against NB, either alone or in combination with common chemotherapeutic agents, including microtubule depolymerization agents.

Material and methods

Plasmids

A pKK223-3 plasmid containing the gene for L-methioninase was kindly provided by Dr. Dennis Carson, University of Southern California (Los Angeles, CA) and Dr. Roger Harrison, University of Oklahoma (Norman, OK).[10]

Tumor cell lines

NB cell line LAN-1 was provided by Dr. Robert Seeger (Children's Hospital of Los Angeles; Los Angeles, CA), NB cell line NMB-7 by Dr. Liao of McMaster University (Hamilton, ON, Canada). SK-N-BE(1)N, SK-N-BE(2)C, SK-N-BE(2)N, SK-N-BE(2)S, LAI-5S and SH-EP-1, 55N and 66N were kindly provided by Dr. Robert Ross, Fordham University (New York, NY). NB cell lines SK-N-LD, SK-N-ED, SK-N-MM were established at Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY). IMR-32 and CHP-212 were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured as previously described.[25]

Mouse feed

The diet D518787 purchased from Dyets (Bethlehem, PA) was methionine and choline deficient and contained 1.7 g/kg DL-homocystine.[6] In this report, it was referred to as Met(−)Hcyss(+)Chl(−).

Production of rMETase

rMETase fermentation was carried out in a 14 L Bioflow 3,000 Fermentor (New Brunswick Scientific, Edison, NJ) using TB (Terrific Broth) medium containing 100 μg/mL ampicillin. Fermentation conditions and cell disruption were performed as previously described.[26] rMETase was captured by QAE-Sepharose FF (Amersham Pharmacia Biotech, Piscataway, NJ) and then eluted with buffer A (20 mmol/L potassium phosphate buffer [pH 7.4], 20 μmol/L pyridoxal 5′-phosphate, 1 mmol/L EDTA [pH 8.0], 0.01% β-mercaptoethanol containing 0.3 mol/L NaCl). Pooled rMETase was heated at 60°C for 15 min. Endotoxin was removed by 1% triton X-114 phase separation,[27] and followed by Detoxi-Gel™ Endotoxin Removing Gel (Pierce, Rockford, IL) to remove

a small amount of endotoxin retained in the rMETase solution. The endotoxin level was determined by Limulus Amebocyte Lysate QCL-1000[®] (Cambrex Bio Science, Walkersville, MD). 2-Ketobutyric acid (Sigma-Aldrich) was used to establish a standard curve for enzyme activity assay.[28] One unit of rMETase was defined as the amount of enzyme that produced 1 μmol of α-ketobutyrate per minute at infinite concentration of L-methionine. Protein was quantified by BCA[TM] Protein Assay Kit (Pierce, Rockford, IL). A TSK-GEL G3000SWxl size exclusion column (30 × 7.8 mm, 5 μ) (Tosoh Bioscience, Montgomeryville, PA) was used for HPLC analysis with 1× PBS as the mobile phase.

WST8 assay for NB cell proliferation

WST-8 assay (Dojindo Molecular Technologies, MA) was validated by the manufacturer using MTT assay in NB cell line IMR-32 in the presence of DNA damaging agents and topoisomerase II inhibitor. Our laboratory also validated this assay using AlamarBlue and direct cell counting (RT-CES system, ACEA Biosciences, San Diego, CA) when LAN-1 and NMB-7 were treated with SN38.

In vitro proliferation inhibition of NB cell lines was carried out in 96-well plates (BD Biosciences, Bedford, MA). Twenty-four hours after the cells were seeded at a density of 4,000–10,000 cells/well, rMETase, chemotherapeutic drugs or their combinations were added to the wells. After 3–5 days of exposure, 1/10 volume WST8 was added and optical density (OD) was read at 450 nm. The proliferation rate of cells was calculated as follows: % proliferation rate = (OD$_{450}$ of treated well − OD$_{450}$ of medium only well)/(OD$_{450}$ of nontreated well − OD$_{450}$ of medium only well) × 100%. Half maximal inhibitory concentration (IC$_{50}$) was calculated by SigmaPlot 8.0 (Systat Software, San Jose, CA) and combination index (CI) was calculated by CompuSyn (CompoSyn, Paramus, NJ).[29]

NB cell apoptosis and cell cycle assay

The *in vitro* apoptosis and cell cycle experiments were carried out in 6-well plates (BD Biosciences). Twenty-four hours after the cells were seeded at a density of 2 to 5 × 10^5 cells/well, rMETase, vincristine or their combinations were added to the wells. After 12–48 hr of treatment, cells were stained with either 7-amino-actinomycin D (7AAD) and Annexin V or propidium iodide (PI) (BD PharMingen, San Diego, CA) for apoptosis assay or stained with PI and MPM-2/CY5 (Upstate Biotechnology, Charlottsville, VA) for cell cycle assay using flow cytometry with BD FACSCalibur System (BD Bioscience). For each sample, 10,000 events in gated area were collected. The data from flow cytometry were analyzed by Flowjo (Windows-version 5.7.2) software (Tree star, Ashland, OR).

Antitumor effect with NB xenografts

All animal experiments were carried out according to an Institutional Animal Care and Use Committee (IACUC)-approved protocol and institutional guidelines for the proper and humane use of animals in research were followed. Athymic female nude mice were purchased from the National Cancer Institute. Mice with 5–10 mm established tumors were randomly separated into groups of 5–10 mice each. Based on pharmacokinetics study of rMETase in mice, after injection of 100 units rMETase, plasma rMETase half-life was 2.06 ± 0.10 hr. When mice were fed methionine-free diet, one injection of 100 units rMETase could keep plasma methionine level nadir to 0.15 ± 0.06 μmol/L at 6 hr and recovered to 13.0% of pretreatment level at 10 hr. One injection of 100 units rMETase could keep plasma methionine level below 5 μM for ~10 hr. That is why we chose twice daily dosing. When rMETase efficacy was tested against NB xenografts, 100 units of rMETase was given iv bid ×4 days/week ×3 weeks as a single agent. Mice were bled 4 hr after rMETase injection, which was carried out on the 4th day of the 1st and 3rd week of treatment. Methionine level

was measured according to the method of Sun *et al.*[30] In combination experiments, rMETase and vincristine were given separately or in combination at same time. One hundred units of rMETase was given iv bid ×2 days/week ×3 weeks and vincristine were given iv qwk ×3 weeks. In these experiments, Met(−)Hcyss(+)Chl(−) diet was given to the animals only during rMETase administration. The weight of mice was used as an index of toxicity. In addition, excessive weight loss may confound the conclusion on tumor shrinkage; thus, 15% maximum weight loss was used as a guideline in designing methioninase dosing and timing of normal feeds. Tumor size and weight were measured twice per week and they were expressed as mean ± SEM (standard error of the mean) relative to tumor size or body weight values on day 0 (start of treatment). Mice were sacrificed when their tumor sizes exceeded 20 mm in diameter.

Results

Fermentation, purification and characterization of rMETase

A 10 L of fermentation culture produced ~70,000 units of rMETase in the supernatant from homogenized *E. coli* cell extracts. The yield of rMETase was about 50%, with 30% loss during the column chromatography and 20% loss during the heat and endotoxin removal. rMETase was stable at 4°C for at least 2 months when stored in buffer A containing 0.3 mol/L NaCl (see Methods). After ultrafiltration, the final concentration of rMETase was about 800 units/mL and endotoxin was less than 1 EU/mL. The enzyme activity ratio was 16.2 units/mg.

SDS-PAGE demonstrated that rMETase was almost pure after one step of ion-exchange chromatography (Fig. 1a). The major band indicated an apparent molecular weight of 43 KD. On size exclusion HPLC, the purified protein exhibited one major peak at a retention time of 15.547 min (Fig. 1b) with 92.5% purity.

Inhibition of in vitro proliferation of NB cells

The growth of 15 NB cell lines was inhibited by rMETase exposure in a dose-dependent manner. IC$_{50}$ values were tabulated in Table I. All cell lines, except SK-N-BE(2)S which had a substrate adherent phenotype, were highly sensitive to rMETase. After 3 days of exposure, methionine levels on the 96-well plate were tested by HPLC. Only the wells with ≤ 0.175 units/mL rMETase had detectable methionine level (≥0.18 ± 0.01 μmol/L); the concentration of methionine in control medium containing 10% calf serum was 62.09 ± 0.94 μmol/L. Among these 15 cell lines, only 66 N showed obvious and reproducible apoptosis induced by rMETase after 48 hr of exposure with a 22.4% increase of Annexin V$^+$/7AAD$^−$ population.

At high concentrations of rMETase, all NB cell lines will eventually die. After cells were treated with rMETase for 24 hr, it will take 3–4 days for the cell to recover by addition of 200 μM methionine as demonstrated by flow cytometry analysis and cell number. These findings suggest that methionine depletion is the major mechanism of cell death.

Combining rMETase and chemotherapeutic drugs on NB in vitro

In vitro efficacy experiments were carried out using SK-N-LD and SK-N-BE(1)N established at diagnosis (p53 wild type), as well as LAN-1 (p53 deleted), SK-N-BE(2)C (p53 mutated) and NMB-7 (p53 wild type) all established at relapse. In these experiments, a fixed concentration of rMETase (~20–50% inhibition of cell growth) was used, while the concentrations of chemotherapeutic drugs were varied so as to kill 0–100% tumor cell in a serial dilution. Combination indices (CIs) calculated in nonconstant ratio mode were shown in Table II and the effects of vincristine with or without rMETase on SK-N-BE(1)N and SK-N-LD proliferation were shown in Fig. 2. In all 5 NB cell lines tested, rMETase synergized with microtubule depolymerization agents (vincristine, vinorelbine, vinblatine and mebendazole), but not microtubule sta-
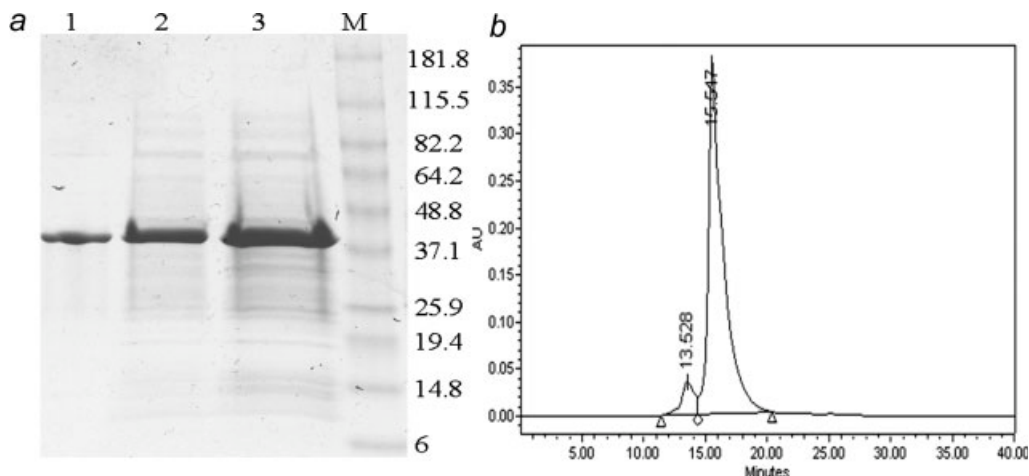
FIGURE 1 – Molecular size and purity of rMETase. (*a*) SDS-PAGE—Lane 1, purified rMETase; Lane 2, supernatant from whole cell extract; Lane 3, whole cell crude extract; Lane 4, marker. (*b*) HPLC size exclusion profile of purified rMETase. The major peak at 15.547 min was rMETase.

TABLE I – INHIBITION OF NB CELL PROLIFERATION BY rMETase

Cell line	IC_{50} (units/mL)
SK-N-BE(1)N	0.04 ± 0.01
SK-N-LD	0.05 ± 0.01
NMB-7	0.08 ± 0.01
SH-EP-1	0.12 ± 0.05
SK-N-BE(2)C	0.13 ± 0.02
LAN-1	0.18 ± 0.01
IMR-32	0.20 ± 0.02
CHP-212	0.20 ± 0.01
66 N	0.22 ± 0.03
55 N	0.27 ± 0.03
SK-N-MM	0.36 ± 0.03
SK-N-ED	0.39 ± 0.03
LAI-5S	0.48 ± 0.02
SK-N-BE(2)N	0.51 ± 0.11
SK-N-BE(2)S	5.12 ± 0.56

IC_{50} (half maximal inhibitory concentration) was expressed as mean $\pm$ SEM (standard error of the mean).

bilizing agents (paclitaxel, docetaxel and Iso-fludelone). Gemcitabine also did not show synergy in these 5 cell lines. Cell lines established at diagnosis (SK-N-LD and SK-N-BE(1)N), but not those established at relapse (LAN-1, SK-N-BE(2)C and NMB-7) showed synergism when rMETase combined with alkylators and topoisomerase inhibitors. In these 5 cell lines, their sensitivity to rMETase exposure was comparable to their sensitivity to chemotherapeutic drugs (data not shown).

rMETase and vincristine on cell cycle arrest

Based on the known function of chemotherapeutic drugs and the CI values (Table II), vincristine was chosen to study the mechanism involved when combined with rMETase. The effect of rMETase with or without vincristine on the cell cycle arrest was examined. After 18 hr of exposure to 0.2 units/mL rMETase, SK-N-LD, SK-N-BE(1)N, LAN-1, SK-N-BE(2)C and NMB-7 showed ~10% increase of G2 population. However, unlike vincristine (2.5ng/mL), rMETase alone did not increase M phase arrest (Fig. 3). When rMETase and vincristine were combined, rMETase decreased the M phase arrest by vincristine, likely the result of a slower tumor cell cycle transition following G2 arrest by rMETase.

Efficacy of rMETase alone on NB xenografts in athymic mice

The schedule of rMETase administration was based on the toxicity of rMETase according to weight loss and pharmacokinetics of rMETase (see Methods). rMETase treatment was generally well tolerated. In xenografts, weight loss after methionine deprivation was the only dose limiting toxicity. When athymic mice planted with NB xenografts were treated with 100 units rMETase iv bid $\times$4 days/week $\times$3 weeks, a ~10% reversible weight loss was observed. Four hours after rMETase injection, plasma methionine concentration was 4.72 ± 0.47 μmol/L among rMETase-treated mice fed with normal chow, in contrast to 61.79 ± 2.14 μmol/L among the untreated mice ($n = 36$). Mice fed with Met(−)Hcyss(+)Chl(−) diet had ~20% reversible weight loss after injection of 100 units rMETase iv bid $\times$4 days/week. Plasma methionine concentration was lowered to 0.40 ± 0.07 μmol/L among the rMETase-treated group, in contrast to 31.39 ± 3.78 μmol/L among the untreated mice ($n = 9$). Hundred units rMETase iv bid $\times$4 days/week $\times$3 weeks demonstrated significant efficacy against LAN-1 and NMB-7 xenografts ($p < 0.01$) (Fig. 4). Methionine levels 4 hr after rMETase injection were comparable among the 2 xenograft models (data not shown).

Combining rMETase and vincristine in the suppression of NB xenografts

On the basis of our *in vitro* study and the CI values (Table II), vincristine was chosen as the best candidate for *in vivo* evaluation of treatment efficacy. Doxorubicin and thiotepa were also tested for comparison. When combined with chemotherapeutic agents, we chose doses of chemotherapeutic drugs with suboptimal antitumor effect so as to avoid a situation where the combined antitumor effect would exceed 100%. The following doses and regimens were chosen: vincristine (0.8 mg/kg)[31] and doxorubicin (6.5 mg/kg)[32] iv qwk $\times$3 weeks, and thiotepa (3 mg/kg)[33] iv $\times$2 days/week $\times$3 weeks were used. For these experiments, 100 units/dose of rMETase was given iv bid $\times$2 days/week $\times$3 weeks in the presence of Met(−)Hcyss(+)Chl(−) diet. Administration of rMETase led to a ~15% body weight loss for rMETase alone and the combination treatment groups; the animals regained their weight prior to the next cycle of treatment. The antitumor efficacy for single agents or in combination with rMETase was evaluated in 2 NB xenografts (Fig. 5). To evaluate *in vivo* efficacy of combination treatments by student's *t*-test, individual tumor growth rate versus time curve was transformed into area under the curve (AUC) by SigmaPlot. AUCs showed that rMETase rendered vincristine more effective than vincristine alone in tumor growth suppression

TABLE II – *IN VITRO* INHIBITION OF PROLIFERATION OF NB CELL LINES EXPOSED TO METHIONINE DEPLETION AND CHEMOTHERAPEUTIC DRUGS AS DETERMINED BY COMBINATION INDEX[1]

Chemotherapeutic agents	Drug functions	SK-N-LD	SK-N-BE(1)N	LAN-1	SK-N-BE(2)C	NMB-7
Vincristine	Microtubule depolymerization	**0.40**	**0.47**	**0.63**	**0.40**	**0.55**
Vinorelbine	Microtubule depolymerization	**0.39**	**0.47**	**0.66**	**0.46**	**0.61**
Vinblatine	Microtubule depolymerization	**0.40**	**0.50**	**0.65**	**0.63**	**0.61**
Mebendazole	Microtubule depolymerization	**0.40**	**0.45**	**0.51**	**0.37**	**0.60**
Paclitaxel	Microtubule stabilization	0.88	1.14	1.91	1.25	1.38
Docetaxel	Microtubule stabilization	0.72	0.81	1.10	1.92	0.99
Iso-fludelone	Microtubule stabilization	0.93	0.71	1.96	0.90	1.14
Etoposide	Topoisomerase II inhibitor	**0.48**	**0.58**	0.98	12.78	1.57
Topotecan	Topoisomerase I inhibitor	**0.51**	**0.35**	0.71	9.25	1.22
SN38	Topoisomerase I inhibitor	**0.59**	**0.51**	1.82	2.24	0.90
Doxorubicin	DNA intercalation, topoisomerase II inhibitor	**0.59**	**0.59**	**0.64**	11.99	0.81
Cisplatin	DNA cross-linking	**0.67**	**0.65**	0.71	1.79	1.37
Thiotepa	Alkylating agent	**0.60**	**0.60**	1.00	2.11	1.34
Gemcitabine	Nucleoside analog	0.85	1.11	1.08	76.99	1.12

Synergism: CI ≤ 0.7 (boldface values).
Additive: $0.90 \leq$ CI ≤ 1.10.
Antagonism: CI > 1.10.
[1]Combination index (CI) was detailed in the "Material and methods" section.
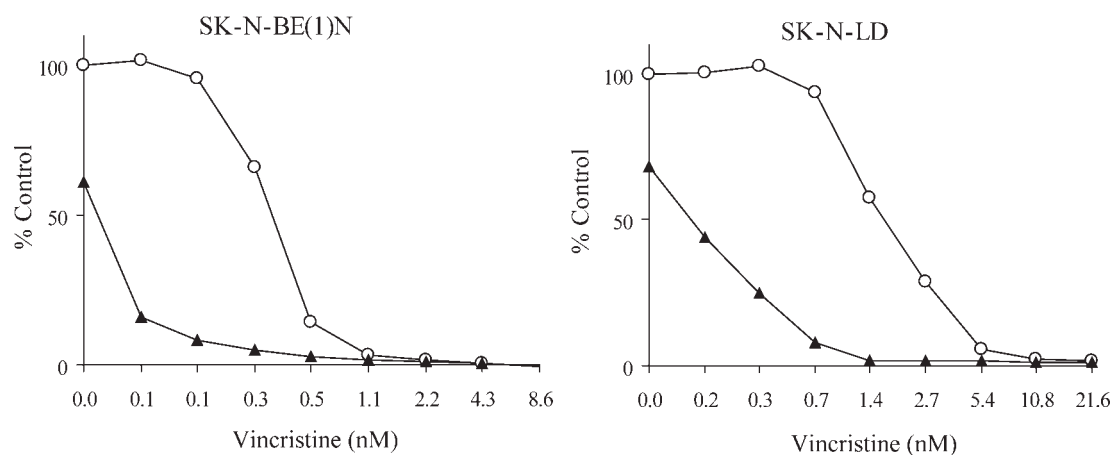

FIGURE 2 – Effect of vincristine with or without rMETase on NB cell proliferation of SK-N-BE(1)N and SK-N-LD. Open circle, vincristine alone; Solid triangle, rMETase + vincristine combined treatment.

($p < 0.001$). In contrast, rMETase did not significantly enhance the efficacy of doxorubicin and thiotepa against tumor growth (data not shown).

Discussion

The biochemical mechanism for methionine dependency has been studied extensively, but its effect on tumors derived from the sympathetic nervous system remains unclear. At high concentrations of rMETase, our studies showed that all NB cell lines will eventually die. At intermediate concentrations, some will survive and some will linger on. Cells can be salvaged (death prevented) when methionine is added back to the medium, suggesting that methionine depletion is the major mechanism of cell death. We interpret these findings to mean that there is differential sensitivity of NB cell lines to methionine deprivation, as well as differences in their inherent resistance to apoptosis and cell death. We have looked for apoptosis at 24, 48 and 72 hr of rMETase treatment and not beyond, because massive cell loss usually has occurred by day 2. Observation at 48 hr after treatment with rMETase seemed to be most optimal time point for showing apoptosis. But we could document apoptosis in only 1 of 15 cell lines. We concluded that apoptosis is not the primary mechanism of cell death in the majority of NB cell lines tested here. This defect in apoptosis may relate

to their caspase 3 and 8 deficiency,[34] especially when *MYCN* was amplified, a genetic aberration typically found in these cell lines.

Methionine deprivation modulates the expression levels of many genes in tumor cells,[16,17] some of which can provide rationales for combination approaches utilizing rMETase and chemotherapeutic drugs. We therefore undertook an analysis of standard chemotherapeutic agents against NB where the mechanisms of drug action were known. We tested for the presence of synergy between these chemotherapeutic agents and methionine deprivation using rMETase. We observed strong synergism between rMETase and vincristine, an M phase specific agent. We expanded these synergy studies using a panel of M phase specific agents (vincristine, vinorelbine, vinblastine, mebendazole, paclitaxel, docetaxel and iso-fludelone) and found synergy only for microtubule depolymerization agents and not microtubule stabilizing agents. The mechanism of action likely involves both the methylation of tubulins and regulation of mitosis related gene by rMETase. *S*-Adenosyl-L-methionine (SAM), produced from methionine and ATP, is the universal methyl donor in prokaryotes and eukaryotes and the reduction of SAM compromises protein methylation. Methionine appears to be critical for the methylation of actins and tubulins in maintaining cellular stability[35] and SAM is known to stabilize microtubule polymerization and reduce micronuclei formation.[36] Thus, it is likely rMETase reduces methylation of tubulins, further destabilizing microtubules when tumor
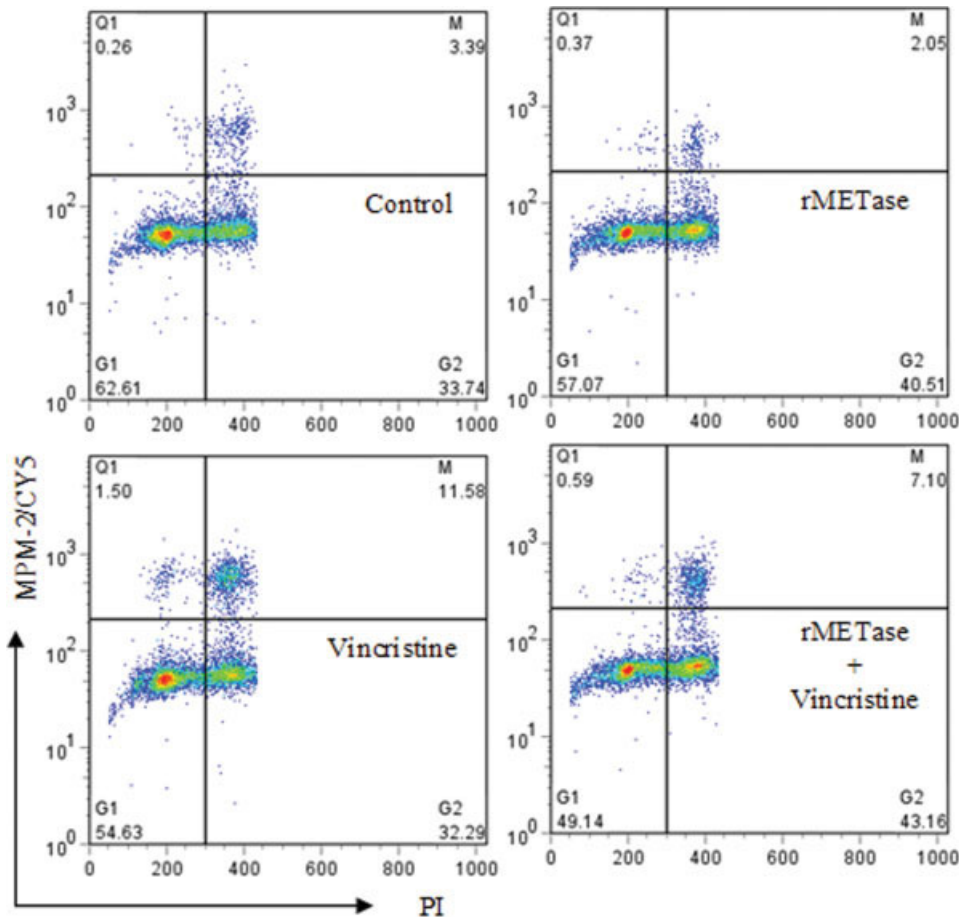
FIGURE 3 – Effect of rMETase with or without vincristine on cell cycle arrest of SK-N-BE(2)C. The flow cytometry results were presented in density plots, where the quadrants were set to include live cells. The lower left quadrant contained G1 phase population; the lower right quadrant contained G2 phase population; and the upper right quadrant contained M phase population. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
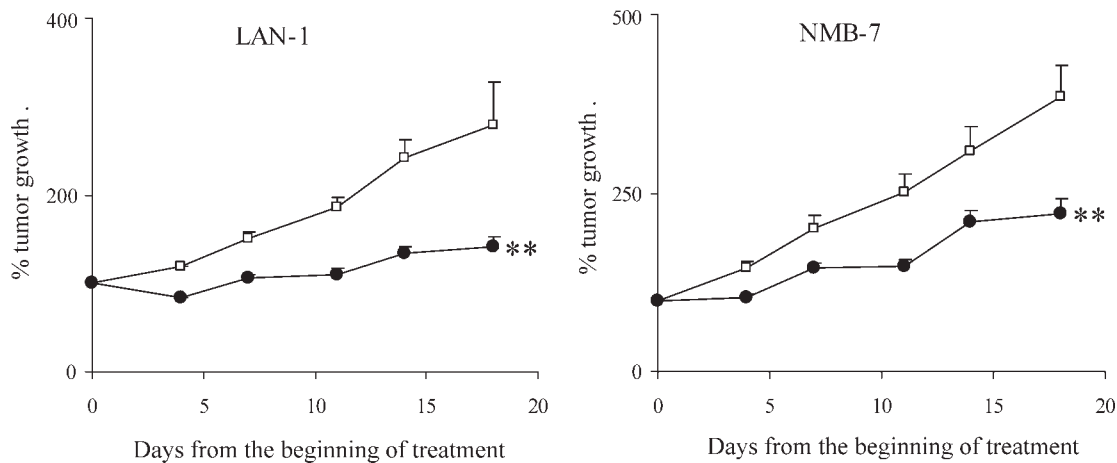

FIGURE 4 – Efficacy of *in vivo* rMETase in NB xenografts. Open square, control ($N = 10$ for LAN-1 and $N = 26$ for NMB-7); Solid circle, rMETase treatment ($N = 10$ for LAN-1 and $N = 34$ for NMB-7); ** $p < 0.01$ when AUC of rMETase group was compared with the control group.

cells are subjected to depolymerization agents. On the other hand, rMETase can up-regulate (CDKN1A, MDA1, etc.) or down-regulate (CDKN2B, Aurora kinase B, etc.).[16,17] The change in their genes expression levels is associated with the loss of mitosis in tumor cells.[16] rMETase-induced genetic alteration shows the multifaceted effect of methionine deprivation,[16,17] which can explain the highly effective synergism between rMETase and a variety of diverse chemotherapies tested.

Down-regulation of O6-methylguanine-DNA methyltransferase[37] and reduction of SAM by methionine deprivation will decrease DNA methylation and affect DNA stability. Less stability of DNA increases the sensitivity of tumor cells to DNA damaging agents.[38] When chemotherapeutic drugs were combined with methionine depletion, cell lines established at diagnosis had substantially smaller CIs and higher sensitivity than those established at relapse. It is highly likely that these resistant cell lines have al-
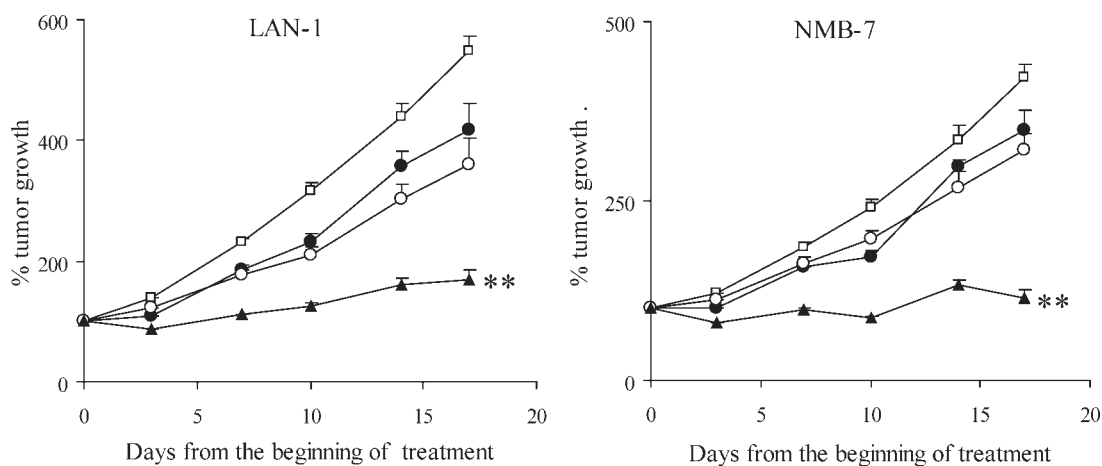
FIGURE 5 – Efficacy of *in vivo* combination treatment with rMETase and vincristine. Open square, control ($N = 9$ for LAN-1 and NMB-7); Solid circle, rMETase alone ($N = 14$ for LAN-1 and $N = 7$ for NMB-7); Open circle, vincristine alone ($N = 8$ for LAN-1 and $N = 7$ for NMB-7); Solid triangle, rMETase + vincristine combined treatment ($N = 14$ for LAN-1 and $N = 13$ for NMB-7); ** $p < 0.001$ when AUC of combination group was compared with other 3 groups, respectively.

ready recruited pathways to repair DNA damage.[39] One might expect the combination of rMETase and DNA damaging agents to be most useful if DNA repair pathways are inhibited. Poly (ADP-ribose) polymerase (PARP) inhibitor is cytotoxic for homologous-recombination (HR)-deficient cells by inhibiting base-excision repair.[40] Our preliminary results suggested that PARP inhibitor synergized with rMETase in suppressing the proliferation of NB cell lines. A more detailed synergism analysis using cell lines established at diagnosis versus at relapse, with known DNA damage repair pathways, will be critical for understanding the underlying mechanism of drug resistance.

For patients with high-risk NB, acquired resistance to chemotherapy and the toxicity of chemotherapy in young children are clinical constraints. Late effects such as second cancer are clearly related to dose and intensity of chemoradiotherapy. Overcoming drug resistance or lowering drug doses to minimize toxicity can have a significant impact on patient survival. Given the toxicity profile and our results on preclinical efficacy of rMETase in human NB, methionine depletion especially when induced by rMETase may provide a useful adjunct to our current therapy of advanced stage disease. rMETase may be most effective when applied at initial diagnosis with diverse classes of chemotherapy. At relapse following exposure to chemotherapy, the synergy appeared to be restricted to microtubule depolymerization agents, a unique class of agents with primarily neurotoxicity, but minimal myelosuppression. The underlying mechanism of this cooperative effect is not currently understood. In contrast, neither microtubule stabilizing agents nor gemcitabine showed synergy despite their *in vitro* activity as single agents and their clinical efficacy in the treatment of NB.

In conclusion, methionine depletion inhibited NB proliferation and arrested tumor cells at G2 phase. However, apoptosis was not the primary mechanism of cell death in the majority of cell lines examined. As a single agent *in vivo*, methionine depletion inhibited NB xenografts growth. In combination therapy, methionine depletion synergized with microtubule depolymerization agents *in vitro* and rendered vincristine more effective than vincristine alone in tumor growth suppression *in vivo*. Synergism between methioninase and DNA damaging agents was dependent on whether cell lines were established at diagnosis or at relapse.

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