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PRECLINICAL STUDIES

Methionine gamma lyase from *Clostridium sporogenes* increases the anticancer effect of doxorubicin in A549 cells and human cancer xenografts

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Summary
The anti-cancer efficacy of methionine γ-lyase (MGL) from *Clostridium sporogenes* (*C. sporogenes*) is described. MGL was active against cancer models in vitro and in vivo. The calculated EC50 values for MGL were 4.4 U/ml for A549, 7.5 U/ml for SK-BR3, 2.4 U/ml for SKOV3, and 0.4 U/ml for MCF7 cells. The combination of doxorubicin (DOX) and MGL was more effective for A549 human lung cancer growth inhibition than either agent alone in vitro and in vivo. MGL reduced the EC50 of doxorubicin from 35.9 μg/mL to 0.01–0.265 μg/mL. The growth inhibitory effect of DOX + MGL on A549 xenografts in vivo was reflective of the results obtained in vitro. The inhibition rate of tumor growth in the combined arm was 57%, significantly higher than that in the doxorubicin ($p = 0.033$)-alone arm.

Keywords Methioninase · Methionine gamma lyase · Doxorubicin · Cisplatin · Combined treatment

Introduction

Methionine γ-lyase (MGL) [EC 4.4.1.11] is a pyridoxal 5′-phosphate-dependent enzyme, which catalyzes γ-elimination of methionine with the production of methyl mercaptan, α-ketobutyric acid, and ammonia. MGL is widely distributed in bacteria and has been isolated from *Pseudomonas putida*, eukaryotic protozoa *Trichomonas vaginalis* and *Entamoeba histolytica*, the fungus *Aspergillus flavipes* and several other microorganisms [1]. The enzyme is promising as an antitumor agent because methionine is required for the growth of malignant cells [2]. MGLs from diverse microorganisms exhibit significant reductions in methionine in vivo and efficacy against a wide spectrum of transplantable animal and human solid tumors [3].

The first MGL was isolated from *Clostridium sporogenes* by Kreis and Hession [4]. This MGL inhibited the growth of P815 cells and the Walker carcinosarcoma 256 in vivo. Compared with a methionine-free diet, this enzyme had greater growth-inhibiting activity and negligible toxicity when evaluated by the weight loss of the host. Semipurified MGL reduced plasma methionine to below 8% of the control [4].

MGL from *P. putida* is the most extensively studied MGL [3]. *P. putida* MGL inhibited the growth of the murine Lewis lung carcinoma in mice [5], human colon cancer xenografts [6] and glioblastoma [7] in many recent studies and in patient-derived xenografts (PDXs) [8–10]. The antitumor activity of the enzyme from *A. flavipes* with respect to several human tumors was observed in vivo [11]. MGL enhanced the efficacy of doxorubicin (DOX) [12], 5-fluorouracil (5-FU) [5], cisplatin (CDDP) [6], and nitrosoureas [7, 13, 14]. MGL from *P. putida* was effectively combined with cisplatin, 5-fluorouracil (5-FU), and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in mouse models of colon cancer, lung cancer, and brain cancer.

Recently, the *MGL* gene from *Clostridium sporogenes* was cloned, and the recombinant enzyme was purified and

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characterized [15]. Previous studies have evaluated the cytotoxic effect of MGL; PC-3 prostate cancer and K562 human chronic erythroblastic leukemia cell cultures were sensitive to MGL from *C. sporogenes*, with IC50 values of 0.1–0.4 and 0.4–1.3 U/ml, respectively [15]. The present study investigated whether MGL from *C. sporogenes* may enhance the cytotoxicity of anticancer agents with a different mechanism of action against human cancer cells A549 i*n vitro*. In this study, we also extended our observations to an in vivo model using xenografts of lung cancer A549, breast cancer MCF7 and SKBR3 cells.

Materials and methods

Bacterial strains and culture conditions

The *Escherichia coli* strain BL21 (DE3) F- *ompT hsdS* B *gal dcm* (DE3) (Novagen) was used to express the *C. sporogenes* MGL gene. The *E. coli* strain K12 AB2463—a *recA*-derivative of *E. coli* K12 with the F-, *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*, *recA13* genotype—was used to produce and store the plasmid.

Bacteria were grown in L-broth and on agarized medium at 37 °C. The transformed strains were cultured on media supplemented with kanamycin at a final concentration of 40 μg/ml. The optical density (OD) of the bacterial suspension was measured at 590 nm using a KFK-2MP photocolorimeter. To test the ability of bacterial strains to synthesize MGL, ZYP-5052 medium was used [16].

The *E. coli* MDG1216 strain was obtained by the transformation of *E. coli* BL21 (DE3) cells with plasmid pET-mgl-Sporog [17]. Plasmid expression was carried out in *E. coli* AB2463 cells. After transformation, the obtained *E. coli* MDG1216 clones were tested for the ability to biosynthesize MGL and were stored in a physiological solution with 40% glycerol in aliquots of 30 μl at −70 °C. The working cell bank was used to prepare the inoculate within the year.

MGL biosynthesis

Inoculate was prepared in medium containing tryptone (11.3 g/l), yeast extract (5.6 g/l) and glycerol (5.3 g/l) by growing the *E. coli* MDG1216 strain in flasks (150 ml) at 37 °C and 200 rpm up to OD = 0.5. The biosynthesis of the pharmaceutical substance was carried out by growing the *E. coli* MDG1216 strain in an ANCUM-2 M fermenter (working volume: 10 l; Institute of Biological Instrumentation of RAS, Pushchino) in Rich medium with extra $(NH_4)_2SO_4$ (23 g/l) and NaOH (1.5 g/l). The total fermentation time was 12.5 h. Thus, on average, 0.45–0.52 kg of wet biomass with an MGL content of up to 45% of the total protein was obtained.

Purification

The cells (200 g) (stored at −70 °C) were thawed and suspended in 10 mM potassium phosphate buffer with 0.001 M Na_4EDTA, 0.01% DTT and 0.01 mM pyridoxalphosphate (pH 7.2; buffer 1) at a ratio of 1:5. The cell biomass was destructed using an APV-1000 disintegrator with the addition of a PMSF solution in dioxane up to a final concentration of 1 mM. The cell lysate was centrifuged, and the supernatant was subjected to heat treatment with the occurrence of 20% (vol.) of ethanol. The admixtures were then precipitated by PEG 6.000.

The obtained supernatant was loaded on a Q-Sepharose column (300 mL) equilibrated with buffer 1. The main peak was eluted from the column by 0.3 M KCl in the same buffer. The collected peak fractions were ultrafiltered (a VivaFlow set) using a membrane module with the cut-off threshold of 50 kDa. The KCl content in the retentate was increased up to 1 M, and the obtained solution was added to a column of Phenyl-SepharoseFF (25 mL) equilibrated with buffer 1 containing 1 M KCl. The target enzyme did not bind to the sorbent under the above conditions and occurred in the breakthrough. The methioninase-containing fraction was concentrated on a set of Amicon filters using the UM-50 membrane. The content of KCl in the retentate was enhanced up to 1.5 M, and the solution was loaded on a column of Phenyl-Sepharose FF (50 mL) equilibrated with buffer 1 containing 1.5 M KCl. Methioninase was eluted from the column by decreasing the salt concentration up to 0.75 M in the buffer.

The MGL solution was desalinated and transferred to a 20-mM sodium phosphate buffer containing 0.15 M sodium chloride and 0.01 mM pyridoxalphosphate, pH 7.2, by ultradiafiltration using the VivaFlow set and a membrane with a 50-kDa cut-off. Tregalose was added to the obtained protein solution at a ratio of 1.5:1 (vol./vol.). The final product was subjected to sterilizing filtration (Fig. 1). The sterile solution was bottled in dark-glass vials (2000 ± 200 MU per vial) and freeze dried (ALPHA 1–4 LD). The ready-made vials were capped, labeled and stored at 2–8 °C.

Enzymatic assay

The reaction mixture consisted of 304 μl of working buffer (100 mM potassium phosphate buffer (pH 8.0), containing 0.01 mM pyrydoxalphosphate and 0.01% dithiothreitol) and 76 μl of 100 mM L-methionine. The reaction was initiated by the addition of 20 μl of purified enzyme (2 mg/ml). The reaction mixture was incubated at 37 °C for 1 min, and the reaction was stopped by adding 100 μl of 1.5 M trichloroacetic acid. The reaction mixture was centrifuged at 10000 g for 5 min at 4 °C to remove the precipitates. The ammonia released in the supernatant was determined using a colorimetric technique by adding 50 μl of Nessler's reagent into the sample containing

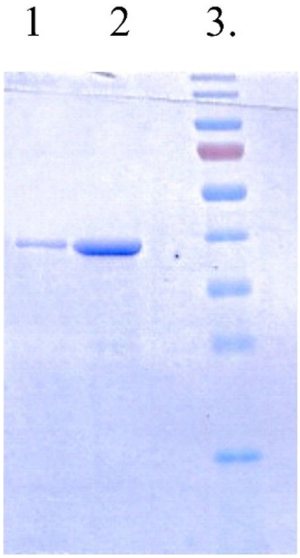

Fig. 1 SDS-PAGE for the homogeneity and purity determination of methioninase. (1) 0.2 μg of MGL. (2) 4 μg of MGL. (3) Protein markers (Fermentas, #SM0671)

50 μl of supernatant and 1150 μl of distilled water. The contents in the sample were vortexed and incubated at room temperature for 12 min, and the OD was measured at 450 nm. The ammonia produced in the reaction was determined based on the standard curve obtained with ammonium sulfate. One unit (U) of methioninase activity is defined as the amount of the enzyme that liberates one μmol of ammonia per min at 37 °C. By this isolation and purification procedure, an enzyme with a specific activity of 25.8–27.7 U/mg was obtained.

Cell lines and cell viability assay

A549 cells were purchased from the ATCC (USA) and were cultured in RPMI 1640 medium (PanEco, Russia) supplemented with 10% fetal bovine serum (HyClone, USA), 25 mM HEPES, 24 mM sodium bicarbonate, glutamine, 100 mg/mL streptomycin, and 100 U/mL penicillin (all were purchased from PanEco, Russia). Cisplatin (CDDP) and doxorubicin (DOX) were purchased from Ebewe Pharma Ges.m.b.H.Nfg.KG (Austria) and Veropharm (Russia), respectively. The flasks and plates were purchased from Nunc. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and pyridoxal-5-phosphate (PLP) were purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS), Trypan blue and dimethylsulfoxide (DMSO) were purchased from PanEco, Russia.

A549 cells were grown in RPMI 1640 medium with supplements in flasks at 37 °C and 5% CO_2. After 80% confluency, the cells in the exponential growth phase were trypsinized, suspended in fresh medium supplemented with 5×10^{-5} M PLP and seeded in 96-well plates at a concentration of 4×10^5 cells/mL. Cells were counted after treatment with 0.4% Trypan blue solution. After 24 h, different concentrations of the tested drugs dissolved in PBS were added to the wells. Cells were exposed to different concentrations of CDDP, DOX, MGL, alone and in combination, for 72 h. In control wells with untreated cells, only PBS was added. The cell viability was estimated by the MTT assay as described previously [18]. The absorbance of DMSO with dissolved MTT-formazan crystals was measured at 540 nm (Multiscan, Finland). The results were expressed as the drug concentration that inhibited 50% cell growth. The EC50 values were calculated from the concentration-effect relationships using Combenefit software.

Animal experiments

Athymic mice (Balb/c nude, female; 18–20 g; 6–8 weeks old) were purchased from N.N. Blokhin Cancer Research Center and were raised in cages maintained at a temperature of 22 ± 2 °C and 65 ± 5% humidity in a controlled animal facility with ad libitum access to water. All animal experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care, as described in the European Economic Community (EEC) guidelines (EEC Directive of 1986; 86/609/EEC), and with approval from the Ethics Committee for Animal Experimentation of N.N. Blokhin Cancer Research Center. A549, MCF7 or SKBR3 cells (2×10^6 cells) were subcutaneously injected into the flanks of the mice for tumor formation. When established tumors of ~100 mm^3 ± 10% in volume were detected, the mice were randomly assigned to different treatment arms as follows: i) 0.2 ml of saline alone, i.p., daily; ii) MGL at 1000 U/kg daily × 10 i.p., as determined by a preliminary experiment (data not published); iii) DOX 2 mg/kg every other day × 5 i.p.; iv) CDDP 1.5 mg/kg every other day × 5 i.p., v) combination of (ii and iii), and vi) combination of (ii and iv). The tumor volume was assessed three times a week using a caliper, measuring three perpendicular diameters, and the body weights were measured every 4 days. The tumor inhibitory ratio was calculated using the following formula: TGI (tumor growth inhibition, %) = [(C–T)/C] × 100, where C is the mean tumor size of the solvent control group and T is the mean tumor size of the treated group.

Analysis of combined drug effects

The drug combination effect was analyzed by the method of Chou and Talalay [19] with calculation of the combination index (CI). The CI indicates antagonism (CI > 1), additivity (CI = 1), or synergism (CI < 1). Additionally, we calculate the modified coefficient of drug interaction (CDI) as described previously [20] where CDI < 1, =1 or > 1 indicates that the drugs are synergistic, additive or antagonistic, respectively. CDI <0.7 indicates that the drug is significantly synergistic.

Finally, average-CDI was used to evaluate the total drug combination effect.

Statistical analysis

Experiments in vitro were repeated until three replicates yielded coefficients $R > 0.9$ for all three median effect lines. The calculations of the CI and average-CDI values were made using Microsoft Office Excel 2010, and EC50 values were calculated using Combenefit software. The Loewe and Bliss models were used for synergy/antagonism drug combination effect analysis.

SPSS 21 software was used for statistical analysis for in vivo experiments. One-way ANOVA was used to compare the efficacy of monotherapy and combined therapy. Post-hoc Dunnett's test was performed to assess differences between the individual groups against one control. Dunnett's T3 test was used to assess differences between the individual groups. A P value <0.05 was considered statistically significant.

Results

EC50 of MGL against A549, SK-BR3, SCOV3 and MCF7 human cancer cell lines

The calculated EC50 values for MGL are 4.4 U/ml for A549, 7.5 U/ml for SK-BR3, 2.4 U/ml for SKOV3, and 0.4 U/ml for MCF7.

MGL synergistically enhances the cytotoxicity of CDDP in A549 cells

Three different mixtures, namely, CDDP+MGL (5 U), CDDP+MGL (2.5 U), and CDDP+MGL (1.25 U), were used to analyze the mutual inhibitory effect of the drug combination compared with MGL and CDDP alone. The concentration of CDDP ranged from 0 to 50 μg/mL, whereas the activity of the enzyme was constant (5 U or 2.5 U or 1.25 U). As shown in Table 1, the CDDP+MGL (5 U) and CDDP+MGL (2.5 U) combinations demonstrated higher cytotoxicity than CDDP alone at low concentrations (0–0.5 μg/mL).

The CI and average CDI values are shown in Table 1. The efficacy of CDDP+MGL (2.5 U) and CDDP+MGL (1.25 U) combinations on A549 cells were synergistic (average CDI: −0.74 and 0.78, respectively). CDDP+MGL (5 U) was additive (average CDI: –0.95). CDDP+MGL (2.5 U) combinations were more synergistic with a lower concentration of CDDP: EC_{50} of CDDP in CDDP+MGL (2.5 U) = 0.002 μg/ml vs 0.01 μg/ml for CDDP+MGL (1.25 U), 0.03 μg/ml CDDP+MGL (5 U), 1.67 μg/ml (CDDP), 4.39 μg/ml (MGL). Key results of this analysis are presented in Fig. 2.

MGL synergistically enhances the cytotoxicity of DOX in A549 cells

To investigate the effects of MGL and DOX on the proliferation of A549 cells, three different mixtures, DOX + MGL (5 U), DOX + MGL (2.5 U), and DOX + MGL (1.25 U), were used. In each mixture, the concentration of DOX ranged from 0 to 100 μg/mL, whereas the activity of the enzyme was constant (5 U or 2.5 U or 1.25 U). As shown in Table 2, all combinations of DOX + MGL demonstrated higher cytotoxicity than DOX alone at 0- to 10-μg/mL concentrations.

The effect of DOX + MGL (1.25 U) can be interpreted as synergistic compared with DOX alone: CI: 0.74; average-CDI: −0.85. The effect of DOX + MGL 2.5 U and DOX + MGL 5 U was defined as additive (Fig. 3).

MGL induces an additive cytotoxic effect in SK-BR3 and SCOV-3 cell lines when combined with CDDP and DOX

CDDP or DOX + MGL combinations were studied in SK-BR-3 and SCOV-3 cell lines. It was found that the DOX + MGL 5 or 2.5 U and CDDP + MGL 5 or 2.5 U combinations had a low synergistic effect against SK-BR3: average-CDI values of DOX + MGL (5 U) = 0.81; DOX + MGL (2.5 U) =0.79; CDDP + MGL (5 U) = 0.71; CDDP + MGL (2.5 U) = 0.84. For SK-BR3, CDDP + MGL (1.25 U) and DOX + MGL

Table 1 Combination effect of CDDP and MGL in A549 cells

Tested drugs	EC_{50}*		CI		Average-CDI	
	m ± M	p				
MGL	4.39 ± 0.07 E	–	–	–	–	–
CDDP	1.67 ± 0.094 μg/mL	–	–	–	–	–
CDDP+MGL 5 U	0.03 ± 0.022 μg/mL	0.0005	1.18	Additive or antagonistic	0.95	Additive
CDDP+MGL 2.5 U	0.002 ± 0.014 μg/mL	0.0004	0.68	Synergistic	0.74	Synergistic
CDDP+MGL 1.25 U	0.01 ± 0.007 μg/mL	0.0004	0.69	Synergistic	0.78	Synergistic

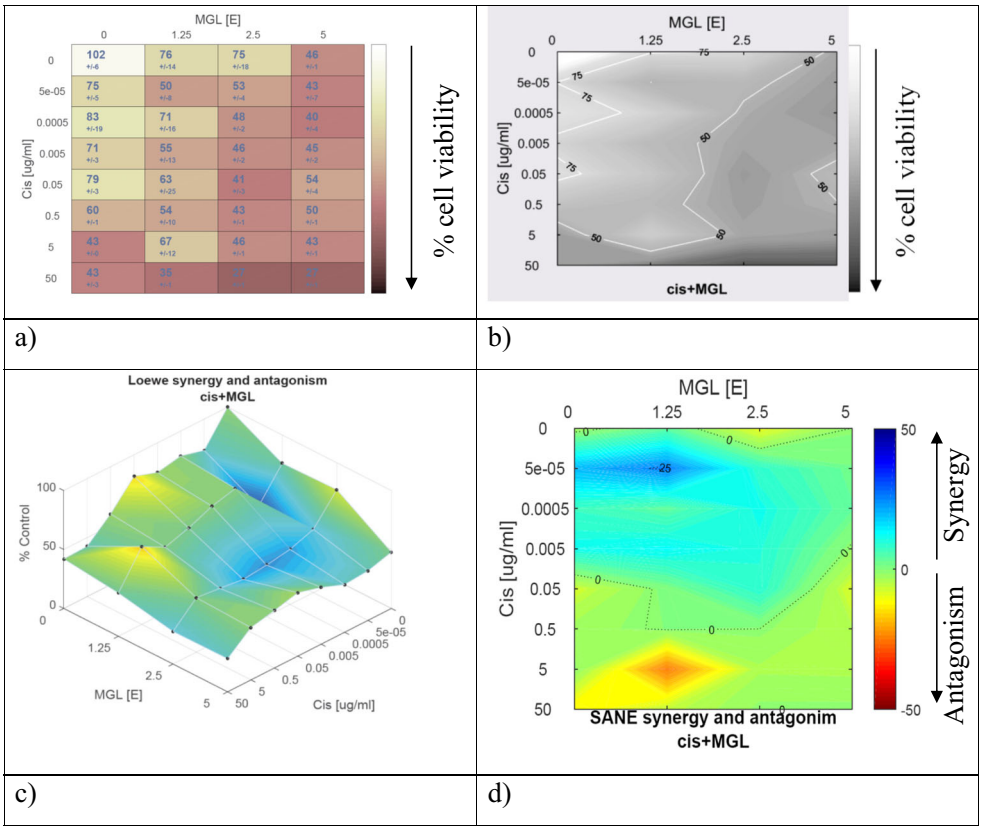

Fig. 2 Efficacy of CDDP and MGL, as determined by % A549 cell viability. **a**) Cell survival after co-incubation with different concentrations of CDDP and MGL. **b**) Contour Sane synergy and antagonism model. **c**) Effect of the concentration of components of the Lowe-mapped surface model. **d**) Contour Loewe synergy and antagonism model. $p < 0.0005$

(1.25 U) showed an additive effect: the average-CDI values were 1.02 and 1.16, respectively (Table 3).

The effect of the combined treatment with the tested cytotoxic agents at 1.25 U MGL and 5 U MGL in SCOV3 cells was defined as antagonistic: the average-CDI values varied from 1.11 to 1.32. The effect of DOX + MGL (2.5 U) and CDDP + MGL (2.5 U) tested in SCOV3 cells was additive: the average-CDI values were equal to 1.08 and 1.09 (Table 4).

MGL inhibits the growth of SK-BR3 xenografts similar to DOX and CDDP

Mice were transplanted with SK-BR3 cells to engineer subcutaneous xenografts. The initial tumor volume in each group was ~110 $mm^3 \pm 10\%$ (range from 98.3 to 120.9 mm^3, day 1, treatment onset). At the end of the treatment (day 10), the average volume of the tumor reached 1172.3 ± 742.1 mm^3 in the control (vehicle) arm, 584.5 ± 215.1 mm^3 in the MGL arm (TGI 50%), 401.3 ± 179.7 mm^3 in the DOX arm (TGI 66%), 560.6 ± 162.1 in the CDDP arm (TGI 52%) and 561.7 ± 247.0 mm^3 and 529.3 ± 144.9 in the combined DOX + MGL and CDDP + MGL arms (TGI 52 and 55%, respectively). No statistically significant differences were observed between the treatment arms (Fig. 4).

MGL inhibits the growth of MCF7 xenografts similar to CDDP or DOX

On day 1 (onset of treatment), the initial xenograft volume reached ~100 mm^3, and the mice were randomly assigned to 6 arms. At the end of treatment (day 10), the volume of the tumor mass was 506.6 ± 269.4 mm^3 in the control (vehicle) arm, 134.5 ± 75.4 mm^3 in the MGL arm (TGI 74%, $p =$ 0,037), 114.5 ± 55.3 mm^3 in the DOX arm (TGI 77%, p =

Table 2 Combination effect of DOX and MGL in A549 cells

Tested drugs	EC50		CI		Average-CDI	
	m ± M, μg/mL	p				
DOX	35,9 ± 2.407	–	–	–	–	–
DOX + MGL 5 U	0.01 ± 0.013	0.002	1.011	Additive	0.97	Additive
DOX + MGL 2.5 U	0.02 ± 0.022	0.001	1.002	Additive	0.89	Additive or synergistic
DOX + MGL 1.25 U	0.265 ± 0.141	0.007	0.740	Synergistic	0.85	Synergistic

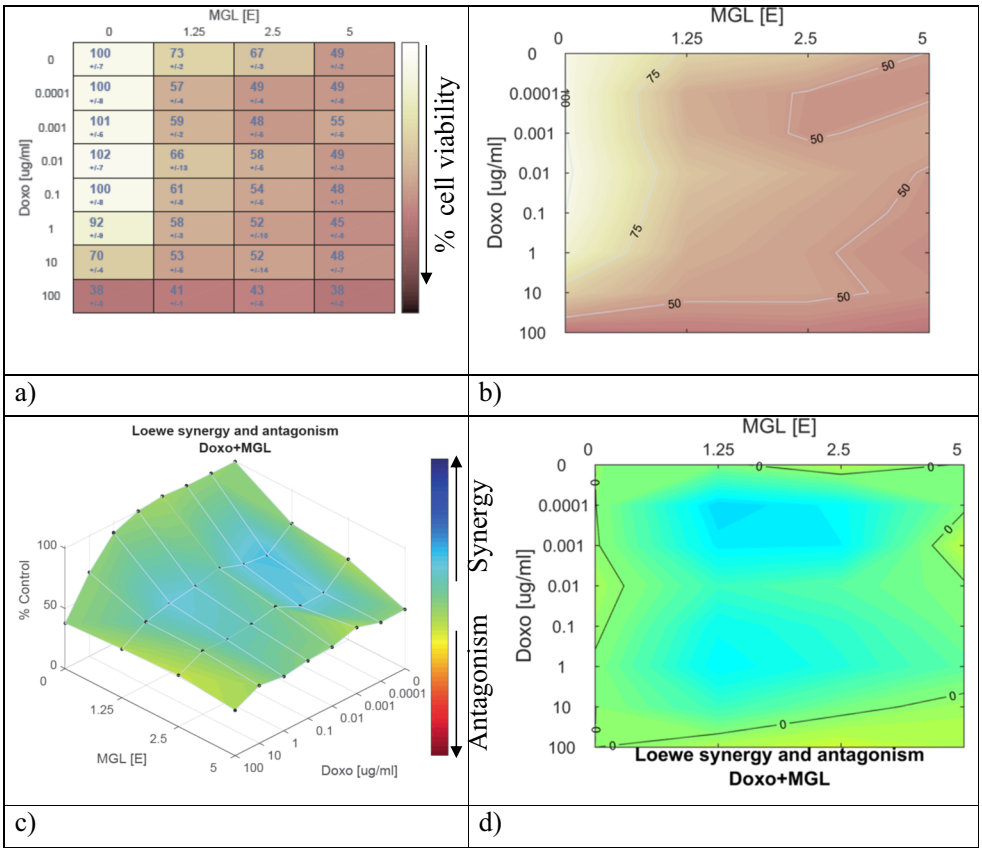

Fig. 3 Efficacy of DOX and MGL as determined by % A549 cell viability. **a**) Cell survival after co-incubation with different concentrations of DOX and MGL. **b**) Contour Sane synergy and antagonism model. **c**) Effect of the concentration of components of the Lowe-mapped surface model. **d**) Contour Loewe synergy and antagonism model. $p < 0.007$

0,029), 162.6 ± 84.4 in the CDDP arm (TGI 68%, $p = 0,046$), and 125.7 ± 80.4 mm^3 and 219.4 ± 68.0 mm^3 in the combined DOX + MGL and CDDP + MGL arms (TGI 75%, $p = 0,033$ and 57%, p = 0,118, respectively). No statistically significant differences between the treatment arms were observed.

MGL in combination with DOX significantly decreased the tumor growth in A549 xenografts

On day 1 (onset of treatment), the initial xenograft volume was ~150 mm^3, and the mice were randomly assigned to 4 arms. Four days after the end of treatment (day 14), the volume of the tumor mass reached 1996.0 ± 200.0 mm^3 in the control (vehicle) arm, 1221.6 ± 177.3 mm^3 in the MGL arm (TGI 39%, $p = 0,045$, Dunnet T3), 1598.7 ± 202.5 mm^3 in the DOX arm (TGI 20%, p = 0,660), and 858.9 ± 123.0 mm^3 in the combined DOX + MGL arm (TGI 57%, $p < 0,001$). Co-treatment with MGL and DOX significantly decreased the substantial tumor volume compared with single treatment with MGL or DOX in A549 tumors. The inhibition rate of tumor growth in the combined arm was 57%, which was significantly greater than that in the DOX arm (p = 0,033), but not in the MGL-alone arm (p = 0,657). None of the mice exhibited signs of physical discomfort during the treatment and follow-up periods. These results suggest that combination of MGL and DOX may significantly inhibit tumor growth in vivo.

Table 3 Cytotoxicity of DOX or CDDP + MGL against SK-BR3

Substance	DOX + MGL			CDDP + MGL		
	EC50		Average-CDI	EC50		Average-CDI
	m ± M	p		m ± M	p	
MGL alone	2.4 ± 0.07 U	–	–	–	–	–
Cytotoxic agent alone	2 ± 0.448 μg/mL	–	–	0.59 ± 0.053 μg/mL	–	–
Cytotoxic agent + MGL 5 U	0.06 ± 0.047 μg/mL	0.023	0.81	0.41 ± 0.081 μg/mL	0.16	0.71
Cytotoxic agent + MGL 2.5 U	0.07 ± 0.012 μg/mL	0.023	0.79	1.87 ± 0.108 μg/mL	0.002	0.84
Cytotoxic agent + MGL 1.25 U	0.29 ± 0.058 μg/mL	0.032	1.02	2.53 ± 0.231 μg/mL	0.004	1.16

Table 4 Cytotoxicity of DOX or CDDP + MGL against SCOV3

Substance	DOX + MGL			CDDP + MGL		
	EC50		Average-CDI	EC50		Average-CDI
	m ± M	p		m ± M	p	
MGL alone	4.4 ± 0.12 U	–	–	–	–	–
Cytotoxic agent alone	0.07 ± 0.03 μg/mL	–	–	1.5 ± 0.057 μg/mL	–	–
Cytotoxic agent + MGL 5 U	0.07 ± 0.034 μg/mL	1	1.27	1.7 ± 0.076 μg/mL	0.126	1.32
Cytotoxic agent + MGL 2.5 U	0.31 ± 0.054 μg/mL	0.03	1.08	0.6 ± 0.01 μg/mL	0.0006	1.09
Cytotoxic agent + MGL 1.25	1.0 ± 0.207 μg/mL	0.021	1.11	0.5 ± 0.007 μg/mL	0.0004	1.28

Discussion

Several amino acid-cleaving enzymes have been studied in recent years, reflecting amino acid dependence as a general metabolic defect in cancer, precluding the cells from growing in media, in which certain amino acids are depleted. These enzymes include novel asparaginases [21–24], lysine alpha-oxidase [25], phenylalanine ammonia-lyase [26] and MGL. Methionine dependence, the essential methionine requirement for cell growth, occurs frequently in many types of human cancer cell lines and animal models of cancer [27–30].

The reduction of plasma methionine below 5 mM arrests human xenograft growth in athymic mice, suggesting that the plasma methionine level < 5 mM may be necessary to achieve a significant anticancer effect in an animal model [13]. We reported previously that the $T_{1/2}$ of MGL from *C. sporogenes* after intravenous injection into mice was 1.31 ± 0.03 h. Mouse plasma methionine decreased to an undetectable level 10 min after 1000 U/kg injection. This effect persisted for 6 h, indicating the feasibility of long-term reduction of methionine to a negligible level (<5 μM) [31].

Combination therapy can improve the treatment efficiency. Several researchers have explored the combined anticancer effects of MGL in the in vitro and in vivo studies. Recombinant MGL from *P. putida* (rMETase) in combination with different chemotherapeutic agents such as CDDP, 5-fluorouracil (5-FU), 1–3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and vincristine has shown efficacy and synergy, respectively, in mouse models of colon cancer, lung cancer, and brain cancer [5–7]. CDDP in combination with rMETase resulted in the tumor regression of Colo 205 cells and growth arrest of SW 620 cells. SW 620 cells were resistant to CDDP alone and only partially sensitive to rMETase alone. However, when SW 620 cells were treated with rMETase and CDDP, tumor growth was arrested. Hence, combining MGL with CDDP synergistically inhibits tumor growth in both CDDP-sensitive and CDDP-resistant tumor models [6]. The potentiating effect of MGL combined with 5-fluorouracil was discovered in the murine model of Lewis lung carcinoma. Combination treatment of the Lewis lung carcinoma with a fixed rMETase dose and increasing doses of 5-fluorouracil resulted in a dose-dependent increase in survival as well as tumor growth inhibition [5]. MGL combinations with vincristine, temozolomide or carmustine were effective against human neuroblastoma and glioblastoma xenografts.

In our study, we found that the combination of DOX and MGL was more effective in A549 tumor growth inhibition than either agent alone. The growth inhibitory effect of

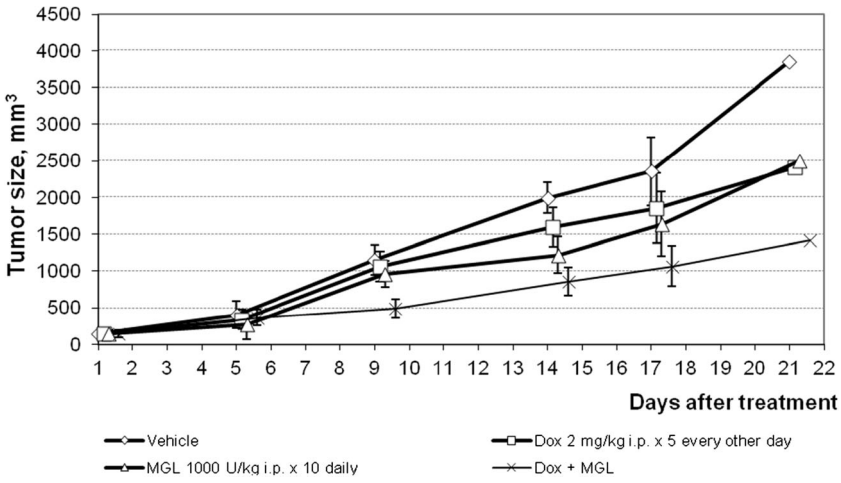

Fig. 4 Efficacy of the DOX + MGL combination in the A549 xenograft model. A549 cells (7×10^6 cells) were injected subcutaneously into both flanks of nude mice (5 mice/group). After tumors were established, the mice were treated with 2 mg/kg of DOX i.p. every other day × 5, and 1000 U/kg MGL i.p. daily × 10 as single agents or in combination. The TGI in the combined arm was 57%, significantly greater than those in the DOX arm ($p = 0{,}033$)

DOX + MGL on A549 xenografts in vivo was reflective of the results obtained in vitro, whereas statistically significant regression of xenografts was not achieved using single treatment with either MGL or DOX. Future studies are required to investigate the cellular mechanisms of potential cytotoxic agents to increase MGL efficacy and optimize the treatment schedule to achieve complete tumor regression.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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