Methotrexate and 5-aminoimidazole-4-carboxamide riboside exert synergistic anticancer action against human breast cancer and hepatocellular carcinoma

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Aim: To investigate the influences of methotrexate (MTX) on the anticancer actions and pharmacokinetics of 5-aminoimidazole-4-carboxamide riboside (AICA riboside) in human breast cancer and hepatocellular carcinoma.

Methods: Human breast cancer cell line MCF-7 and human hepatocellular carcinoma cell line HepG2 were examined. The cell proliferation was assessed using a sulforhodamine B assay. Western blotting and radioactivity assays were used to analyze the phosphorylation of AMPK. The DNA synthesis was analyzed with BrdU incorporation. Nude mice bearing MCF-7 cell xenografts were used for in vivo study. MTX (50 mg/kg, ip, per week) and AICA riboside (200 mg/kg, ip, every other day) were administered the animals for 2 weeks. The concentrations of AICA riboside and its active metabolite AICA ribotide in the plasma and tumors were measured with HPLC.

Results: Synergistic cytotoxicity in vitro was observed with MTX (0.1, 0.5, and 1 μmol/L) combined with AICA riboside (0.25–1 mmol/L) in MCF-7 cells, and with MTX (0.5 and 1 μmol/L) combined with AICA riboside (0.5 and 1 mmol/L) in HepG2 cells. MTX (1 μmol/L) significantly enhanced the AICA riboside-induced AMPK activation and BrdU incorporation in both MCF-7 and HepG2 cells. Co-treatment with MTX and AICA riboside exerted more potent inhibition on the tumor growth in nude mice than either drug alone. After injection of AICA riboside (200 mg/kg, iv) in nude mice bearing MCF-7 xenografts, MTX (50 mg/kg, iv) significantly increased the concentrations of AICA riboside and its active metabolite AICA ribotide in tumors.

Conclusion: MTX and AICA riboside exert synergistic anticancer action against MCF-7 and HepG2 cells in vitro and in vivo. MTX increases the concentration of AICA riboside and its active metabolite AICA ribotide in tumors in vivo.

Keywords: methotrexate; 5-aminoimidazole-4-carboxamide riboside; antitumor agents; human breast cancer; human hepatocellular carcinoma; drug synergism; pharmacokinetics; AMP-activated protein kinase; DNA synthesis

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Introduction

5-Aminoisimidazole-4-carboxamide riboside (AICA riboside), a cell permeable nucleoside, was the first compound reported to activate AMP-activated protein kinase (AMPK)⁴. AICA riboside is taken up into cells by adenosine transporters and is then converted by adenosine kinase to the monophosphorylated derivative AICA ribotide, which mimics the effect of AMP in activation of AMPK², ³. AMPK is a sensor of cellular energy status, and is activated under conditions of an elevated ratio of AMP to ATP. Activated AMPK inhibits tumor cell proliferation through activating elongation factor-2 and inhibiting key metabolic enzymes such as acetyl-CoA carboxylase 1, glycerol phosphate acyl transferase and HMG-CoA reductase⁴, ⁵. In addition, AMPK is associated with liver kinase B1 (LKB1), mammalian target of rapamycin (mTOR), p53 and other tumor suppressors⁶–⁸. AICA riboside has an inhibitory effect on the proliferation of a variety of human cancer cell lines such as B-cell chronic lymphocytic leukemia cells⁹, chronic myelogenous leukemia cells¹⁰, gastric cancer cells¹¹, cervical cancer cells¹², prostate cancer cells¹³ and hepatocellular carcinoma cells¹⁴. AICA riboside also has
potent antitumor activity in nude mice bearing MDA-MB-231 and C6 cell xenografts\(^{[15, 16]}\). The first patient was enrolled in a phase I/II trial of AICA riboside treatment for B-cell chronic lymphocytic leukemia in 2008\(^{[17]}\).

As a result of the rapid biotransformation of AICA ribotide to 10-formyl AICA ribotide, which participates in the de novo purine synthesis pathway, the most obvious disadvantage of AICA riboside is its poor pharmacokinetic profile, with a short half-life \((t_{1/2})\) of 1.4 h in healthy volunteers\(^{[18]}\). This shortcoming may be overcome by coadministration of another drug that inhibits de novo purine synthesis. Methotrexate (MTX), a classical folic acid antagonist, has been one of the most widely used anticancer agents since 1948\(^{[19]}\). MTX polyglutamates inhibit de novo purine biosynthesis through their action on the key enzymes of de novo purine biosynthesis, including AICA ribotide transformylase, which converts AICA ribotide to 10-formyl AICA ribotide\(^{[20]}\).

Combined chemotherapy is a common practice in the treatment of cancer and can achieve better therapeutic effects than single drug treatment and can also reduce side effects and drug resistance\(^{[21, 22]}\). Beckers et al have reported that MTX enhances the antianabolic and antiproliferative effects of AICA riboside\(^{[23]}\). The present study was aimed at investigating the combined effects of MTX and AICA riboside on tumor cell proliferation in vitro and in vivo and the influence of MTX on the pharmacokinetics of AICA riboside in nude mice bearing MCF-7 cell xenografts.

**Materials and methods**

**Reagents**

AICA riboside and AICA ribotide were obtained from Toronto Research Chemicals (Toronto, Canada). MTX and sulforhodamine B were purchased from Sigma-Aldrich (Milwaukee, WI, USA). RPMI-1640 medium was obtained from Macgene Biotech Co Ltd (Beijing, China), and fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Cell culture BrdU ELISA kit was obtained from CycLex Co Ltd (Nagano, Japan). Antibody against β-actin was obtained from Abmart (Shanghai, China). Antibody against phospho-Thr172 AMPK α and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). SAMS peptide was obtained from GenScript USA Inc (Piscataway, NJ, USA).

**Cell culture**

The human breast cancer cell line MCF-7 and human hepatocellular carcinoma cell line HepG2 were purchased from the Cell Bank of the Cancer Institute & Hospital, Chinese Academy of Medical Science (Beijing, China). MCF-7 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and HepG2 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37°C in a 5% CO\(_2\) incubator.

**In vitro cytotoxicity assay**

The sulforhodamine B (SRB) assay was performed according to the method developed by Vichai\(^{[24]}\). MTX and AICA riboside were dissolved in pH 7.4 PBS. MCF-7 and HepG2 cells were plated at a density of 1×10\(^4\) cells per well in 96-well plates. After incubation for 24 h, cells were pretreated with 0.1, 0.5, or 1 μmol/L MTX. Four hours later, cells were exposed to 0.125, 0.25, 0.5, and 1 mmol/L AICA riboside. At the time of MTX addition, cells in one plate were fixed in situ with cold 10% \((w/v)\) trichloroacetic acid (TCA), to represent a measurement of the cell population at the time of drug addition \((OD_{d0})\). After incubation for another 48 h, cells were fixed in situ with cold 10% TCA. The plates were washed with tap water, stained with 0.4% SRB \((w/v)\), dissolved in 1% acetic acid, and washed with 1% acetic acid. The protein-bound dye was subsequently dissolved in 10 mmol/L Tris. The absorbance at 540 nm was read on a colorimetric plate reader (Bio-Rad, USA).

Percentage of cell survival was calculated as followed:

\[
\text{Percentage of cell survival} = \frac{\text{mean } OD_{\text{sample}} - \text{mean } OD_{d0}}{\text{mean } OD_{\text{control}} - \text{mean } OD_{d0}} \times 100
\]

If \(OD_{\text{sample}}\) was below \(OD_{d0}\), cell killing had occurred, and the percentage of cells killed was expressed by the following equation:

\[
\text{Percentage of cells killed} = 100 - \frac{\text{mean } OD_{\text{sample}}}{\text{mean } OD_{d0}} \times 100
\]

**Drug interaction analysis**

The combined effect was analyzed using published methods\(^{[25, 26]}\) based on the principles described by Chou and Talalay\(^{[27]}\). The expected OD value for the combined effect of MTX and AICA riboside was calculated as follows:

\[
\text{Expected OD value for the combined effect} = \frac{\text{observed } OD_{\text{MTX}}}{\text{observed } OD_{d0}} \times \frac{\text{observed } OD_{\text{AICA riboside}}}{\text{OD}_{\text{control}}}
\]

The combination index (CI) was calculated as the ratio of (observed OD value)/(expected OD value)\(^{[28]}\). Synergism was defined as CI<1, whereas CI>1 indicated antagonism, and CI=1 meant addition. CI<0.7 indicated that the drugs were significantly synergistic.

**Immunoblot analysis**

MCF-7 and HepG2 cells were pretreated with 1 μmol/L MTX or vehicle (pH 7.4 PBS), and treated with AICA riboside 4 h later, and then after incubation for 3 h, cells were lysed in RIPA buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with 1 mmol/L protease inhibitor phenylmethylsulfonylfluoride (PMSF) and 1% phosphatase inhibitors mixture for 30 min on ice. The protein concentration was determined with a BCA protein assay kit (Beyotime Biotechnology, Haimen, China). Proteins were resolved by 10% SDS-PAGE and transferred to polyvinylidenedifluoride membranes. The membranes were
AMPK activity assay
Measurement of AMPK activity was carried out as described previously[28]. MCF-7 or HepG2 cells were treated as described for immunoblot analysis, and lysed in homogenization buffer (20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, and 1% Triton X-100) with 1 mmol/L protease inhibitor PMSF and 1% phosphatase inhibitors mixture for 30 min on ice. Fifty microliters of 25% polyethylene glycol 6000 (PEG 6000) was added to 450 μl of 0.1 M HEPES, 80 mmol/L NaCl, 8 mmol/L mannitol, 800 μmol/L EDTA, 2, 40 mmol/L dithiothreitol, and then centrifuged at 1110×g for 10 min. The resultant pellets were dissolved and assayed for AMPK activity with SAMS at 11 750×g of 25% PEG 6000 was added to 450 μl of 0.1 M HEPES, 80 mmol/L NaCl, 8 mmol/L mannitol, 800 μmol/L EDTA, 800 μmol/L dithiothreitol, and 0.01% Brij-35. After incubation for 15 min at 30 °C, the reaction mixture was spoted onto Whatman P81 paper. The papers were washed with 1% phosphoric acid (v/v) four times and 32P was counted onto Whatman P81 paper. The papers were washed with 32P and frozen at -80 °C. Tumor growth inhibition studies
MCF-7 cells were inoculated in female BALB/c nude mice as described above. When the tumor volume reached ~1000 mm³, the mice were randomly divided into groups of 24. One group was injected iv with 50 mg/kg MTX via the tail vein, and 4 h later, 200 mg/kg AICA riboside was administered iv. Another group was administered with the same dose of AICA riboside alone. Blood samples were obtained at 5, 10, 15 and 30 min, and 1, 2, 3, and 4 h after injection of AICA riboside, and then centrifuged at 1110×g for 10 min at 4 °C. The upper plasma was collected and stored at -80 °C before analysis. Mice were sacrificed immediately following blood collection, and tumor samples were removed from the mice and frozen at -80 °C. PBS (250 μL, pH 2.5) was added per 250 mg tumor tissue and tumor tissue was homogenized. The concentrations of AICA riboside and its active metabolite AICA ribotide in plasma and tumors were assayed by an HPLC method developed by Cheng et al.[29]. The pharmacokinetic parameters of AICA riboside and its active metabolite AICA ribotide in nude mouse plasma and tumors were estimated by non-compartment analysis. $C_{\text{max}}$ was the observed maximum concentration. Mean residence time (MRT) was calculated as the ratio of the area under the first moment versus time curve (AUMC$_{0-\infty}$) to the area under the concentration versus time curve (AUC$_{0-\infty}$). $t_{1/2}$ was calculated as 0.693×MRT, and systemic clearance was obtained from the equation $\text{CL} = D/AUC_{0-\infty}$, where $D$ was the dose of AICA riboside. The apparent volume of distribution was calculated from the equation $V = D \times \text{AUMC}_{0-\infty}/\text{AUC}_{0-\infty}$. Statistical analysis
Data were expressed as mean±SD. Statistical analyses among groups were performed using one-way analysis of variance with Fisher’s least significant test, and statistical analyses between two groups were done using student’s t-test. Relative tumor volumes were compared using Mann-Whitney U test followed by Bonferroni’s multiple-comparison test. A value of
Results

Combined MTX and AICA riboside displayed more potent cytotoxicity against tumor cells than either drug alone

MCF-7 and HepG2 cells were pretreated with MTX, and 4 h later were exposed to AICA riboside for a further 48 h. Cell viability was evaluated using the SRB assay (Figure 1). The percentage of MCF-7 cell survival after treatment with 0.125, 0.25, 0.5, and 1 mmol/L AICA riboside was 66.64%±5.00%, 85.13%±5.99%, 65.74%±5.42%, and 17.02%±4.64%, respectively. When MCF-7 cells were pretreated with 1 μmol/L MTX (the percentage of cell survival after treatment with 1 μmol/L MTX was 27.74%±0.97%), the percentage of cell survival after treatment with 0.125, 0.25, 0.5, and 1 mmol/L AICA riboside was 8.46%±0.87%, -3.92%±0.29%, -16.48%±1.04%, and -21.41%±3.40% (P<0.001 versus the MTX or AICA riboside group), respectively. For HepG2 cells, the percentage of cell survival after treatment with 0.25, 0.5, and 1 mmol/L AICA riboside was 47.25%±4.55%, 49.85%±4.60%, and 47.20%±8.18%, respectively. When HepG2 cells were pretreated with 1 μmol/L MTX (the percentage of cell survival after treatment with 1 μmol/L MTX was 13.26%±0.90%), the percentage of cell survival after treatment with 0.25, 0.5, and 1 mmol/L AICA riboside was -1.80%±0.12%, -24.58%±3.89%, and -51.46%±6.28% (P<0.001 versus MTX or AICA riboside group), respectively. The combination of 0.1 or 0.5 μmol/L MTX with AICA riboside also exhibited more potent cytotoxicity than either drug alone. Percentage of cell survival <0 means that the treatment could kill tumor cells[24]. We showed that MTX or AICA riboside used alone mainly inhibited tumor cell proliferation, while combined treatment killed the tumor cells.

Synergistic cytotoxicity of combined MTX and AICA riboside

A CI<1 indicates a synergistic interaction between MTX and AICA riboside. All combinations displayed synergistic cytotoxicity against MCF-7 cells except that of 0.1 μmol/L MTX and 0.125 mmol/L AICA riboside (Figure 2). For HepG2 cells, the CI for 1 mmol/L AICA riboside and 0.5 or 1 μmol/L MTX was 0.50 and 0.44, respectively, indicating that these combinations exerted significant synergism. The results suggest that the combination of MTX and AICA riboside has a synergistic effect on the inhibition of proliferation of MCF-7 and HepG2 cell lines.

MTX enhanced the ability of AICA riboside to activate AMPK

MCF-7 or HepG2 cells were exposed to AICA riboside for 3 h, and then phosphorylation at Thr172 of the α catalytic subunit of AMPK was investigated by Western blot analysis.

Figure 1. Combined effects of MTX and AICA riboside on inhibition of MCF-7 (A) and HepG2 (B) cell proliferation in vitro. Data are presented as mean±SD (n=6). 'P<0.01 versus control group. "P<0.01 versus either MTX or AICA riboside treated group.

Figure 2. Synergistic effect of MTX and AICA riboside on inhibition of MCF-7 (A) and HepG2 (B) cell proliferation in vitro. CI<1 means synergism.
with phospho-AMPK-specific antiserum. As shown in Figure 3A, phosphorylation of AMPK increased with increasing AICA riboside concentration. In addition, when cells were pretreated with 1 μmol/L MTX for 4 h and then exposed to AICA riboside, the phosphorylated AMPK increased significantly compared with that of cells without MTX pretreatment. The results demonstrated that MTX pretreatment potently increased the ability of AICA riboside to phosphorylate AMPK in MCF-7 and HepG2 cells.

The activity of AMPK was determined by analyzing the level of incorporation of $^{32}$P into SAMS peptide, which is a specific substrate of AMPK. Treatment of MCF-7 or HepG2 cells with AICA riboside resulted in increased AMPK activity, and pretreatment with 1 μmol/L MTX enhanced the ability of AICA riboside to activate AMPK (Figure 3B). AMPK activity of MCF-7 cells after incubation with 0.125, 0.25, 0.5 and 1 mmol/L AICA riboside for 3 h was 51.63±5.59, 58.51±2.25, 73.68±0.82, and 80.64±11.74 cpm μg protein$^{-1}$ min$^{-1}$, respectively, and when cells were pretreated with 1 μmol/L MTX for 4 h, AMPK activity of MCF-7 cells after exposure to AICA riboside with the above concentrations was 66.43±14.16, 89.43±6.62, 99.10±0.98, and 87.75±0.61 cpm μg protein$^{-1}$ min$^{-1}$, respectively. Similarly, the AMPK activity of HepG2 cells when cotreated with AICA riboside and MTX was higher than that treated with AICA riboside alone.

**Combined effect of MTX and AICA riboside in blocking DNA synthesis**

It is known that MTX and its polyglutamates block de novo nucleotide synthesis[30]. AICA riboside has also been shown to inhibit DNA synthesis[15]. Incorporation of the thymidine analog BrdU was measured to investigate the effect of MTX and AICA riboside on blocking tumor cell DNA synthesis. Treatment of MCF-7 or HepG2 cells with AICA riboside caused a potent and dose-dependent reduction in BrdU incorporation, and combination of AICA riboside and 1 μmol/L MTX resulted in greater reduction of BrdU incorporation (Figure 4). The relative BrdU incorporation of MCF-7 cells after treatment with 0.125, 0.25, 0.5, and 1 mmol/L AICA riboside for 24 h was 96.60%±1.83%, 89.43%±8.06%, 57.69%±7.43%, and 32.43%±2.62%, respectively, and when cells were pretreated with 1 μmol/L MTX for 4 h, the relative BrdU incorporation after exposure to 0.125, 0.25, 0.5, and 1 mmol/L AICA riboside for 24 h was 56.93%±3.01%, 54.40%±4.50%, 40.19%±4.04%, and 2.96%±1.25%, respectively ($P<0.005$ versus either MTX or AICA riboside treated group). The relative BrdU incorporation in HepG2 cells after treatment with 0.25, 0.5, and 1 mmol/L AICA riboside for 24 h was 96.62%±8.09%, 60.31%±11.01%, and 26.21%±1.42%, respectively, and when cells were pretreated with 1 μmol/L MTX for 4 h, the relative BrdU incorporation after treatment with 0.25, 0.5, and 1 mmol/L AICA riboside for 24 h was 35.81%±2.71%, 28.93%±3.95%, and 19.46%±3.05%, respectively.

**Synergistic effect of MTX and AICA riboside on tumor growth in vivo**

The *in vivo* antitumor efficacy of combined MTX and AICA riboside was evaluated in female BALB/c nude mice bearing MCF-7 cell xenografts. Figure 5 shows the relative tumor volume ($\text{mean±SD}$) of different treatment groups over time. The combination of MTX and AICA riboside achieved a superior antitumor effect than either drug alone. MTX and AICA riboside alone inhibited tumor growth by 55.00%±0.79% and 42.16%±12.15% at d 14, respectively. The combination of MTX and AICA riboside alone inhibited tumor growth by 55.00%±0.79% and 42.16%±12.15% at d 14, respectively. Combination of MTX and AICA riboside showed more potent antitumor activity with 79.92%±34.61% inhibition of tumor growth.
MTX increased AICA riboside and its active metabolite in tumor tissue

As shown in Figure 6, MTX had no influence on concentration of AICA riboside and AICA ribotide in plasma, but increased concentration of both AICA riboside and AICA ribotide in tumors, especially for AICA ribotide. The $C_{\text{max}}$ of AICA riboside and its active metabolite AICA ribotide in tumors after intravenous administration of 200 mg/kg AICA riboside alone was 4.49±0.82 μg/mL and 8.33±0.49 μg/mL, respectively, while the $C_{\text{max}}$ of AICA riboside and AICA ribotide in tumors after iv injection of the same dose of AICA riboside at 4 h after iv administration of 50 mg/kg MTX was 8.56±1.06 μg/mL.

**Figure 4.** The combined effect of MTX and AICA riboside on inhibition of cancer cell DNA synthesis. Data are presented as mean±SD (n=3). $^cP<0.01$ versus control group. $^dP<0.05, ^eP<0.01$ versus either MTX or AICA riboside treated group.

**Figure 5.** Effect of MTX, AICA riboside and their combination on the growth of MCF-7 cell xenografts in BALB/c nude mice. Data are presented as mean±SD (n=8). $^bP<0.05, ^cP<0.01$ versus control group. $^fP<0.01$ versus either MTX or AICA riboside treated group.

**Figure 6.** Concentration-time curves of AICA riboside (A) and its active metabolite AICA ribotide (B) in plasma, and AICA riboside (C) and AICA ribotide (D) in tumor.
(P<0.01) and 19.80±1.32 μg/mL (P<0.001), respectively. Pharmacokinetic parameters are shown in Tables 1 and 2. The AUC_{0-t} of AICA riboside and AICA ribotide in tumors after iv administration of 200 mg/kg AICA riboside alone was 2.23±0.48 and 14.86±1.67 μg·h/mL, respectively. However, when the same dose of AICA riboside was administered iv at 4 h after 50 mg/kg MTX, the AUC_{0-t} of AICA riboside and AICA ribotide in tumors was 4.36±0.89 (P<0.05) and 38.95±3.03 μg·h/mL (P<0.001), respectively.

Table 1. Pharmacokinetic parameters of AICA riboside and its active metabolite AICA ribotide in plasma of nude mice bearing MCF-7 xenografts after iv administration of AICA riboside (200 mg/kg) or iv injection of the same dose of AICA riboside administered 4 h after iv MTX (50 mg/kg) (n=3).

| Parameter | AICA riboside | MTX and AICA riboside |
|-----------|---------------|-----------------------|
| AUC_{0-∞} (μg·h/mL) | 8.01±1.96 | 7.58±0.82 |
| MRT_{0-∞} (h) | 0.64±0.04 | 0.65±0.14 |
| C_{max} (μg/mL) | 1.53±0.18 | 1.51±0.16 |

Note: AUC is the area under the concentration versus time curve; MRT is mean residence time; t_{1/2} is half-life; CL is systemic clearance; V is the apparent volume of distribution; and C_{max} is the observed maximum concentration.

Table 2. Pharmacokinetics parameters of AICA riboside and its active metabolite AICA ribotide in tumor of nude mice bearing MCF-7 xenografts after iv administration of AICA riboside (200 mg/kg) or iv injection of the same dose of AICA riboside administered 4 h after iv MTX (50 mg/kg) (n=3).  ^{p<0.05,^{p<0.01 versus AICA riboside group.}

| Parameter | AICA riboside | MTX and AICA riboside |
|-----------|---------------|-----------------------|
| AUC_{0-∞} (μg·h/mL) | 18.9±0.42 | 1.58±0.16 |
| MRT_{0-∞} (h) | 1.2±0.04 | 1.04±0.20 |
| C_{max} (μg/mL) | 4.49±0.82 | 8.56±1.06 |

Note: AUC is the area under the concentration versus time curve; MRT is mean residence time; t_{1/2} was half-life; CL is systemic clearance; V is the apparent volume of distribution; and C_{max} is the observed maximum concentration.

Discussion

In most cases, combination chemotherapy is more effective than monotherapy\(^{16,32}\). Combination therapy can reduce side effects and prevent the emergence of resistance to anticancer drugs when drugs with different mechanisms of action are used in combination\(^{21,22}\). MTX is a classical folic acid antagonist\(^{19}\), whereas AICA riboside activates AMPK and subsequently inhibits energy-consuming processes, such as DNA synthesis, protein translation and lipogenesis\(^{15}\). The antitumor mechanisms of MTX and AICA riboside are complementary. Moreover, MTX can inhibit AICA ribotide transformylase, which converts AICA ribotide to 10-formyl AICA ribotide\(^{20}\). This provides a reasonable rationale for using MTX and AICA riboside for combination chemotherapy.

The main aims of this study were to investigate the combined effects of MTX and AICA riboside on tumor cell growth in vitro and in vivo, and to evaluate the influence of MTX on both AICA riboside and its active metabolite concentration in plasma and tumors. This is believed to be the first study to demonstrate that the combination of MTX and AICA riboside synergistically inhibits cancer cell growth in vitro and in vivo, and MTX increases AICA riboside and its active metabolite concentration in tumors.

Although AICA riboside is well tolerated\(^{18}\), the clinical use of AICA riboside is limited by the large amount needed to exert its effects\(^{35}\) and its poor pharmacokinetic profile. These shortcomings are associated in part with the rapid metabolism of AICA ribotide, which is converted to 10-formyl AICA ribotide by AICA ribotide transformylase. MTX polyglutamates can inhibit AICA ribotide transformylase activity. An in vitro study has shown that incubation of cells with 1 μmol/L MTX for 4 h resulted in accumulation and polyglutamylation of MTX in cells\(^{30}\). In the present study, MCF-7 and HepG2 cells were pretreated with 1 μmol/L MTX for 4 h before exposure to AICA riboside, and we investigated the influence of MTX on the ability of AICA riboside to activate AMPK. Western blotting and the AMPK activity assay (Figure 3) showed that MTX enhanced the ability of AICA riboside to activate AMPK. Interestingly, the results of Western blotting and the AMPK activity assay (Figure 3) showed that MTX also activated AMPK. A previous study has reported that MTX treatment blocked de novo purine biosynthesis, resulting in accumulation of the intermediate AICA ribotide\(^{31}\). We speculate that activation of AMPK by MTX may be attributed to both its inhibitory effect on AICA ribotide transformylase and its promotion of AICA ribotide accumulation.

The in vitro cytotoxicity assay (Figure 1) showed that the combination of MTX and AICA riboside exhibited more potent cytotoxicity against MCF-7 or HepG2 cells than either drug alone. MTX or AICA riboside alone only inhibited cancer cell proliferation, but the combination of MTX with AICA riboside resulted in cell killing. Combined treatment with 1 μmol/L MTX and 1 mmol/L AICA riboside for 48 h killed 49.02%±7.79% MCF-7 cells and 64.28%±7.84% HepG2 cells. CI analysis (Figure 2) demonstrated that combined MTX and AICA riboside exhibited a broad range of synergism in inhib-
iting the proliferation of MCF-7 and HepG2 cells.

In the MCF-7 cell xenograft model, the combination of MTX and AICA riboside had a superior antitumor effect to MTX or AICA riboside alone (Figure 5). Both MTX and AICA riboside were administered with a fixed schedule and dose. The observed synergism can be improved by modulating dosage and frequency of administration based on the pharmacokinetics and pharmacodynamics of both drugs.

MTX enhanced the AICA riboside and AICA ribotide concentration in tumors, but had no influence on their concentration in plasma (Figure 6). This could be because MTX is transported into cells via the reduced folate carrier and undergoes polyglutamation by folylpolyglutamate synthetase in cells, and MTX polyglutamates potently inhibit AICA ribotide transformylase, which converts AICA ribotide to 10-formyl AICA ribotide.[20, 35].

Visentin et al have reported that pretreatment of HeLa cells with AICA riboside results in augmentation of MTX initial rates and net uptake in cells[36]. There may be an interaction between MTX and AICA riboside, and further studies on the influence of AICA riboside on the antitumor efficacy and pharmacokinetics of MTX are needed.

In conclusion, MTX enhances the anticancer potency of AICA riboside and significantly increases the concentration of both AICA riboside and its active metabolite AICA ribotide in tumors. MTX acts synergistically with AICA riboside to inhibit the proliferation of cancer cells and tumor growth in nude mice.

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Author contribution

Wei LU, Zai-quan LI, and Tian-yan ZHOU designed the research; Xiao-liang CHENG, Bo LI, and Meng-yao LI performed the research; LIANG LI and Xiao-liang CHENG analyzed the data; and Xiao-liang CHENG, Tian-yan ZHOU, and Wei LU wrote the paper.

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