

# Preclinical rationale for synergistic interaction of pemetrexed and cytotoxic nucleoside analogues

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**Abstract.** Cytotoxic nucleoside analogues are widely used in cancer chemotherapy. We used the cytosine arabinoside (Ara-C)-resistant erythroleukaemia cell line K562 and the Ara-C-sensitive myeloid leukaemia cell line HL60 to examine the differential expression of molecular markers. We found increased expression levels of deoxycytidine kinase (dCK) and human equilibrative nucleoside transporter 1 (hENT1) and decreased levels of multidrug resistance protein 5 (ABCC5) and ribonucleoside reductase subunit M1 (RRM1) expression in Ara-C-sensitive HL60 cells. We previously established the pemetrexed (MTA)-resistant small cell lung cancer cell lines PC6/MTA-0.4 and PC6/MTA-1.6 and found that MTA-resistant cells are more sensitive to gemcitabine (GEM) and Ara-C compared with parental PC-6 cells. We examined the molecular markers for GEM and Ara-C sensitivity in MTA-resistant cells and found increased gene expression of dCK and hENT1. Furthermore, treatment with MTA resulted in increased expression of dCK and hENT1 and decreased expression of ABCC5 and RRM1, concomitant with the alteration of the resistance to Ara-C in Ara-C-resistant K562 cells. These results provide evidence that the chemotherapeutic activity of the combination of MTA and cytotoxic nucleoside analogues is synergistic with regard to the alteration of metabolic molecules.

## Introduction

Cytotoxic nucleoside analogues are widely used in cancer chemotherapy for colorectal, breast, head and neck, non-small

cell lung, pancreatic cancer and leukaemia. Most antitumour 2'-deoxycytidine analogues, such as gemcitabine (GEM) and cytosine arabinoside (Ara-C), have common antitumour mechanisms and metabolic pathways (1). These nucleosides must first be transported into the cell via a nucleoside transporter. The most active uptake has been found to be via the human equilibrative nucleoside transporter 1 (hENT1) and efflux has been found to occur via the ATP-binding cassette (ABC) transporter (2-4). Once inside a cell, these nucleosides are phosphorylated by deoxycytidine kinase (dCK) to the active triphosphate form (5), which is then most likely incorporated into DNA. The active diphosphate metabolite inhibits DNA synthesis indirectly through the inhibition of ribonucleoside reductase (RR). RR has two subunits (RRM1 and RRM2) and converts ribonucleoside 5'-diphosphates to the deoxyribonucleotide 5'-diphosphates that are essential for DNA synthesis. Therefore, the mechanism of sensitivity and/or resistance to 2'-deoxycytidine analogues is likely to be multifactorial, involving decreased intracellular accumulation and the alteration of metabolism by the aforementioned molecules (1-7).

Pemetrexed (MTA) is a multitarget antifolate cytotoxic agent available for use in the treatment of non-small cell lung cancer (NSCLC) and malignant pleural mesothelioma (8,9). We previously reported that MTA pretreatment altered the expression of dCK, resulting in the enhanced cytotoxicity of GEM in GEM-resistant NSCLC cells (4). In the present study, we have further examined the molecules involved in the synergistic interaction of MTA and 2'-deoxycytidine analogues.

## Materials and methods

**Cell lines and chemicals.** The human erythroleukaemia cell line K562 and the myeloid leukaemia cell line HL60 were used in this study. Cells from the MTA-resistant human small cell lung carcinoma cell lines PC6/MTA-0.4 and PC6/MTA-1.6 were established from parental PC-6 cells as described previously (10,11). PC6/MTA-0.4 cells were cultured with 0.4  $\mu$ M MTA and PC6/MTA-1.6 cells with 1.6  $\mu$ M MTA. The 5-fluorouracil (5-FU)-resistant subline PC-6/FU23-26, selected from PC-6 human small cell lung cancer cells as described previously, was used as a control (12). The cells were cultured in RPMI-1640 (or, for A549, in Dulbecco's modified Eagle's

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**Key words:** cytotoxic nucleoside analogue, pemetrexed, deoxycytidine kinase, human equilibrative nucleoside transporter 1, multidrug resistance protein 5, ribonucleoside reductase subunit M1

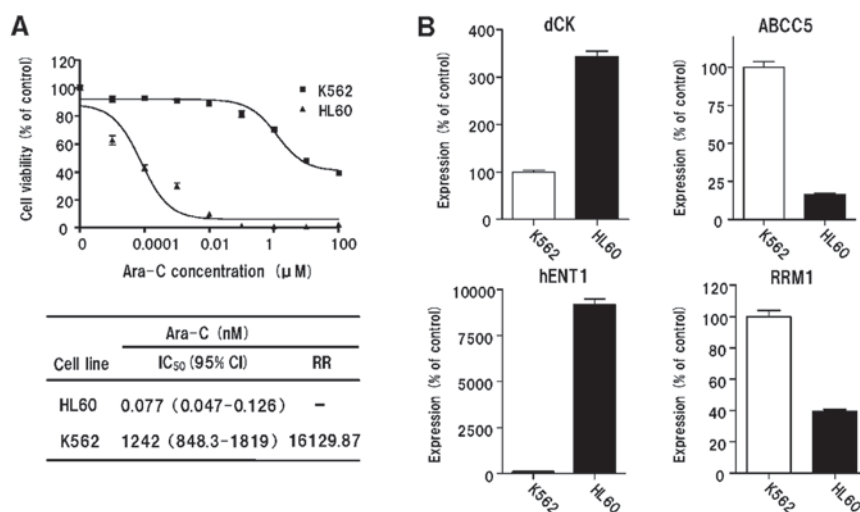


Figure 1. Cytotoxicity of Ara-C and expression levels of dCK, ABCC5, hENT1 and RRM1 genes in K562 and HL60 cells. (A) Cytotoxicity of Ara-C in K562 and HL60 cells. (B) Expression levels of dCK, ABCC5, hENT1 and RRM1 in K562 and HL60 cells genes, as determined by real-time PCR. Ara-C, cytosine arabinoside; dCK, deoxycytidine kinase; ABCC5, multidrug resistance protein 5; hENT1, human equilibrative nucleoside transporter 1; RRM1, ribonucleoside reductase subunit M1; IC<sub>50</sub>, concentration for 50% cell survival; CI, confidence interval; RR, resistance rate.

medium) supplemented with 10% heat-inactivated FBS and 1% (v/w) penicillin/streptomycin in a humidified chamber (37°C, 5% CO<sub>2</sub>). GEM and MTA were provided by Eli Lilly Pharmaceuticals (Indianapolis, IN, USA). Ara-C was purchased from Wako Pure Chemical Industries (Osaka, Japan).

**Total RNA extraction and quantitative RT-PCR.** Total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany). Quantitative real-time RT-PCR was performed in a volume of 20 µl with a Taqman One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems, Foster City, CA, USA) using the StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Melting curve analysis was used to control for the specificity of the amplification products. The number of transcripts was calculated from a standard curve obtained by plotting the input of four different known transcript concentrations versus the PCR cycle number at which the detected fluorescence intensity reached a fixed value. The PCR program consisted of 45 cycles of 94°C for 15 sec and 60°C for 1 min. The experiment was performed in triplicate. The primer and Taqman probe sets (Taqman Gene Expression Assays, Inventoried) for dCK (Hs00176127\_m1), hENT1 (Hs01085706\_m1), multidrug resistance protein 5 (ABCC5; Hs00981087\_m1), RRM1 (Hs00168784\_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs99999905\_m1) were purchased from Applied Biosystems (sequences not disclosed). Expression was normalised to the housekeeping gene GAPDH.

**Concentration of GEM and Ara-C for cell survival.** Cells were cultured at 5,000 cells/well in 96-well tissue culture plates. To assess cell viability, ten-fold dilutions of the anticancer drug were added stepwise 2 h after plating, and the cultures were incubated at 37°C for 96 h. At the end of the culture period, 20 µl of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] solution (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega,

Madison, WI, USA) was added, the cells were incubated for a further 4 h and the absorbance was measured at 490 nm using an ELISA plate reader. Mean values were calculated from three independent experiments performed in quadruplicate. Chemosensitivity is expressed as the drug concentration for IC<sub>50</sub>, determined from the concentration-effect correlation using Graph Pad Prism version 4 (GraphPad Software, San Diego, CA, USA).

**Statistical analysis.** The differences in cell viability between samples were evaluated using the Student's unpaired t-test. The level of significance was set at 5%, using a two-sided analysis.

## Results

**Gene expression levels of dCK, hENT1, ABCC5 and RRM1 in leukaemia cells.** Previously, we examined dCK, hENT1, ABCC5 and RRM1 expression in GEM-resistant NSCLC cells, and found that alteration of these molecules was associated with sensitivity and/or resistance to GEM (3,4,7). Therefore, to examine the differential expression of dCK, hENT1, ABCC5 and RRM1 for Ara-C sensitivity, we used two leukaemia cell lines: Ara-C-resistant erythroleukaemia K562 cells and Ara-C-sensitive myeloid leukaemia HL60 cells. We found that the levels of expression of ABCC5 and RRM1 were lower in HL60 cells than in K562 cells. By contrast, the expression levels of hENT1 and dCK were higher in HL60 cells than in K562 cells (Fig. 1).

**Sensitivity to GEM and Ara-C in MTA-resistant cells.** The sensitivities of parental and MTA-resistant cells to GEM and Ara-C are summarised in Fig. 2. Both MTA-resistant cell lines, PC6/MTA-0.4 and PC6/MTA-1.6, were more sensitive to GEM and Ara-C than the parental PC6 cells.

**Gene expression levels of dCK, hENT1, ABCC5 and RRM1 in MTA-resistant cells.** We examined the level of gene expression of dCK, hENT1, ABCC5 and RRM1 in parental

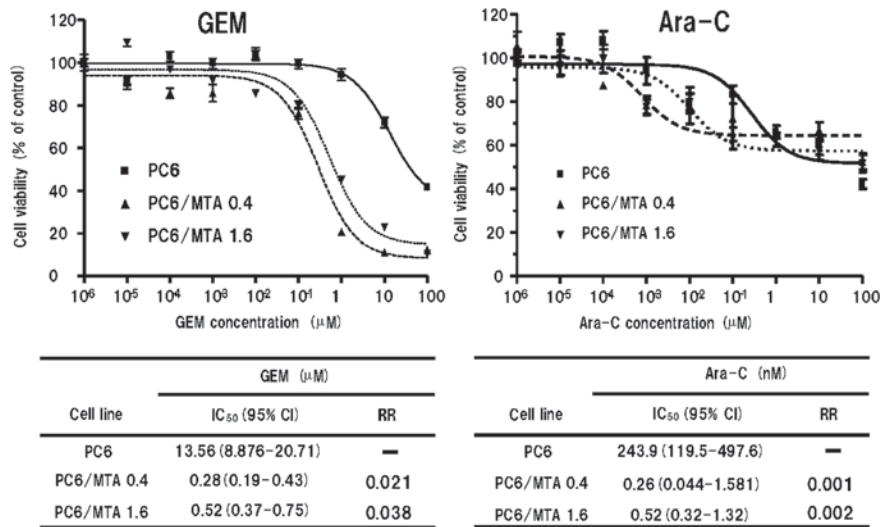


Figure 2. GEM and Ara-C sensitivity in MTA-resistant cells. Cytotoxicity of GEM and Ara-C in PC6, PC6/MTA-0.4 and PC6/MTA-1.6 cells. GEM, gemcitabine; Ara-C, cytosine arabinoside; MTA, pemetrexed; IC<sub>50</sub>, concentration for 50% cell survival; CI, confidence interval; RR, resistance rate.

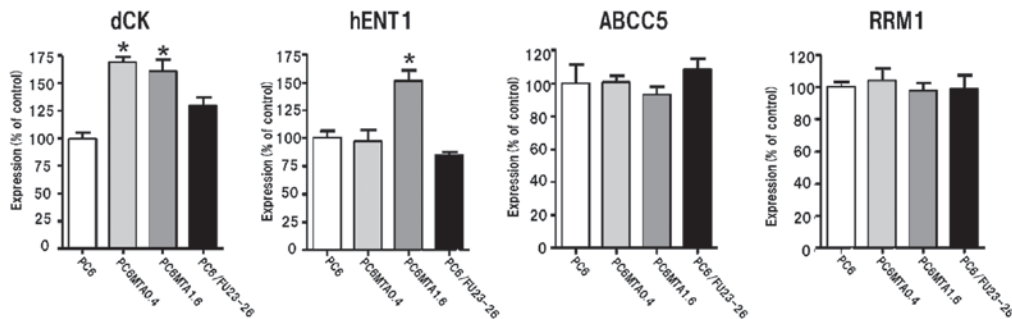


Figure 3. Expression levels of dCK, ABCC5, hENT1 and RRM1 genes in PC-6, PC-6/MTA-0.4, PC6/MTA-1.6 and PC6/5FU23-26 cells, as determined by real-time PCR. \*P<0.05. dCK, deoxycytidine kinase; ABCC5, multidrug resistance protein 5; hENT1, human equilibrative nucleoside transporter 1; RRM1, ribonucleoside reductase subunit M1.

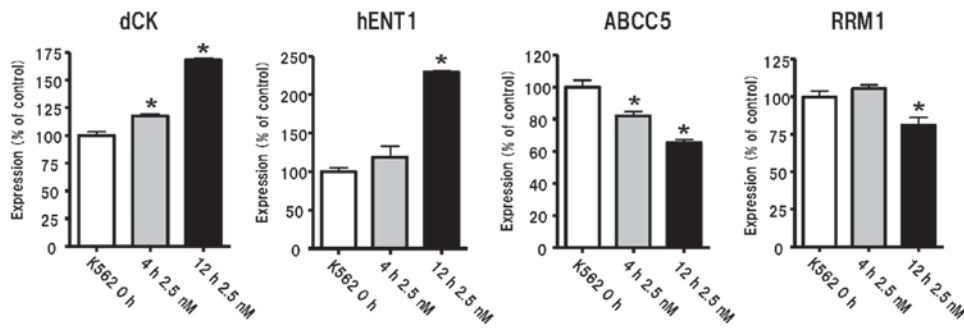


Figure 4. Modification of dCK, ABCC5 and hENT1 expression by MTA in K562 cells. Time-dependent modification of dCK, ABCC5, hENT1 and RRM1 gene expression in K562 cells following pemetrexed treatment at 2.5 nM for 4 and 12 h. \*P<0.05. dCK, deoxycytidine kinase; ABCC5, multidrug resistance protein 5; hENT1, human equilibrative nucleoside transporter 1; MTA, pemetrexed.

and MTA-resistant cells. As shown in Fig. 3, the expression levels of hENT1 and dCK in MTA-resistant cells were significantly higher than in the parental cells (P<0.05). We used 5-FU-resistant PC6/FU23-26 cells as a control as the pyrimidine analogue 5-FU, while also an antimetabolite drug, differs from 2'-deoxycytidine analogues in that it requires intracellular conversion (12). The expression levels of hENT1

and dCK in PC6/FU23-26 cells were not changed compared with PC6 cells. These results indicate that the alteration of these molecules affects sensitivity to GEM and Ara-C in MTA-resistant cells.

*Modification of Ara-C cytotoxicity by MTA treatment.* We examined gene expression levels of dCK, hENT1, ABCC5

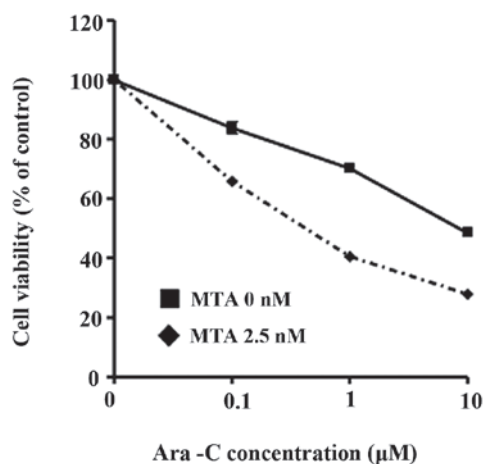


Figure 5. Modification of Ara-C cytotoxicity by MTA in K562 cells. Pretreatment with MTA at 2.5 nM for 12 h altered the cytotoxicity of Ara-C in K562 cells. Ara-C, cytosine arabinoside; MTA, pemetrexed.

and RRM1 in K562 cells following treatment with 2.5 nM MTA for 4 and 12 h. We found increased levels of expression of hENT1 and dCK and decreased expression of ABCC5 and RRM1 ( $P < 0.05$ , Fig. 4). We also found that pretreatment with 2.5 nM of MTA for 12 h altered the cytotoxicity of Ara-C in K562 cells (Fig. 5).

## Discussion

We found that the gene expression levels of dCK, hENT1, ABCC5 and RRM1 are different in Ara-C-sensitive and -resistant leukaemia cells.

We previously reported that ABCC5, hENT1, dCK and RRM1 expression levels are associated with sensitivity and/or resistance to GEM (4). hENT1 and ABCC5 are associated with drug uptake and efflux, meaning that increased expression of hENT1 and decreased expression of ABCC5 may result in increased intracellular concentration of cytotoxic nucleoside analogues, which is an important factor influencing their cytotoxicity. By contrast, dCK mediates the rate-limiting step, which is the first phosphorylation, in the process that converts anticancer nucleoside analogues to their active triphosphate form (2). Increased RRM1 increases the size of deoxynucleoside triphosphate (dNTP) pools that competitively inhibit the incorporation of the triphosphate cytotoxic nucleoside analogue into DNA (2). These increased dNTP pools further downregulate the activity of dCK via a negative-feedback pathway. The diphosphate forms of cytotoxic nucleoside analogues inhibit RRM1, resulting in a decrease in dNTP pools (6). Therefore, increased expression of dCK and decreased expression of RRM1 may result in the increased activation of anticancer nucleoside analogues. Alteration of the expression of dCK, hENT1, ABCC5 and RRM1 affects the intracellular concentration and activation of GEM and Ara-C.

Previous studies have revealed that the downregulation of dCK increased the resistance of human leukaemia cells to cladribine and clofarabine (13,14). We previously showed that treatment with MTA enhances dCK expression, concomi-

tant with altering the cytotoxicity of GEM (4). Indeed, the clinical administration of MTA followed by GEM met the protocol-defined efficacy criteria, and was less toxic than administering GEM followed by MTA (15). Therefore, we investigated the effect of treatment with MTA on Ara-C sensitivity. We found that MTA-resistant cells were more sensitive to GEM and Ara-C concomitant with increased expression of dCK and hENT1. Further, pretreatment with MTA enhanced not only dCK expression, but also ABCC5 and hENT1 expression concomitant with altering the sensitivity to Ara-C in Ara-C-resistant K562 cells. Thus, the combination of Ara-C and MTA exhibits order-dependent synergistic cytotoxic activity, suggesting the efficacy of gene-expression modulation by chemotherapy combinations. Ara-C is currently used to treat haematological malignancies, especially as induction chemotherapy for acute myeloid leukaemia (AML) (16). High-dose Ara-C is also used to treat relapsed or refractory AML, and induction chemotherapy with high-dose Ara-C has been shown to improve overall survival in clinical trials (17). Although high-dose Ara-C is most effective for relapsed or refractory AML, toxicity, especially non-haematological toxicity such as severe infection, emesis and central nervous system toxicity, is common (16,17). While MTA has not been approved for haematological malignancies, its toxicity is mild when supplemented with folic acid and vitamin B12 (18). Our *in vitro* study shows the efficacy of the combination of MTA and Ara-C, suggesting a new treatment option for AML chemotherapy.

We found that the mechanisms of the synergistic interaction of MTA and cytotoxic nucleoside analogues are multifactorial. Future studies should examine whether these factors may be used as predictive markers for cytotoxic nucleoside analogues.

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