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**A new mechanism of methotrexate action revealed by target screening with affinity
beads**

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Running title: Regulation of dCK activity by MTX

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The abbreviations used are:

DHFR, dihydrofolate reductase; ara-C, 1- β -D-arabinofuranosylcytosine; GMA, glycidyl methacrylate; DMF, N,N-dimethylformamide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; His-dCK, polyhistidine-tagged dCK; LDH, lactate dehydrogenase; ara-CMP, 1- β -D-arabinofuranosylcytosine monophosphate.

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Abstract

Methotrexate (MTX) is the anti-cancer and anti-rheumatoid drug that is thought to block nucleotide synthesis and cell cycle by inhibiting dihydrofolate reductase (DHFR) activity. Recently, we developed novel affinity matrices, termed SG beads, that are easy to manipulate and compatible with surface functionalization. Using the matrices, here we present evidence that deoxycytidine kinase (dCK), an enzyme that acts in the salvage pathway of nucleotide biosynthesis, is another target of MTX. MTX modulates dCK activity differentially depending on substrate concentrations. 1- β -D-arabinofuranosylcytosine (ara-C), a chemotherapy agent often used in combination with MTX, is a nucleoside analog whose incorporation into chromosome requires prior phosphorylation by dCK. Remarkably, we show that MTX enhances incorporation and cytotoxicity of ara-C through regulation of dCK activity in Burkitt's lymphoma cells. Thus, this study provides new insight into the mechanisms underlying MTX actions and also demonstrates the usefulness of the SG beads.

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Introduction

Identification of drug target proteins provides important clues to the mechanism of the action of the drug. Affinity chromatography using a chemical compound as ligand is potentially a very useful method for this purpose. However, conventional matrices for affinity chromatography are chemically unstable and incompatible with various fixation procedures, and purification efficiency is often low. Previously, we developed a matrix composed of a styrene and glycidyl methacrylate (GMA) copolymer core and a GMA polymer surface, termed SG beads. This matrix shows a number of excellent features, such as chemical and physical stability, high capacity for ligand fixation, low nonspecific protein binding, and high purification efficiency, and has proven useful for identification of novel ligand-binding proteins (Kawaguchi et al., 1989; Wada et al., 1995; Shimizu et al., 2000; Hiramoto et al., 2002). For example, using SG beads to which the anti-inflammatory agent E3330 was fixed, we identified an E3330 "receptor" protein and revealed the molecular basis for the effects of the drug (Shimizu et al., 2000; Hiramoto et al., 2002; Nishi et al., 2002).

MTX is a folate antagonist that inhibits DHFR activity and is used for the treatment of malignancies, such as leukemia. Notable for its anti-inflammatory and immunosuppressive effects (Genestier et al., 2000; Alarcon, 2000), MTX is currently used also in the treatment of refractory rheumatoid arthritis (RA). While high-dose MTX is used for the treatment of malignancies, low-dose MTX having no substantial anticarcinogenic effects is successfully used for the treatment of RA (Weinblatt et al., 1985). This raises the possibility that MTX targets another protein in addition to DHFR, although little is known about such an alternative mechanism.

It is widely accepted that a combination of MTX and ara-C is effective for the treatment of leukemia and lymphoma, such as refractory or biologically aggressive non-Hodgkin's lymphoma, including Burkitt's lymphoma/leukemia (Thomas et al., 1999;

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Lee et al., 2001). ara-C is a nucleoside analog, the 5'-hydroxyl group of which needs to be mono-, di-, and tri-phosphorylated by various enzymes including dCK (Arner and Eriksson, 1995). The produced ara-C triphosphate is incorporated into the chromosome and exerts its antiproliferative activity in cells. Although it has been reported that MTX enhances ara-C-induced cytotoxicity or the intracellular conversion of ara-C into the active, triphosphate form in leukocytes (Edelstein et al., 1975; Roberts et al., 1979), the precise mechanism for their synergistic effects remains largely unknown.

To understand mechanisms underlying apparently diverse functions of MTX, we sought to identify a new MTX-interacting protein by affinity chromatography using SG beads. Here we show that dCK, an enzyme that converts nucleosides to nucleoside monophosphates and acts in the salvage pathway of nucleotide biosynthesis, is another target of MTX. MTX modulates dCK activity differentially depending on substrate concentrations. We present evidence that MTX enhances incorporation and cytotoxicity of ara-C through regulation of dCK activity. Thus, this study provides new insight into the mechanisms underlying MTX actions and also demonstrates the usefulness of the SG beads.

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Materials and Methods

Preparation of MTX-fixed SG beads. Details of the preparation of SG beads were described previously (Kawaguchi et al., 1989). For preparation of SGN beads, SG beads (1 g) were suspended in 100 ml of 3 M NH_4OH (pH 11), incubated at 70°C for 24 h, and washed with distilled water. For preparation of SGNEGDE beads, SGN beads were incubated with 100 mmol of ethylene glycol diglycidyl ether (pH 11) at 30°C for 24 h. SGNEGDE beads were stored at 4°C in the dark.

For preparation of MTX-fixed SGNE-OH beads, SGNEGDE beads were first hydrolyzed to generate SGNE-OH beads. The SGNE-OH beads (10 mg/ml suspension in N,N-dimethylformamide (DMF)) were then incubated with an equal volume of 20 mM MTX (Nacalai Tesque) in DMF in the presence of water-soluble carbodiimide and 10% triethylamine at room temperature for 24 h. Subsequently, the MTX-fixed SGNE-OH beads were washed with DMF and suspended in distilled water to 10 mg/ml.

For preparation of MTX amino derivative-fixed SGNE-COOH beads, SGNEGDE beads were first aminated by incubation with 3 M NH_4OH (pH 11) to generate SGNEGDE-NH₂ beads. The SGNEGDE-NH₂ beads were then succinylated by incubation with DMF containing 0.5 M succinic anhydride at room temperature for 12 h (SGNE-COOH beads). MTX amino derivative (Fig.1A) was newly synthesized by Kanto Kagaku. SGNE-COOH bead (5 mg) were incubated with 500 μl of 0.25 mM MTX amino derivative in DMF in the presence of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride and N-hydroxysuccinimide at room temperature for 1 h. Residual reactive groups were blocked with 1 M ethanolamine, and the beads were suspended in 10 mg/ml of distilled water.

Affinity purification of MTX-target proteins. Cytoplasmic extracts of THP-1 cells (American Type Culture Collection) were prepared from a 4-1 culture (4×10^9 cells) according to the Dignam method. MTX-fixed beads or control beads (0.1 mg) were

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equilibrated with binding buffer (10 mM Tris-HCl (pH 7.4), 10% glycerol, 0.1 M NaCl, 0.2 mM EDTA, 1 mM DTT) and incubated with 100 μ l of the cytoplasmic extracts at 4°C for 4 h with occasional agitation. After washing with binding buffer, bound proteins were eluted with SDS-PAGE sample buffer and subjected to SDS-PAGE and silver staining or immunoblotting. For the latter, protein samples were transferred to a polyvinylidene difluoride membrane (Millipore) after electrophoresis, and the membrane was incubated with 5% nonfat milk for 1 h and then with polyclonal antibodies against dCK (produced in rabbits immunized with His-dCK protein) and DHFR (BD Biosciences) for 2 h. Proteins of interest were visualized using an enhanced chemiluminescence system (Amersham).

Preparation of recombinant dCK. The cDNA encoding human dCK was obtained by reverse transcription-polymerase chain reaction using total RNA prepared from THP-1 cells. *Xho*I and *Bam*HI sites were introduced at the 5'- and 3'-ends of the coding sequence, respectively, and the PCR product was cloned into the bacterial expression vector pET-14b (Novagen). The sequences of the PCR primers used are 5'-CCCGCTCGAGGCCACCCCGCCCAAGAGAAGCT-3' and 5'-CACGGATCCTCACAAAGTACTCAAAACTCTTT-3'. The sequence of the cloned cDNA was verified by DNA sequencing. The polyhistidine-tagged dCK protein was expressed in *Escherichia coli* BL21 (DE3). Bacterial pellets were resuspended and lysed in 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, and 1 mM phenylmethylsulfonyl fluoride by freezing-thawing cycles and sonication on ice. Cleared lysates were subjected to Ni²⁺ affinity chromatography (Qiagen). After washing the column, bound His-dCK was eluted with the same buffer containing 0.5 M imidazole. Subsequently, the eluate was desalted using a PD-10 gel filtration column (Amersham), and its protein concentration was determined by the Bradford method.

dCK kinase assays. Since it was reported that recombinant His-dCK expressed in *E. coli* is catalytically similar to endogenous dCK (Usova and Eriksson, 1997), the

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recombinant protein was used for the kinase assays. Reactions (200 μ l) containing purified His-dCK and various concentrations of a nucleoside (dCyd or ara-C) and UTP in 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM MgCl₂, 2 mM dithiothreitol, and 0.5 mg/ml BSA were incubated at 37°C for 5 or 15 min and stopped by heating at 95°C for 1 min. Then, the nucleoside monophosphate contents in the reaction mixtures were analyzed using a high performance liquid chromatography system (Waters) equipped with a DEAE-5PW column (Tosoh). Bound nucleoside monophosphates were eluted with 5 mM phosphate buffer (pH 8) at a flow rate of 1 ml/min and quantified on the basis of their A_{270s} using known amounts of nucleoside monophosphates as standards. Results shown are representative of two or more independent experiments.

Determination of cytotoxicity. Jurkat or HS-Sultan cells (American Type Culture Collection) were plated in 48-well plates at a concentration of 4×10^5 cells/ml in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS and with or without 100 μ M hypoxanthine and 16 μ M thymidine. MTX and ara-C were added at various concentrations 24 h and 25 h later, respectively, and the cells were further incubated for 24 to 36 h. Injured cells were then quantified with the LDH cytotoxicity detection kit (Takara), which measures the release of LDH into culture supernatant using a tetrazolium/formazan assay. Percentages of injured cells were calculated by comparing drug-treated and -untreated cells with control cells that were lysed with 1% Triton X-100.

Determination of ara-C incorporation into the chromosome. ara-C incorporation into the chromosome was determined as described (Azuma et al., 2001) with some modifications. Jurkat or HS-Sultan cells (1×10^6 cells/ml) were cultured in RPMI 1640 medium containing 100 μ M Hx and 16 μ M Thd. Three hours after addition of MTX at various concentrations, a constant amount of [³H] ara-C (Moravek Biochemicals) and various amounts of cold ara-C were added to final concentrations of 10 nM, 100 nM, and 1 μ M. After an additional 7-h incubation, cells were collected and lysed in one

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packed-cell-volume of 6% trichloroacetic acid with vigorous mixing on ice. After centrifugation, pellets containing chromosomal DNA were washed with 70% ethanol and dissolved in PBS, and radioactivities of aliquots of the samples were measured with a liquid scintillation counter (Packard).

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Results

One-step affinity purification of MTX-binding proteins using SG beads. To identify novel MTX-binding proteins, we prepared two types of SG beads to which MTX was immobilized through different functional groups, either using the amino group of an MTX amino derivative or using the two carboxyl groups of MTX itself (Fig. 1A). In a previous study, affinity chromatography using MTX fixed through the γ -carboxyl group of its glutamate moiety resulted in a high-yield purification of DHFR. However, since the α -carboxyl group of the glutamate moiety is involved in MTX binding to DHFR according to the co-crystal structure (Bolin et al., 1982), MTX coupling through its carboxyl groups may disturb the MTX-DHFR interaction. To avoid this problem, we synthesized the MTX amino derivative bearing an amino group instead of the γ -carboxyl group and fixed it to SGNE-COOH beads, the SG beads carrying carboxyl groups, through the amino group by amidation (Fig. 1A). Since leukocytes are sensitive to MTX with respect to both its anti-cancer and anti-inflammatory effects, cytoplasmic extracts of the monocytic leukemia cell line THP-1 were used as source materials. After incubation with the cytoplasmic extracts, the beads were washed, and bound proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and silver staining. As a result, a 23 kDa protein was purified specifically (Fig. 1B), and immunoblot analysis revealed that this protein is DHFR (Fig. 1E).

We also fixed authentic MTX to SGNE-OH beads, the SG beads carrying hydroxyl groups, through the two carboxyl groups by esterification (Fig. 1A). To our surprise, the same procedure resulted in purification of an abundant 30 kDa protein and small amount of DHFR, only detectable by immunoblotting (Fig. 1, C and E). The interaction between the 30 kDa protein and MTX is specific because the protein did not bind to control beads, because the interaction was inhibited by preincubation of the extracts with excess amounts of free MTX, and the protein was eluted from the beads

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with free MTX (Fig. 1, C and D). Mass spectrometric analysis showed that anhydration occurred between the two carboxyl groups of MTX during its fixation to the beads (data not shown). Since, in general, a hydroxyl group reacts with one of two acyl groups in carboxylic anhydride at an equal efficiency, both the α - and γ -carboxyl groups were likely used for fixation, and perhaps this resulted in a low yield of DHFR.

Identification of the 30 kDa MTX-binding protein as dCK. The 30 kDa protein was purified on a large scale using the MTX-fixed SGNE-OH beads and subjected to proteolytic digestion and tandem mass spectrometric analysis. Four peptide sequences (STFVNILK, NGGNVLQMMYEKPER, LKDAEKPVLEFFER, and NEEQGIPLEYLEK) were identified, and a database search revealed that they correspond to parts of the amino acid sequence of dCK (Fig. 2A). The identity was further confirmed by immunoblot analysis using anti-dCK antibody (Fig. 2B). As a next step, we expressed and purified polyhistidine-tagged dCK (His-dCK) from bacteria, and tested whether the recombinant protein binds to MTX in the absence of any other factor. As shown in Fig. 2C, purified His-dCK specifically bound to the MTX-fixed SGNE-OH beads, demonstrating that dCK directly binds to MTX.

dCK is an enzyme in the salvage pathway of nucleotide biosynthesis that phosphorylates the 5'-hydroxyl groups of deoxyribonucleosides, such as deoxycytidine (dCyd) to produce nucleoside monophosphates. dCK is also involved in the conversion of synthetic nucleosides with antitumor activities, such as ara-C (Arner and Eriksson, 1995), to active, phosphorylated forms and is therefore important in the exhibition of their antitumor activities. dCK is expressed in all types of cells, especially in leukocytes and lymphocytes including monocytes and T cells at high levels (Arner and Eriksson, 1995). Since ara-C is often used in combination with MTX for the treatment of leukemia and lymphoma, we speculated that dCK may contribute the synergistic action of ara-C and MTX.

MTX positively and negatively regulates dCK activity. First, we

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investigated the effect of MTX on the activity of purified recombinant dCK. It has been proposed that dCK utilizes UTP, rather than ATP, as a phosphate donor in cells, based on the observation that the K_m values for nucleosides with UTP as a phosphate donor are lower than those with ATP (White and Capizzi, 1991; Shewach et al., 1992; Krawiec et al., 1995; Godsey et al., 2006). Therefore, we used UTP as a phosphate donor in dCK kinase assays. Reactions containing His-dCK, one of nucleosides (dCyd or ara-C) as a phosphate acceptor, UTP as a phosphate donor, and MTX as a potential effector were incubated, and then the reaction products were subjected to anion exchange chromatography and quantified on the basis of their A_{270S} . As shown in Fig. 3, A and B, the initial velocity plots against the initial concentration of dCyd or ara-C substrate showed that dCK activity is suppressed at high nucleoside concentrations. This phenomenon was noted previously (White and Capizzi, 1991; Hughes et al., 1997). Remarkably, MTX dose-dependently enhanced dCK activity at high, inhibitory concentrations of nucleoside substrates. On the other hand, it reduced dCK activity at lower substrate concentrations (Fig. 3, A and B). These data demonstrated that MTX both positively and negatively regulates dCK activity. Substrate concentrations at which the MTX's effects were reversed were relatively low ($\sim 8 \mu\text{M}$). Thus, phosphorylation of dCyd and ara-C is activated, rather than repressed, by MTX over a broad range of substrate concentrations. Fig. 3C shows, as an example, MTX's effects on dCK activity at a nucleoside concentration of $20 \mu\text{M}$. The observed K_m and V_{max} for dCyd and ara-C and K_i values for MTX, calculated from double reciprocal plots, were presented in Table I. For comparison, we investigated the effects of folate, leucovorin and tetrahydrofolate, which are structurally similar to MTX, on dCK activity. These molecules have no apparent effect on dCK activity, unable to measure their K_i values (data not shown). These results suggest that the regulatory effect on dCK is specific to MTX.

MTX enhances ara-C-induced cytotoxicity independently of its effects on

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DHFR. We next investigated whether MTX regulates dCK activity in cells. As cells deficient in dCK activity are resistant to ara-C (Chottiner et al., 1991; Owens et al., 1992), dCK is essential for ara-C-induced cytotoxicity. In other words, ara-C-induced cytotoxicity can be regarded as an index of intracellular dCK activity. After incubation of Jurkat cells with various concentrations of ara-C and MTX, lactate dehydrogenase (LDH) activity released from injured cells was measured. As reported previously (Edelstein et al., 1975; Roberts et al., 1979), MTX enhanced ara-C-induced cytotoxicity in a dose-dependent manner (Fig. 4A). MTX may exert its cytotoxic effects through DHFR by inhibiting the *de novo* pathway of nucleotide biosynthesis, or through dCK by increasing the pool of ara-C monophosphate and its incorporation into the chromosome. To distinguish these possibilities, we supplemented culture medium with hypoxanthine (Hx) and thymidine (Thd) to circumvent the DHFR inhibition. As expected, addition of Hx and Thd fully reversed MTX-induced cytotoxicity (Fig. 4B), but had no substantial effect on ara-C-induced cytotoxicity (Fig. 4C), indicating that Hx and Thd eliminated DHFR-mediated cytotoxic effects of MTX. Even under these conditions, MTX dose-dependently enhanced ara-C-induced cytotoxicity, although to a lesser extent (Fig. 4D). On the other hand, folate, leucovorin and tetrahydrofolate had no substantial effect on ara-C-induced cytotoxicity (Fig. 4, E, F and G), suggesting that the regulatory effect on dCK is specific to MTX. Since combination therapy with high-dose ara-C and MTX is often prescribed for Burkitt's lymphoma (Thomas et al., 1999; Lee et al., 2001), Burkitt's lymphoma HS-Sultan cells were subjected to the same analysis. Ara-C-induced cytotoxicity was even more significantly enhanced by MTX in HS-Sultan cells than in Jurkat cells (Fig. 4H). Taken together, these results suggest that MTX enhances ara-C-induced cytotoxicity independently of its effects on DHFR.

MTX differentially affects ara-C incorporation into the chromosome depending on ara-C concentrations. The above results predict that ara-C incorporation into the chromosome increases when MTX is co-administered with high-

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dose ara-C, whereas its incorporation decreases when MTX is co-administered with low-dose ara-C. To test this idea, we incubated Jurkat or HS-Sultan cells in the presence of various concentrations of MTX and [³H] ara-C, and measured radioactive incorporation into the chromosome. Hx and Thd were included in the medium to eliminate DHFR-mediated cytotoxic effects of MTX. In Jurkat cells, MTX dose-dependently decreased or increased ara-C incorporation at low (0.01 μM) or high (1 μM) concentrations of ara-C, respectively (Fig. 5A), and this tendency was more prominent in HS-Sultan cells (Fig. 5B). These results are fully consistent with the finding that MTX has dual roles in the regulation of the activity of purified recombinant dCK, and strongly suggest that MTX exerts its effects in cells, in part, through dCK. Finally, we examined dCK protein levels by immunoblotting and found that dCK is expressed at a higher level in HS-Sultan cells than in Jurkat cells (Fig. 5C), which may account for the difference of MTX responses between these cells.

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Discussion

This study demonstrated the usefulness of our developed SG beads. Using the MTX-fixed SG beads, we identified dCK as a novel MTX target protein. With recent advancements in human genomics, 5,000-10,000 proteins have been obtained as potential targets for drugs (Drews, 2000), while combinatorial chemistry allows development of chemical libraries for drug screening (Webb, 2005). For drug discovery, various chemogenomic approaches to study potential interactions between a large number of chemical compounds and potential targets are being explored (Austin et al., 2004; Ganter et al., 2005). We expect that SG beads technology will provide high quality chemogenomic information.

At high concentrations of ara-C, MTX markedly increased ara-C-induced cytotoxicity and ara-C incorporation into the chromosome in Burkitt's lymphoma HS-Sultan cells, which were observed even when its inhibitory effects on DHFR were reversed (Fig. 4 and 5). Moreover, dCK activity and ara-C incorporation into the chromosome were similarly regulated by the presence of MTX (Fig. 3 and 5). These results strongly suggest that in the presence of high concentrations of ara-C, MTX exerts its cytotoxic effects, in part, through dCK by increasing the pool of ara-C monophosphate (ara-CMP) and its incorporation, as summarized in Fig.6. At lower nucleoside concentrations, MTX inhibited the conversion of nucleosides to nucleoside monophosphates by dCK. Since MTX had no significant effect on cell viability when its DHFR-mediated action was reversed, the inhibition of the salvage pathway of nucleotide biosynthesis by MTX alone does not seem to have a significant cytotoxic effect. However, this route of MTX actions may contribute to the significant cytotoxicity together with its main action on the *de novo* pathway of nucleotide biosynthesis (Fig.6).

The high concentrations of ara-C used in this study are similar to those used in

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clinical setting. According to recent studies, regimens such as Hyper-CVAD and CALGB-9251 that involve high-dose administration of ara-C and MTX in combination with other anti-cancer agents show encouraging results with aggressive cases of Burkitt's leukemia/lymphoma (Thomas et al., 1999; Lee et al., 2001). Although high-dose ara-C (>10 μM) is generally administered to leukemia patients, an excess administration of ara-C is not effective (White and Capizzi, 1991; Plunkett et al., 1987; Muus et al., 1987). This problem may arise from the fact that ara-C inhibits dCK activity at high concentrations. In any case, we believe that the dCK-mediated action of MTX is the molecular mechanism underlying existing chemotherapeutic regimens involving high-dose ara-C and MTX.

dCK activity was inhibited by high nucleoside concentrations, indicating the existence of negative allosteric regulation. This phenomenon is commonly known to be the case as product inhibition of enzymes. One possible explanation was as follows: In addition to a primary (catalytic) nucleoside-binding site, dCK has a secondary nucleoside-binding site responsible for allosteric regulation. Nucleoside binding to the secondary site inhibits the interaction between UTP and the phosphate donor-binding site. According to a recent crystal structure, however, human dCK has only one nucleoside binding site and one phosphate donor binding site (Sabini et al., 2003). Since dCK forms a homodimer (Bohman and Eriksson, 1988; Datta et al., 1989; Chottiner et al., 1991), we assume that the allosteric regulation is due to negative homotropic cooperativity, *i.e.*, two nucleoside binding sites on a dimer act as the primary and secondary binding sites, respectively. One such example is cytochrome c oxidase, which has only one substrate binding site and changes its form from monomer to dimer as its concentration increases. Only the dimeric form shows biphasic kinetics as a function of substrate concentration (Bolli et al., 1985).

MTX inhibited or enhanced the incorporation of low or high nucleoside concentrations, respectively. This dual effect of MTX can be explained simply as

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follows: MTX blocks nucleoside actions through the both nucleoside binding sites. At low nucleoside concentrations, a nucleoside acts only as a substrate, and MTX prevents its action, thereby inhibiting the enzymatic reaction. In contrast, at high nucleoside concentrations, the nucleoside acts as both a substrate and an allosteric inhibitor, and MTX selectively prevents the latter action, thereby enhancing the enzymatic reaction. Because the latter action may be mediated by the lower affinity interaction between the nucleoside and the secondary binding site, it is reasonable to speculate that MTX selectively removes the allosteric inhibition.

MTX modulated dCK activity at a relatively high concentration (>300 μM) when purified enzyme was used (Fig. 3) but at as low as 10 μM in culture (Fig. 5). In addition, ara-C's dual effects on dCK activity were reversed at lower concentrations in culture than in enzyme kinetics (0.1 μM versus 8 μM ; Fig. 3 and 5). These results suggest the possibility that in the cell the dCK's affinities to MTX and ara-C are higher than those of purified recombinant enzyme. MTX polyglutamates, the intracellular metabolites of MTX (Genestier et al., 2000), may have a higher affinity to dCK than unmodified MTX, which is known to be the case for thymidylate synthase (Allegra et al., 1985). However, MTX di- or tri-glutamate had no apparent effect on dCK activity (data not shown) and the polyglutamation is unlikely to be involved in the dCK's affinity to MTX. Recent studies indicate that dCK is phosphorylated (Wang and Kucera, 1994; Smal et al., 2006) and activated in a calcium-dependent manner (Keszler et al., 2004). Moreover, the dCK activity that is enhanced by 2-chloro-2'-deoxyadenosine, a positive effector of dCK, is returned to its basal level by treatment with protein phosphatase 2A (Smal et al., 2004). Possible post-translational modifications of dCK may change its affinity to substrates and MTX in the cell. Alternatively, the reduced folates are bound intracellularly to a number of different enzymes, which is likely to cause sequestration of folates and/or regulation of enzyme activities (Anguera et al., 2006). It is possible that such complex behaviors of folates affect intracellular MTX

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action.

MTX target proteins were isolated differentially depending on ligand fixation methods. For example, the use of MTX-fixed SGNE-OH beads resulted in an efficient purification of dCK, but not of DHFR. Although this reason is not clear at present, steric hindrance caused by MTX fixation is likely responsible for the low yield of DHFR. In addition, there may be competition between dCK and DHFR for binding to MTX. Further analysis for the MTX's interactions with dCK and DHFR may allow the development of a novel MTX derivative that selectively binds and modulates dCK without affecting DHFR. Such a MTX derivative may be clinically useful, because when used in combination with high-dose ara-C, the MTX derivative would have negligible cytotoxic effects through DHFR in most cells and still enhance ara-C-induced cytotoxicity through dCK, especially in lymphoma/leukemia cells expressing high levels of dCK (Arner and Eriksson, 1995). Moreover, the ability of the MTX derivative to specifically enhance dCK activity may also be particularly useful for the treatment of ara-C-resistant patients in which dCK expression has been reduced (Kakihara et al., 1998).

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Footnotes

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Figure Legends

Figure 1 One-step affinity purification of MTX-binding proteins using SG beads.

(A) Diagrams for MTX fixation to SG beads. Chemical structures of MTX, MTX amino derivative, and functional groups on the surface of derivatized SG beads are shown. (B) THP-1 cytoplasmic proteins that bound to control beads (lane 1) or MTX amino derivative-fixed SGNE-COOH beads (lanes 2, 3) were eluted with SDS and analyzed by SDS-PAGE and silver staining. In lane 3, 100 μ M free MTX was added to the cytoplasmic extracts before purification. An arrowhead indicates a 23 kDa MTX-binding protein. (C) THP-1 cytoplasmic proteins that bound to control beads (lane 1) or MTX-fixed SGNE-OH beads (lanes 2-4) were eluted with SDS and analyzed by SDS-PAGE and silver staining. In lanes 3 and 4, 30 μ M and 100 μ M free MTX was added to the cytoplasmic extracts before purification. An arrowhead indicates a 30 kDa MTX-binding protein. (D) Purification and analysis were performed as in (C) except that elution was carried out with a buffer containing 0 mM (lane 1), 0.1 mM (lane 2), or 1 mM (lane 3) MTX. An arrowhead indicates the 30 kDa MTX-binding protein. (E) Identification of the 23 kDa protein as DHFR. Various eluate fractions (lanes 1, 2, 4-6) shown in (B) and (C), and THP-1 cytoplasmic extracts (lane 3) were subjected to immunoblot analysis using anti-DHFR antibody.

Figure 2 Identification of the 30 kDa MTX-binding protein as dCK.

(A) Amino acid sequence of dCK. Four peptides derived from tryptic digests of the 30 kDa protein are underlined. (B) Endogenous dCK binds to MTX. THP-1 cytoplasmic extracts (lane 1) and cytoplasmic proteins that bound to control beads (lane 2) or MTX-fixed SGNE-OH beads (lanes 3, 4) were analyzed by SDS-PAGE and immunoblotting using anti-dCK antibody. In lane 4, 100 μ M free MTX was added to the cytoplasmic extracts before purification. (C) Recombinant His-dCK binds to MTX. His-dCK that was

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expressed and purified from *E. coli* was subjected to affinity purification and analysis as in (B).

Figure 3 MTX positively and negatively regulates dCK activity. (A, B) dCK activity was measured using 2 to 32 μM dCyd (A), or ara-C (B) as a phosphate acceptor and 1 mM UTP as a phosphate donor in the presence or absence of MTX (0-900 μM). Initial velocities were plotted against initial concentrations of each phosphate acceptor. (C) Reactions containing dCK, one of the phosphate acceptors at 20 μM , 1 mM UTP, and MTX at an indicated concentration were incubated for 15 min at 37°C, and then concentrations of monophosphate products were determined. Data indicate the mean and S.E.M. of three separate experiments.

Figure 4 MTX enhances ara-C-induced cytotoxicity independently of its effects on DHFR. (A) Jurkat cells were treated with various concentrations of ara-C and MTX. After 24 h, cytotoxicity was determined by LDH activity that was released into culture supernatant. Cytotoxicity levels were expressed as percentages of injured cells. (B, C) Jurkat cells were treated with various concentrations of MTX (B) or ara-C (C) in the presence or absence of 100 μM Hx and 16 μM Thd for 36 h, and then cytotoxicity was determined. (D-G) Jurkat cells were treated with various concentrations of ara-C and either MTX (D), folic acid (E), leucovorin (F) or tetrahydrofolate (G) in the presence of 100 μM Hx and 16 μM Thd for 36 h, and then cytotoxicity was determined. (H) HS-Sultan cells (solid lines) were used for the same analysis as in (D), and the results were compared with those of Jurkat cells (broken lines). All the data indicate the mean and S.E.M. of three separate experiments.

Figure 5 MTX differentially affects ara-C incorporation into the chromosome depending on ara-C concentrations. (A, B) Jurkat cells (A) or HS-Sultan cells (B)

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were treated with various concentrations of MTX and [³H] ara-C for several hours. Genomic DNA was then prepared, and radioactive incorporation was determined by liquid scintillation counting. At each concentration of ara-C, radioactive incorporation in the absence of MTX was set to 1. Experiments were performed at least three times, and means and S.E.M.s are shown. The paired t-test was used to compare the extents of ara-C incorporation with or without MTX. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (C) Jurkat and HS-Sultan cell extracts were subjected to immunoblot analysis using anti-actin and anti-dCK antibodies.

Figure 6 Summary of effects of MTX on cytotoxicity.

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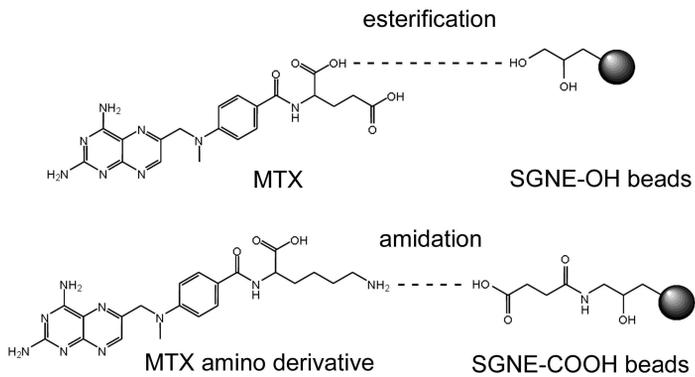
Table I Kinetic parameters of dCK and its inhibition constants for MTX

Substrate	K _m (μM)	V _{max} (μmol/min/mg)	K _i (μM)
dCyd	0.906 ± 0.17	0.0562 ± 0.011	197 ± 19
ara-C	2.55 ± 0.37	0.827 ± 0.24	96.1 ± 15

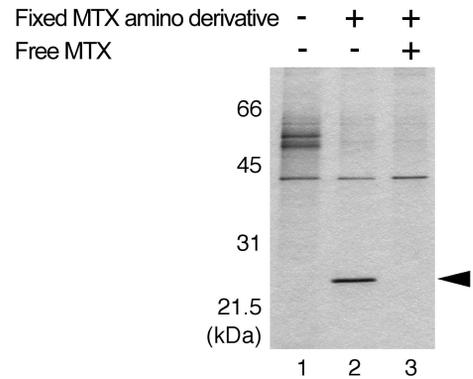
ATP (1 mM) was used as a phosphate donor.

Figure 1

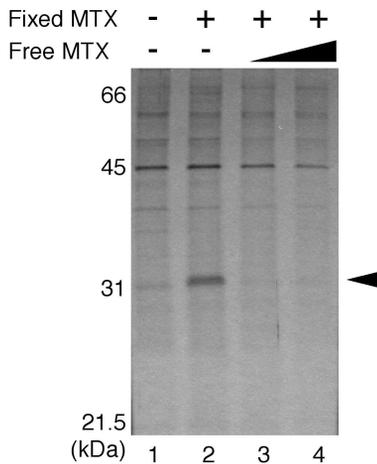
A.



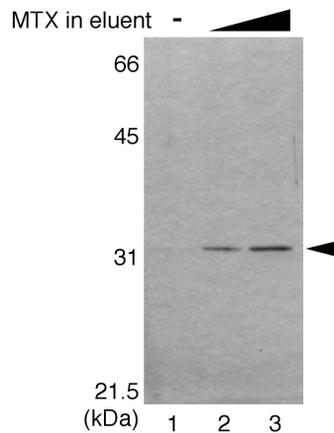
B.



C.



D.



E.

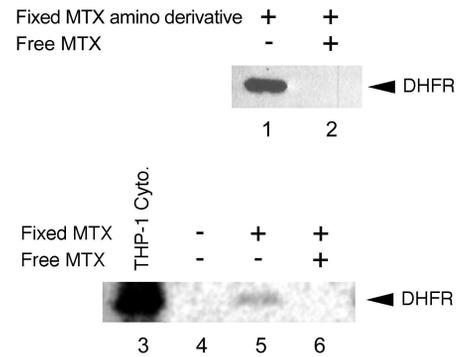
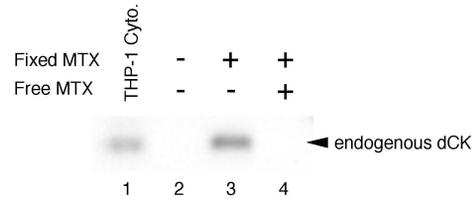


Figure 2

A.

1- MATPPKRSCPSFSASSEGTRIKKISIEGNI -30
 31- AAGKSTFVNILKQLCEDWEVVPEPVARWCN -60
 61- VQSTQDEFEELTMSQKNGGNVLQMMYEKPE -90
 91- RWSFTFQTYACLSRIRAQLASLNGKLDKDAE -120
 121- KPVLEFFERSVYSDRYIFASNLYESECMNET -150
 151- EWTIYQDWDWMNMQFGQSELDGIIYLQA -180
 181- TPETCLHRIYLRGRNEEQGIPLYLEKLHY -210
 211- KHESWLLHRTLKTNFDYLQEVPIILTDVNE -240
 241- DFKDKYESLVEKVKFLSTL -260

B.



C.

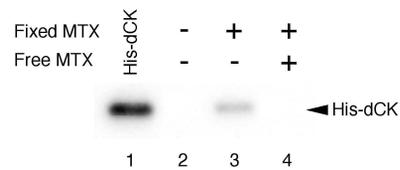


Figure 3

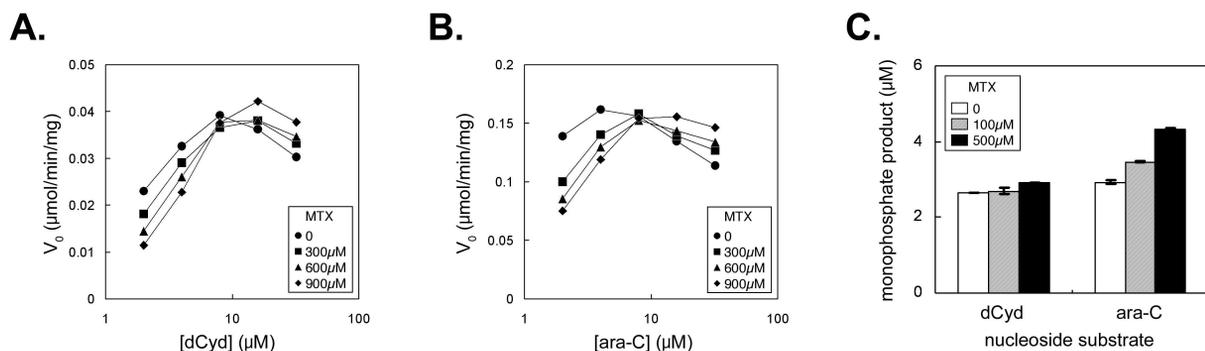


Figure 4

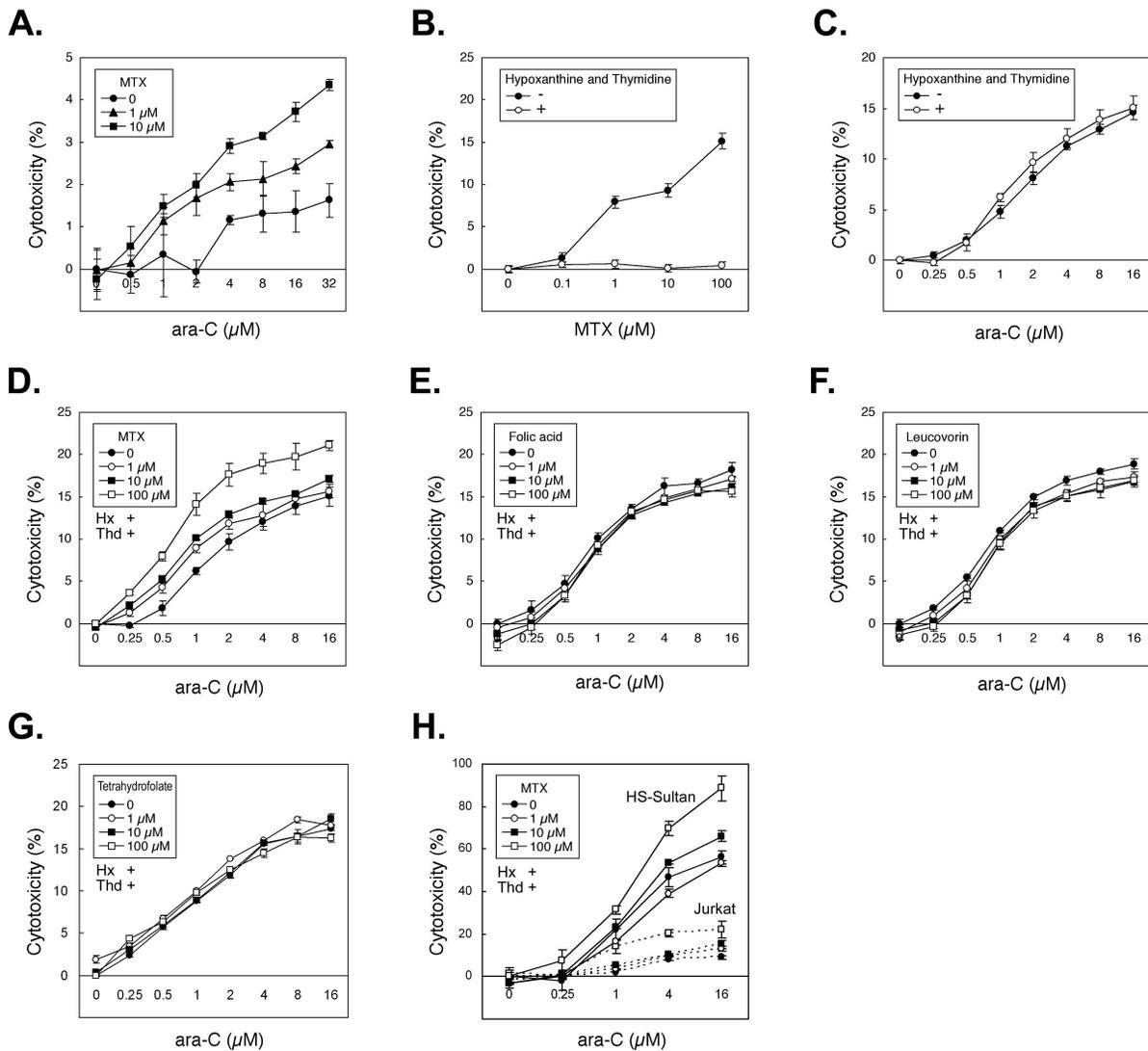


Figure 5

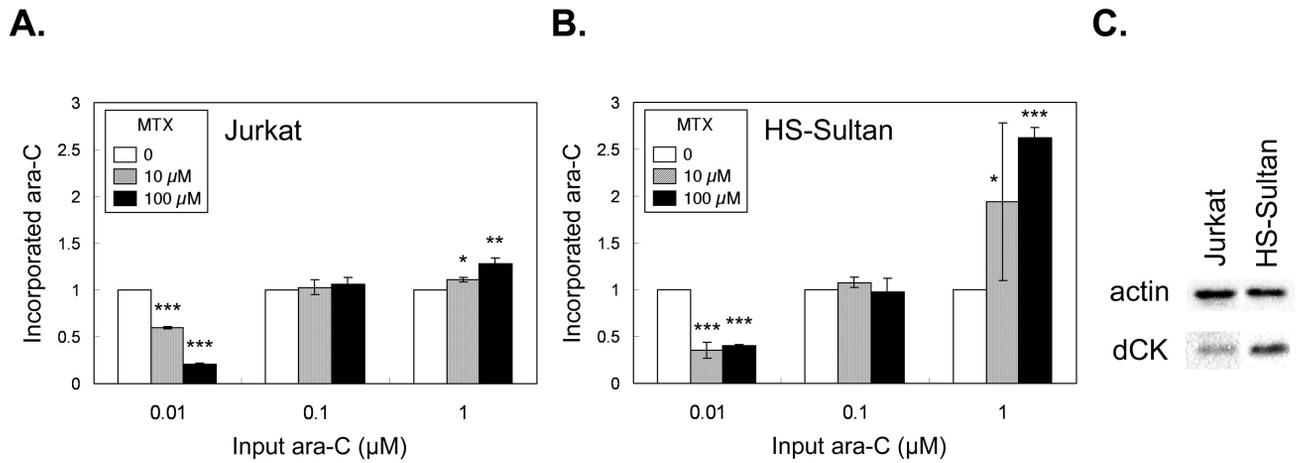


Figure 6