[CANCER RESEARCH 58, 1165–1169, March 15, 1998]

# The Antiproliferative Activity of DMDC Is Modulated by Inhibition of Cytidine Deaminase

**Hiroyuki Eda, Masako Ura, Kaori F.-Ouchi, Yutaka Tanaka, Masanori Miwa, and Hideo Ishitsuka[1]**

*Cytostatics Group, Nippon Roche Research Center, Kamakura, Kanagawa 247-8530, Japan*

## ABSTRACT

**We showed that the efficacy of the new 2′-deoxycytidine (2′-dCyd) analogue antimetabolite 2′-deoxy-2′-methylidenecytidine (DMDC) correlates well with tumor levels of cytidine (Cyd) deaminase in human cancer xenograft models. DMDC was highly effective in tumors with higher levels of Cyd deaminase, whereas lower levels yielded only slight activity. In contrast, gemcitabine (2′,2′-difluorodeoxycytidine), which has action mechanisms similar to those of DMDC, is only slightly active in tumors with higher levels of the enzyme. In the present study, we investigated the roles of Cyd deaminase in the antitumor activity of the two 2′-dCyd antimetabolites in 13 human cancer cell lines. Tetrahydrouridine, an inhibitor of Cyd deaminase, reduced the antiproliferative activity of DMDC ($P = 0.0015$). Furthermore, tumor cells transfected with the gene of human Cyd deaminase become more susceptible to DMDC both *in vitro* and *in vivo*. These results indicate that Cyd deaminase is indeed essential for the activity of DMDC. In contrast, the antiproliferative activity of gemcitabine was increased to some extent by tetrahydrouridine ($P = 0.0277$), particularly in tumor cell lines with higher levels of Cyd deaminase. This suggests that higher levels of Cyd deaminase may inactivate gemcitabine. Among nucleosides and deoxynucleosides tested, only dCyd, a natural substrate of both Cyd deaminase and dCyd kinase, suppressed the antiproliferative activity of DMDC by up to 150-fold. Because the $V_{max}/K_m$ of DMDC for dCyd kinase was 8-fold lower than that for dCyd, the activation of DMDC to DMDC monophosphate (DMDCMP) by dCyd kinase might be competitively inhibited by dCyd. In addition, the dCyd concentrations in human cancer xenografts were inversely correlated with levels of Cyd deaminase activity. It is therefore suggested that higher levels of Cyd deaminase reduce the intrinsic cellular concentrations of dCyd in tumors, resulting in efficient activation of DMDC to DMDCMP by dCyd kinase. These results indicate that the efficacy of DMDC may be predicted by measuring the activity of Cyd deaminase in tumor tissues before treatment starts and that DMDC may be exploited in a new treatment modality: tumor enzyme-driven cancer chemotherapy.**

## INTRODUCTION

DMDC[2] is a new 2′-dCyd analogue antimetabolite with mechanisms of action similar to those of gemcitabine (1–5). Both antimetabolites are incorporated into cells and phosphorylated by enzymes including dCyd kinase (EC 2.7.1.74). The nucleoside triphosphates of these compounds inhibit DNA polymerase and the nucleoside diphosphates inactivate ribonucleotide reductase, a key enzyme for the biosynthesis of deoxyribonucleotides. They are reported to be highly effective against various types of solid tumors in animals (6–8), although their parent compound 1-β-D-arabinofuranosylcytosine is mainly effective against leukemia. We previously observed that these two compounds have different antitumor spectra in human cancer xenograft models (9). DMDC was highly effective in tumors with higher levels of Cyd deaminase (EC 3.5.4.5) but only slightly effective in tumors with lower enzyme levels. In contrast, gemcitabine was less effective in tumors with higher levels of Cyd deaminase. It is therefore of interest to clarify the mechanisms responsible for this difference in efficacy to ensure the rational use of these antineoplastic agents based on tumor levels of Cyd deaminase.

The major characteristic difference between DMDC and gemcitabine is their susceptibility to Cyd deaminase (9–11). Gemcitabine is reported to be susceptible to this enzyme and metabolized to an inactive molecule by deamination, whereas DMDC is highly resistant. The different efficacies of DMDC and gemcitabine in tumors with higher levels of Cyd deaminase might be explained by different susceptibilities to the enzyme. However, it is not yet known why DMDC showed only slight activity in tumors with lower levels of Cyd deaminase. In the present study, we first demonstrated that the susceptibility of tumor cells to DMDC and gemcitabine can be modulated by changing Cyd deaminase activity. We then investigated the roles of Cyd deaminase and its natural substrate dCyd affecting DMDC. This study clearly shows that Cyd deaminase inhibition and Cyd deaminase gene transfection oppositely affect the antitumor activity of DMDC and gemcitabine and suggests that dCyd plays a key role in determining the susceptibility of a tumor to DMDC.

## MATERIALS AND METHODS

**Cells and Culture.** The human tumor cell lines used were obtained from the following institutions: human colon cancer HCT116, COLO205, HT29, DLD-1, WiDr, human pancreatic cancer AsPC-1, and BxPC-3 from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan); human prostate cancer PC-3, human bladder cancer 5637, and Scaber from the American Type Culture Collection (Rockville, MD); human gastric cancer MKN1, MKN28, and MKN45 from Immunobiological Laboratories (Fujioka, Japan); human colon cancer CXF280 and human breast cancer MAXF401 from Prof. H. H. Fiebig, Freiburg University (Freiburg, Germany); human non-small cell lung cancer LX-1 from Dr. Tashiro, the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo, Japan); human ovarian cancer Nakajima from Dr. Adachi, Niigata University School of Medicine (Niigata, Japan); and human bladder cancer T24 from Dr. Akaza, the University of Tsukuba, Institute of Clinical Medicine (Tsukuba, Japan). Human cervical cancer Yumoto clone 17 was established in this laboratory from the original Yumoto line obtained from Dr. Tokita, Chiba Cancer Center (Chiba, Japan; Ref. 12). CXF280, MAXF401, and LX-1 were maintained by continuous passage in BALB/c-nu/nu mice. The other lines were maintained in *in vitro* culture with the following media containing 10–15% fetal bovine serum: HCT116 and HT29 in McCoy's 5A medium; Nakajima in MEM; Scaber and WiDr in MEM supplemented with nonessential amino acids; PC-3 in F12K medium; and AsPC-1, BxPC-3, COLO205, DLD-1, MKN1, MKN28, MKN45, Yumoto clone 17, and T24 in RPMI 1640. We confirmed that all cell lines used were mycoplasma negative with a *Mycoplasma* detection kit (Boehringer Mannheim, Mannheim, Germany).

**Susceptibility of Tumor Cells.** A single suspension of tumor cells (0.7–$3 \times 10^3$ cells/well) was added to the serially diluted DMDC or gemcitabine in a microtest plate. The cells were then cultured at 37°C for 6 days until the cell number in the control culture had increased more than 10-fold. The degree of cell growth was measured with a Cell Counting Kit (DOJINDO, Osaka, Japan).

Received 8/4/97; accepted 1/16/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Cytostatics Group, Nippon Roche Research Center, 200 Kajiwara, Kamakura, Kanagawa 247-8530, Japan. Phone: 81-467-47-2220; Fax: 81-467-45-6782.

[2] The abbreviations used are: DMDC, 2′-deoxy-2′-methylidenecytidine; Cyd, cytidine; dCyd, deoxycytidine; 5′-dFCyd, 5′-deoxy-5-fluorocytidine; DSA, 1-decane sulfonic acid; THU, 3,4,5,6-tetrahydrouridine; 5′-dFUrd, 5′-deoxy-5-fluorouridine.

The $IC_{50}$ of test compounds was expressed as the concentration at which cell growth was inhibited by 50% as compared with the control.

**Cyd Deaminase Assay.** Cultured cancer cells were collected and sonicated in 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM $MgCl_2$, and 50 μM potassium phosphate. This homogenate was then centrifuged at 105,000 × *g* for 90 min. The supernatant was dialyzed overnight at 4°C against 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM β-mercaptoethanol and used as a source of crude Cyd deaminase. All procedures were done at 4°C. The protein concentration was determined by the method of Lowry *et al.* (13). The enzyme activity was determined by measuring 5′-dFUrd and 5-fluorouracil generated from the enzyme substrate 5′-dFCyd. A reaction mixture (100 μl) for the enzyme activity contained 50 mM Tris-acetate buffer (pH 7.4), 2 mM 5′-dFCyd, and crude enzyme (0.08–0.8 mg protein). The reaction was carried out at 37°C for 60 min and then terminated by the addition of 300 μl of methanol. After removal of the precipitate by centrifugation (7,000 × *g* for 3 min.), a portion of the reaction mixture (50 μl) was added to 200 μl of 10 mM sodium phosphate buffer (pH 6.8) containing 20 μM 5-chlorouracil as the internal standard and then applied onto a high-performance liquid chromatography column (ERC-ODS-1171). The solvent system used was as follows: 10 mM sodium phosphate buffer (pH 6.8) containing 5 mM DSA: methanol (85:15; v/v). The amount of 5′-dFUrd and 5-fluorouracil produced was measured by an UV monitor (265 nm). Cyd deaminase activity was expressed as nmol 5′-dFCyd deaminated·mg protein$^{-1}$·min$^{-1}$.

**Cyd Deaminase Gene Transfection.** A full-length human Cyd deaminase cDNA was identified from a human placenta cDNA library as described elsewhere (14). Cyd deaminase cDNA was ligated into the *Hin*dIII site of pRc/CMV (Invitrogen, San Diego, CA). This vector also carries the neomycin-resistant gene as a selection marker. The recombinant plasmid was transfected into the HCT116 single cell clone (HCT116 CB) by using LipofectAMINE reagent (Life Technologies, Inc., Gaithersburg, MD). As a control, HCT116 cell lines were transfected with the vector pRc/CMV that did not contain the Cyd deaminase cDNA. The gene-transfected and control cells were cultured in a selection medium containing 1 mg/ml Geneticin (G-418, Wako pure chemical, Osaka, Japan) and cloned by the limiting dilution method. These isolated clones were confirmed to be free of mycoplasma with a *Mycoplasma* detection kit (Boehringer Mannheim, Germany).

**Animal Experiments.** Five-week-old male nude mice were obtained from SLC Inc. (Hamamatsu, Japan). Vector- or Cyd deaminase gene-transfected human colon cancer HCT116 cells were inoculated s.c. into the nude mice ($6.5 \times 10^6$ cells/mouse). DMDC was dissolved in 5% gum arabic and administered p.o. to mice daily 5 times a week for 2 weeks, whereas gemcitabine was dissolved in saline (pH 7.0) and administered i.v. to mice twice a week for 2 weeks. Drug administration was started on the day when the tumor volumes reached approximately 300 $mm^3$. The length (*a*) and width (*b*) of the tumors were measured, and the volume was estimated by using the following formula: volume ($mm^3$) = *a* (mm) × $b^2$ (mm)/2.

**dCyd Concentration in Tumor Tissues.** dCyd concentrations were measured as described elsewhere (15, 16) with minor modifications. Tumor tissues were homogenized in 10% trichloroacetic acid (20% w/v). The supernatant (4 ml) was shaken with 8 ml of dichloromethane/tri-*n*-octylamine (100/15; v/v) and then centrifuged at 1500 × *g* for 5 min. The supernatant (3.5 ml) containing dCyd and dCyd nucleotides was applied to a Sep Pak Plus QMA cartridge to remove dCyd nucleotides and eluted with 5 ml of distilled water. The pass-through fraction was evaporated under reduced pressure, and the residue was dissolved in 2 ml of a buffer containing 2.5 mM $KH_2PO_4$ and 5 mM DSA. The solution was applied to a Bond Elute $C_{18}$ cartridge and eluted with 1 ml of 4% (v/v) methanol in the same buffer and 1 ml of 50% (v/v) methanol in the same buffer. The dCyd fraction (pass-through) was then applied to a Sep Pak Plus QMA cartridge to remove DSA and was eluted with 5 ml of distilled water. The pass-through fraction was evaporated under reduced pressure. The residue was dissolved in 400 μl of 25% (v/v) methanol and filtered through a 0.45 μm filter. A portion of the solution was diluted with distilled water, and injected on to an high-performance liquid chromatography column (ERC-ODS-1171). The solvent system used was as follows: 2.5 mM $KH_2PO_4$ containing 5 mM DSA:methanol (80:20). The amount of dCyd was measured by an UV monitor (265 nm).

Table 1 *Effect of THU on the antiproliferative activity of DMDC and gemcitabine and the activities of Cyd deaminase and dCyd kinase in human cancer cell lines*

Cells were plated into 96-well plates ($0.8–3 \times 10^3$ cells) and incubated for 6 days. The degree of cell growth was measured with a Cell Counting Kit (DOJINDO). The $IC_{50}$ of test compounds was expressed as the concentration at which cell growth was inhibited by 50% as compared with the control. Cell growth inhibition of THU alone: −3.13 ± 3.67%.

| Cell line | Drug | $IC_{50}$ (nM), mean ± SD | | | Cyd deaminase activity (nmol/mg/min), mean ± SD | dCyd kinase activity (nmol/mg/hr), mean ± SD |
|---|---|---|---|---|---|---|
| | | THU[a] (−) | THU (+) | THU (+/−) | | |
| Scaber (Bladder) | Gemcitabine | 23.9 ± 0.94 | 7.72 ± 1.27 | 0.32 | 14.7 ± 1.51 | 1.337 ± 0.114 |
| | DMDC | 135 ± 13.5 | 1100 ± 45.7 | 8.13 | | |
| AsPC-1 (Pancreas) | Gemcitabine | 18.9 ± 5.51 | 10.8 ± 2.11 | 0.57 | 11.8 ± 0.35 | 1.541 ± 0.291 |
| | DMDC | 52.7 ± 19.7 | 500 ± 100 | 9.49 | | |
| PC-3 (Prostate) | Gemcitabine | 8.56 ± 0.67 | 3.65 ± 0.52 | 0.43 | 7.54 ± 0.37 | 1.246 ± 0.106 |
| | DMDC | 125 ± 6.2 | 510 ± 20.7 | 4.07 | | |
| BxPC-3 (Pancreas) | Gemcitabine | 5.86 ± 0.70 | 4.26 ± 0.18 | 0.73 | 5.78 ± 0.33 | 1.344 ± 0.086 |
| | DMDC | 54.4 ± 2.06 | 432 ± 80.9 | 7.94 | | |
| 5637 (Bladder) | Gemcitabine | 6.09 ± 0.81 | 4.05 ± 0.20 | 0.67 | 4.13 ± 0.04 | 1.190 ± 0.056 |
| | DMDC | 167 ± 8.6 | 806 ± 127.8 | 4.82 | | |
| MKN1 (Gastric) | Gemcitabine | 6.40 ± 1.02 | 5.62 ± 0.54 | 0.88 | 4.03 ± 0.08 | 1.184 ± 0.333 |
| | DMDC | 190 ± 34.6 | 674 ± 270.6 | 3.55 | | |
| COLO205 (Colon) | Gemcitabine | 21.9 ± 3.03 | 9.06 ± 0.57 | 0.41 | 3.40 ± 0.18 | 2.111 ± 0.064 |
| | DMDC | 148 ± 4.9 | 342 ± 13.6 | 2.32 | | |
| MKN45 (Gastric) | Gemcitabine | 1.78 ± 0.22 | 2.12 ± 0.14 | 1.19 | 3.04 ± 0.12 | 1.071 ± 0.084 |
| | DMDC | 71.9 ± 7.26 | 164 ± 46.1 | 2.28 | | |
| HCT116 (Colon) | Gemcitabine | 4.55 ± 0.15 | 4.69 ± 0.30 | 1.03 | 2.35 ± 0.04 | 2.029 ± 0.134 |
| | DMDC | 196 ± 18.2 | 290 ± 30.1 | 1.48 | | |
| MKN28 (Gastric) | Gemcitabine | 14.6 ± 2.26 | 17.4 ± 0.53 | 1.20 | 0.64 ± 0.01 | 0.297 ± 0.064 |
| | DMDC | 3580 ± 248.5 | 5830 ± 1090 | 1.62 | | |
| Yumoto C-17 (Cervix) | Gemcitabine | 3.67 ± 0.34 | 2.74 ± 0.21 | 0.75 | 0.40 ± 0.02 | 0.854 ± 0.086 |
| | DMDC | 201 ± 16.4 | 248 ± 18.7 | 1.23 | | |
| HT29 (Colon) | Gemcitabine | 7.63 ± 0.26 | 7.77 ± 0.32 | 1.02 | 0.07 ± 0.00 | 1.035 ± 0.175 |
| | DMDC | 695 ± 29.0 | 708 ± 60.7 | 1.02 | | |
| DLD-1 (Colon) | Gemcitabine | 9.66 ± 0.92 | 8.57 ± 0.73 | 0.89 | 0.07 ± 0.01 | 0.954 ± 0.048 |
| | DMDC | 760 ± 103.5 | 804 ± 60.2 | 1.06 | | |

[a] Inhibitor of Cyd deaminase; 50 μM.

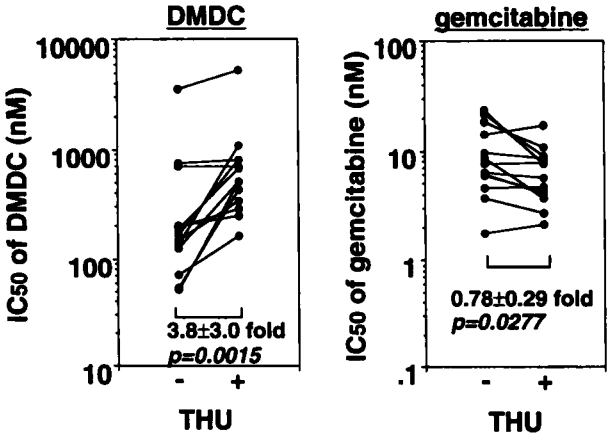


Fig. 1. Modulation of the antiproliferative activity of DMDC and gemcitabine by THU, an inhibitor of Cyd deaminase, in 13 human cancer cell lines. *Data points*, means of three determinations.

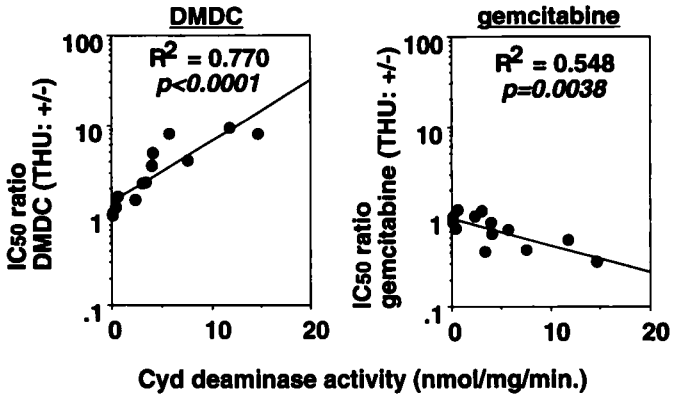


Fig. 2. THU modulates the efficacy of DMDC and gemcitabine to a greater extent in human cancer cell lines with higher levels of Cyd deaminase. *Data points*, means of three determinations.

**dCyd Kinase Assay.** The enzyme assay and the kinetic study were carried out as described elsewhere (17). Briefly, the standard reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 7.6), 10 μl of cell supernatant, 7 mM $MgCl_2$, 2 mM DTT, 7 mM ATP, 1 mM phosphoenol pyruvate, 10 IU of pyruvate kinase, 10 mM NaF, 0.4 mM THU (inhibitor of Cyd deaminase; Ref. 18), 0.5–400 μM [2-$^{14}$C]-dCyd (125 or 2072 MBq/mmol), or 10–320 μM [methylidene-$^{14}$C]DMDC (56.1 or 740 Mbq/mmol). Enzyme sources were prepared by the same method as for the Cyd deaminase assay. The cell supernatant fraction of HCT116 was used for the enzyme kinetic study. This mixture was incubated for 5–60 min at 37°C. The reaction was terminated by adding an equal volume of cold methanol, and the precipitated protein was sedimented by centrifugation. A sample of the supernatant (50 μl) was applied to DE81 DEAE paper. Unreacted dCyd or DMDC was removed by three successive washes in 10 ml of 5 mM ammonium formate for each DEAE paper, followed by a 5-min wash in distilled water and then in 95% ethanol. After being dried, the discs were placed in scintillation vials and counted in 5 ml of Aquasol. The kinetics parameters were determined from double reciprocal plots of enzyme activity as a function of changing substrate concentration.

**Reagents.** Gemcitabine and DMDC were obtained from Dr. D. Keith, Hoffmann La-Roche, Inc. (Nutley, NJ) and from Yamasa Co. (Choshi, Chiba, Japan), respectively. THU was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA). Other chemicals were purchased commercially. [2-$^{14}$C]dCyd was purchased from Amersham Corp. (Tokyo, Japan). [methylidene-$^{14}$C]DMDC was obtained from Yoshitomi Pharmaceutical Industries Ltd. (Osaka, Japan).

**Statistics.** Statistical analyses were performed using the Bonferroni/Dunn test, simple regression analysis, the Wilcoxon test, and the Mann-Whitney *U* test. Differences were considered to be significant at $P < 0.05$.

## RESULTS

**Susceptibility of Tumor Cells Modulated by a Cyd Deaminase Inhibitor.** To validate our previous finding of the difference in susceptibility of tumors to DMDC and gemcitabine (9), we tried to demonstrate that their antiproliferative activity is oppositely modified by the Cyd deaminase inhibitor THU. Table 1 and Fig. 1 show $IC_{50}$s of DMDC and gemcitabine against 13 human cancer cell lines in the presence or absence of THU. The susceptibility to DMDC was decreased by the Cyd deaminase inhibitor by 3.8-fold (mean; $P = 0.0015$). In contrast, the inhibitor increased the susceptibility to gemcitabine to some extent (0.78 ± 0.29-fold; $P = 0.0277$), probably by inhibiting the deamination of the compound. Fig. 2 shows a comparison of the degree to which susceptibility to DMDC and gemcitabine is modulated by THU and Cyd deaminase activity in the human cancer cell lines. The effect of the modulation, either the decrease in susceptibility to DMDC or the increase in that to gemcitabine, is more obvious in those tumor cell lines with higher levels of Cyd deaminase activity. There was no correlation between the degree of the susceptibility modulation by THU and dCyd kinase activity in these cell lines.

Table 2 *Antiproliferative activity of DMDC and gemcitabine in HCT116 cell lines transfected with the Cyd deaminase gene*

Cells were plated into 96-well plates ($0.7 \times 10^3$ cells) and incubated for 6 days. The degree of cell growth was measured with a Cell Counting Kit (DOJINDO). The $IC_{50}$ of test compounds was expressed as the concentration at which cell growth was inhibited by 50% as compared with the control.

| Cell line | $IC_{50}$ (nM), mean ± SD | | Cyd deaminase activity (nmol/mg/min), mean ± SD |
|---|---|---|---|
| | DMDC | Gemcitabine | |
| Parent clone | | | |
| HCT116 CB | 190.47 ± 10.26 | 4.71 ± 0.14 | 2.02 ± 0.09 |
| Vector transfectant | | | |
| CBV14 | 136.43 ± 8.71 | 4.00 ± 0.13 | 1.86 ± 0.05 |
| CBV70 | 153.83 ± 2.39 | 4.17 ± 0.12 | 2.84 ± 0.11 |
| Cyd deaminase transfectant | | | |
| CBC212a | 41.69 ± 4.71 | 4.28 ± 0.12 | 15.47 ± 2.29 |
| CBC700a | 52.95 ± 2.44 | 3.86 ± 0.21 | 13.63 ± 0.39 |
| CBC106a | 82.37 ± 2.52 | 3.13 ± 0.26 | 7.50 ± 0.31 |
| CBC104b | 93.86 ± 11.63 | 5.19 ± 0.18 | 5.56 ± 0.12 |

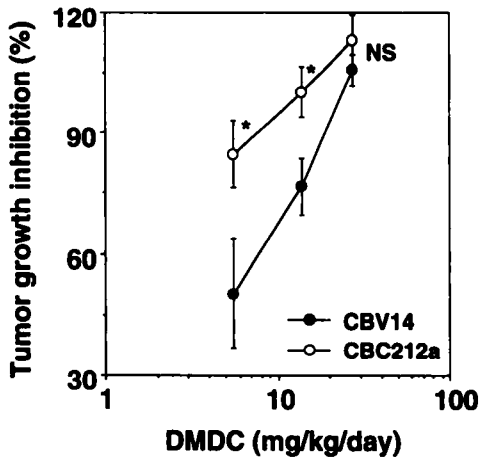


Fig. 3. Antitumor activity of DMDC in human colon cancer HCT116 transfected with the Cyd deaminase gene. ●, HCT116 CBV14 (vector transfectant); ○, HCT116 CBC212a (Cyd deaminase transfectant). Vector-transfected or Cyd deaminase gene-transfected human colon cancer HCT116 cells were inoculated s.c. into the nude mice ($6.5 \times 10^6$ cells/mouse). DMDC was administered p.o. five times a week for 2 weeks. Drug administration was started on the day when the tumor volumes reached approximately 300 $mm^3$. The length (*a*) and width (*b*) of the tumors were measured, and the volume was estimated by using the following formula: volume ($mm^3$) = *a* (mm) × $b^2$ (mm)/2. *, $P < 0.05$ compared with CBV14; *NS*, not significant (Bonferroni/Dunn test).

Table 3 *dCyd levels in human cancer xenografts are inversely correlated with levels of Cyd deaminase activity*

| Human cancer xenografts | Cyd deaminase activity (nmol/mg/min), mean ± SD | dCyd level (nmol/g tissue) | Efficacy of DMDC[a] |
|---|---|---|---|
| AsPC-1 | 15.8 ± 2.3 | <0.4 | ++ |
| CXF280 | 3.0 ± 1.0 | <0.4 | ++ |
| HCT116 | 2.9 ± 0.6 | <0.4 | ++ |
| HT29 | 2.8 ± 1.6 | <0.4 | + |
| LX-1 | 1.9 ± 0.5 | <0.4 | ++ |
| BxPC-3 | 1.8 ± 0.7 | <0.4 | + |
| DLD-1 | 0.34 ± 0.03 | <0.4 | − |
| Yumoto C-17 | 0.20 ± 0.06 | 0.4 ± 0.11 | − |
| Nakajima | 0.022 ± 0.002 | 3.5 ± 2.7 | − |
| MAXF401 | 0.015 ± 0.002 | 2.5 ± 0.49 | + |
| T24 | <0.013 | 12.9 ± 2.3 | − |

[a] Efficacy is expressed in terms of therapeutic index. Therapeutic index = most tolerated dose/$ED_{50}$. Therapeutic index: ++, ≥10; +, <10 and ≥2; −, <2.

**Modulation of the Susceptibility by Cyd Deaminase Gene Transfection Both *in Vitro* and *in Vivo*.** We investigated whether Cyd deaminase gene transfection modifies the susceptibility of recipient tumor cells to DMDC and gemcitabine. In the HCT116 human colon cancer cells, the Cyd deaminase gene transfection increased the enzyme activity by several-fold to 8-fold as compared with that of the cells transfected with vector alone (Table 2). In such Cyd deaminase gene transfectants, the susceptibility to DMDC was increased by 2–4-fold and was significantly correlated with the level of Cyd deaminase activity ($R^2$=0.968). In contrast, the susceptibility to gemcitabine was not changed by the gene transfection. In an *in vivo* experiment, the tumor transfected with Cyd deaminase gene (HCT116 CBC212a) also became more susceptible to DMDC than the tumor transfected with the vector alone (HCT116 CBV14; Fig. 3), whereas the susceptibility to gemcitabine was not changed by Cyd deaminase gene transfection (data not shown).

**Inverse Correlation between Cyd Deaminase Activity and dCyd Levels in Tumors.** One possible role of Cyd deaminase in modulating the efficacy of DMDC is to reduce the concentration of a natural substrate, dCyd, which is a competitive inhibitor of the DMDC activation by dCyd kinase. To ascertain this possibility, we investigated the correlation between dCyd levels and Cyd deaminase activity. Table 3 shows a comparison of these two factors in the human cancer xenografts used in the previous study (9). There appears to be inverse correlation between these factors; *i.e.*, the higher the Cyd deaminase levels, the lower the dCyd concentrations. Cyd deaminase might metabolize dCyd to yield lower levels of this natural substrate in tumors.

We also found that dCyd indeed inhibits the antiproliferative activity of DMDC in three representative human cancer cell lines (Table 4). We determined $IC_{50}$s of DMDC in the presence and absence of pyrimidines and calculated the degree to which the antiproliferative activity of DMDC had been reduced. Among various nucleosides and deoxynucleosides tested, only dCyd greatly reduced the antiproliferative activity of DMDC by up to 150-fold in HCT116 and DLD-1 cells. However, dCyd reduced the antiproliferative activity of DMDC only 6-fold in PC-3 cells, which have a high level of Cyd deaminase activity. In contrast, other pyrimidines reduced or enhanced the activity only slightly. Purines also reduced or enhanced the activity only slightly (data not shown).

**Substrate Activity for dCyd Kinase.** We investigated the reason why the efficacy of DMDC is so affected by Cyd deaminase activity in tumors. Table 5 shows parameters of enzyme kinetics for dCyd kinase, by which dCyd and DMDC are activated to their corresponding nucleoside monophosphates. The $V_{max}/K_m$ of DMDC for dCyd kinase was 8-fold lower than that of dCyd. In addition, the $K_i$ of DMDC for the enzyme was higher than that of gemcitabine (433 *versus* 74 μM). Thus, activation of DMDC to DMDCMP would be affected by dCyd to a greater extent than that of gemcitabine to its monophosphate form.

## DISCUSSION

We observed in the previous study that the new nucleoside antimetabolites DMDC and gemcitabine have different antitumor spectra (9), although their mechanisms of action are reported to be similar (1–5). The efficacy of DMDC was well correlated with Cyd deaminase levels in tumors. In contrast, gemcitabine was less effective in tumors with higher levels of the enzyme. In the present study, we observed that the susceptibilities of tumor cells to DMDC and gemcitabine are changed by modulating Cyd deaminase activity. An inhibitor of Cyd deaminase, THU, decreased the susceptibility of tumor cells to DMDC, but it increased the susceptibility to gemcitabine. In addition, the relative increase in enzyme activity by resulting from transfection of the enzyme gene made recipient tumor cells more susceptible to DMDC both *in vitro* and *in vivo*. These results validated our previous findings that Cyd deaminase activity is essential for the efficacy of DMDC, whereas it is a negative factor for gemcitabine efficacy.

It has been reported that tumor cells become resistant or refractory to 2′-dCyd analogues, including gemcitabine and DMDC, as a result of losing their dCyd kinase activity (19, 20), by which 2′-dCyd analogues are phosphorylated. However, we observed no positive correlation between dCyd kinase activity and the susceptibility of human cancer cell lines to either DMDC or gemcitabine (9). The present study suggested that Cyd deaminase activity in tumor cells and tissues affects their

Table 5 *Phosphorylation kinetics of dCyd and DMDC for dCyd kinase*

A cell supernatant fraction of HCT116 human colon cancer cells was used for the enzyme kinetic study. The kinetics parameters were determined from double reciprocal plots of enzyme activity as a function of changing substrate concentration.

| Substrates | $K_m$ (μM) | $V_{max}$ (pmol/mg protein/min) | $V_{max}/K_m$ |
|---|---|---|---|
| dCyd | 2.26 | 43.0 | 19.0 |
| DMDC | 32.4 | 75.0 | 2.31 |

Table 4 *dCyd reduces antiproliferative activity of DMDC*

Cells were plated into 96-well plates (0.8–3 × $10^3$ cells) and incubated for 6 days. The degree of cell growth was measured with a Cell Counting Kit (DOJINDO). The $IC_{50}$ of test compounds was expressed as the concentration at which cell growth was inhibited by 50% as compared with the control. Cell growth inhibition rates of nucleoside or deoxynucleoside alone (50 μM) were lower than 8% except for thymidine against PC-3 cell (28.6% of growth inhibition).

| Nucleosides (50 μM) | $IC_{50}$ of DMDC, nM (Fold: $IC_{50}$ of DMDC with nucleoside/$IC_{50}$ of DMDC alone) | | |
|---|---|---|---|
| | HCT116 | DLD-1 | PC-3 |
| None | 157.7 ± 5.8 | 694.4 ± 168.6 | 100.3 ± 7.6 |
| Deoxycytidine | 16760 ± 1430 (106.34)[a] | 99730 ± 10860 (147.39)[a] | 619.3 ± 65.4 (6.23)[a] |
| Cytidine | 333.5 ± 27.6 (2.11) | 1389.0 ± 214.0 (2.04) | 114.3 ± 9.7 (1.14) |
| Deoxyuridine | 87.6 ± 7.6 (0.56) | 608.0 ± 37.1 (0.91) | 118.8 ± 8.9 (1.19) |
| Uridine | 140.0 ± 9.7 (0.89) | 548.4 ± 55.3 (0.82) | 98.7 ± 6.1 (0.99) |
| Thymidine | 67.8 ± 3.6 (0.43) | 842.2 ± 97.2 (1.24) | 127.0 ± 42.7 (1.29) |

[a] $P > 0.05$ *versus* DMDC alone (Bonferroni/Dunn test).

susceptibility to DMDC. DMDC and dCyd are phosphorylated to their corresponding nucleoside monophosphates by dCyd kinase. However, dCyd should competitively inhibit the activation of DMDC to DMDCMP by this enzyme, because the $V_{max}/K_m$ of DMDC is much smaller than that of dCyd. In fact, we observed in the present study that dCyd greatly reduced the antiproliferative activity of DMDC. Therefore, it is likely that Cyd deaminase reduces the concentration of dCyd and consequently increases the susceptibility of tumor cells and tumor tissues to DMDC. This possibility is supported by observation that the dCyd concentrations in tumors are inversely correlated with the levels of Cyd deaminase activity. Conversely, dCyd may only slightly affect the efficacy of gemcitabine, because the $V_{max}/K_m$ of gemcitabine for dCyd kinase reaction is comparable to that of dCyd (21).

In separate experiments, only gemcitabine is susceptible to Cyd deaminase, but DMDC was highly resistant to this enzyme (2). The $V_{max}/K_m$ for Cyd deaminase for dCyd, gemcitabine, and DMDC is 559, 117, and 0.159, respectively (9). Therefore, it would naturally follow that gemcitabine is less effective in tumors with higher levels of the enzyme, probably because of its inactivation by the enzyme. Neff and Blau (22) reported the Cyd deaminase gene transfection conferred resistance to gemcitabine by up to 3-fold. In the present study, however, the susceptibility of the human colon cancer cell line HCT116 to gemcitabine was not decreased by Cyd deaminase gene transfection. The enzyme up-regulation alone may not be sufficient for this tumor cell line to become resistant to gemcitabine. Another explanation is that this cell line may have high dCyd kinase activity. Because dCyd kinase has a higher affinity for gemcitabine than does Cyd deaminase (10), it is more likely that gemcitabine was phosphorylated than deaminated in the cell line used in the present study.

We have indicated that Cyd deaminase activity in tumors may be a useful factor for predicting the efficacy of DMDC. There are few cytotoxic agents of which the activities have been well correlated with tumor levels of particular enzymes in animal tumor models. DMDC is one of these cytotoxic agents, and its efficacy should be clinically assessed in types of cancers expressing higher levels of Cyd deaminase activity. If DMDC proves to be highly effective in such cancers, this unique characteristic will provide us with new treatment strategies. That is, the efficacy could be predicted by measuring Cyd deaminase activity in tumors before treatment starts, or the efficacy of DMDC could be optimized by selecting patients based on the Cyd deaminase levels in tumors. This tumor enzyme level-driven chemotherapy could also avoid unnecessary treatment with DMDC. Our further investigation into the preclinical efficacy of DMDC will focus on using its mechanisms of action to identify new methods of optimizing its efficacy, thereby increasing its value as a therapeutic agent within a novel disease management strategy.

## REFERENCES


1. Baker, C. H., Banzon, J., Bollinger, J. M., Stubbe, J., Samano, V., Robins, M. J., Lippert, B., Jarvi, E., and Resvick, R. 2′-Deoxy-2′-methylenecytidine and 2′-deoxy-2′, 2′-difluorocytidine 5′-diphosphates: potent mechanism-based inhibitors of ribonucleotide reductase. J. Med. Chem., *34:* 1879–1884, 1991.
2. Matsuda, A., Azuma, A., Nakajima, Y., Takenuki, K., Dan, A., Iino, T., Yoshimura, Y., Minakawa, N., Tanaka, M., and Sasaki, T. Design of new types of antitumor nucleosides: the synthesis and antitumor activity of 2′-deoxy-(2′-*C*-substituted) cytidines. *In:* C. K. Chu and D. C. Baker (eds.), Nucleosides and Nucleotides as Antitumor and Antiviral Agents, pp. 1–22. New York: Plenum Press, 1993.
3. Heinemann, V., Hertel, L. W., Grindey, G. B., and Plunkett, W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. Cancer Res., *48:* 4024–4031, 1988.
4. Huang, P., Chubb, S., Hertel, L. W., Grindey, G. B., and Plunkett, W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. Cancer Res., *51:* 6110–6117, 1991.
5. Heinemann, V., Xu, Y-Z., Chubb, S., Sen, A., Hertel, L. W., Grindey, G. B., and Plunkett, W. Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2′, 2′-difluorodeoxycytidine. Mol. Pharmacol., *38:* 567–572, 1990.
6. Braakhuis, B. J. M., van Dongen, G. A. M. S., Vermorken, J. B., and Snow, G. B. Preclinical *in vivo* activity of 2′,2′-difluorodeoxycytidine (gemcitabine) against human head and neck cancer. Cancer Res., *51:* 211–214, 1991.
7. Hertel, L. W., Boder, G. B., Stan Kroin, J., Rinzel, S. M., Poore, G. A., Todd, G. C., and Grindey, G. B. Evaluation of the antitumor activity of gemcitabine (2′, 2′-difluorodeoxycytidine). Cancer Res., *50:* 4417–4422, 1990.
8. Yamagami, K., Fujii, A., Arita, M., Okumoto, T., Sakata, S., Matsuda, A., Ueda, T., and Sasaki, T. Antitumor activity of 2′-deoxy-2′-methylidenecytidine, a new 2′-deoxycytidine derivative. Cancer Res., *51:* 2319–2323, 1991.
9. Ishitsuka, H., Miwa, M., Eda, H., Ura, M., Ouchi, K., Foley, L. H., and Keith, D. D. High susceptibility of human cancer xenografts with higher levels of cytidine deaminase to a new 2′-deoxycytidine antimetabolite DMDC. Proc. Am. Assoc. Cancer Res., *38:* 315, 1997.
10. Bouffard, D. Y., Laliberte, J., and Momparler, R. L. Kinetic studies on 2′,2′-difluorodeoxycytidine (gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. Biochem. Pharmacol., *45:* 1857–1861, 1993.
11. Takenuki, K. Matsuda, A., Ueda, T., Sasaki, T., Fujii, A., and Yamagami, K. Design, synthesis, and antineoplastic activity of 2′-deoxy-2′-methylidenecytidine. J. Med. Chem., *31:* 1063–1064, 1988.
12. Tamura, S., Fujimoto-Ouchi, K., Mori, K., Endo, M., Matsumoto, T., Eda, H., Tanaka, Y., Ishitsuka, H., Tokita, H., and Yamaguchi, K. Involvement of human interleukin 6 in experimental cachexia induced by a human uterine cervical carcinoma xenograft. Clin. Cancer Res., *1:* 1353–1358, 1995.
13. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265–275, 1951.
14. Watanabe, S-I., and Uchida, T. Expression of cytidine deaminase in human solid tumors and its regulation by 1α,25-dihydroxyvitamin $D_3$. Biochim. Biophys. Acta, *1312:* 99–104, 1996.
15. Chan, T., Lakhchaura, B. D., and Hsu, T-F. Differences in deoxycytidine metabolism in mouse and rat. Biochem J., *210:* 367–371, 1983.
16. Pillwein, K., Jayaram, H. N., and Weber, G. Effect of ishema on nucleosides and bases in rat liver and hepatoma 3294A. Cancer Res., *47:* 3092–3096, 1987.
17. Harkrader, R. J., Jackson, R. C., Ross, D. A., and Weber, G. Increase in liver and kidney deoxycytidine kinase activity linked to neoplastic transformation. Biochem. Biophys. Res. Commun., *96:* 1633–1639, 1980.
18. Hanze, A. R. Nucleic Acids IV. The catalytic reduction of pyrimidine nucleosides (human liver deaminase inhibitors). J. Am. Chem. Soc., *89:* 6720–6725, 1967.
19. Ruiz van Haperen, V. W. T., Veerman, G., Eriksson, S., Boven, E., Stegmann, A. P. A., Hermsen, M., Vermorken, J. B., Pinedo, H. M., and Peters, G. J. Development and molecular characterization of a 2′, 2′-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. Cancer Res., *54:* 4138–4143, 1994.
20. Miura, S., Tanaka, M., Yoshimura, Y., Satoh, H., Sakata, S., Machida, H., Matsuda, A., and Sasaki, T. *In vitro* and *in vivo* antitumor activity of a novel nucleoside, 4′-thio-2′-deoxy-2′-methylidenecytidine. Biol. Pharm. Bull. (Tokyo), *19:* 1311–1315, 1996.
21. Plunkett, W., Huang, P., Searcy, C. E., and Gandhi, V. Gemcitabine: preclinical pharmacology and mechanisms of action. Semin. Oncol., *23* (Suppl. 10): 3–15, 1996.
22. Neff, T., and Blau, C. A. Forced expression of cytidine deaminase confers resistance to cytosine arabinoside and gemcitabine. Exp. Hematol., *24:* 1340–1346, 1996.