High Susceptibility of Human Cancer Xenografts with Higher Levels of Cytidine Deaminase to a 2'-Deoxycytidine Antimetabolite, 2'-Deoxy-2'-methylidenecytidine

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ABSTRACT

2'-Deoxy-2'-methylidenecytidine (DMDC) is a new 2'-deoxycytidine (dCyd) antimetabolite. The present study compared its antitumor activities with those of 2',2'-difluorodeoxycytidine (gemcitabine) in 15 human cancer xenograft models. DMDC was highly resistant to cytidine (Cyd) deaminase, which deaminates the dCyd analogues to inactive molecules, whereas gemcitabine was susceptible to the enzyme. Given p.o., high antitumor activity with therapeutic index of more than 10 was found with DMDC in 7 of 15 xenograft lines. In contrast, gemcitabine given i.v. or p.o. was highly effective in 4 of 15 human cancer xenograft lines. The antitumor spectrum of these compounds was quite different, although their molecular targets are reported to be similar. DMDC was highly effective in tumors with higher levels of Cyd deaminase activity, whereas it showed only slight activity in those with lower levels of Cyd deaminase. In contrast, gemcitabine appeared to be less effective in tumors with high levels of Cyd deaminase. We also investigated the correlation with the susceptibility of the two dCyd antimetabolites and dCyd kinase activity in tumors, but none was observed. Cyd deaminase activity was found to be high in tumor tissues from various types of human cancers thus far tested, such as colorectal cancer and non-small cell lung cancer. Such cancer types or individual patients who have tumors with high activity of the enzyme may be targets for DMDC therapy.

INTRODUCTION

The dCyd2 analogue gemcitabine (2',2'-difluorodeoxyctydine) is reported to have broad antitumor spectrum against solid tumors in animals (1, 2), although its parent drug Ara-C is effective mainly against leukemia (3). Gemcitabine, as well as Ara-C, inhibit DNA polymerases after their conversion to the nucleoside triphosphate form (4, 5). Gemcitabine also inactivates ribonucleotide reductase, an activity not found with Ara-C (6). In clinical trials, gemcitabine has been shown to be effective in pancreatic cancer, NSCLC, and breast cancer (7–9). DMDC, a dCyd analogue of very similar chemical structure to that of gemcitabine, also inhibits DNA polymerase and inactivates ribonucleotide reductase and has a broad antitumor spectrum against solid tumors in animals (10–12). A major characteristic difference between gemcitabine and DMDC is susceptibility to Cyd deaminase; gemcitabine is metabolized to the inactive molecule by Cyd deaminase (13), whereas DMDC is reported to be resistant to this enzyme (14).

Cyd deaminase activity was reported to be high in various types of cancer in humans (15), suggesting that DMDC may have broader antitumor activity in patients than does gemcitabine. Therefore, it was of interest to compare the antitumor spectrum of these compounds with different susceptibilities to Cyd deaminase. In the present study, we confirmed that DMDC is indeed highly resistant to the enzyme, and then we compared the antitumor activity of these dCyd analogues in human cancer xenografts with various levels of Cyd deaminase. The results show that they have different antitumor spectra based on the Cyd deaminase activity in tumors. We also investigated the correlation between the susceptibility of the cancer xenografts to the dCyd analogues and their dCyd kinase activity, which is essential for the activation of the analogues by conversion to their corresponding nucleoside monophosphates. Finally, we discuss possible therapeutic advantages of DMDC, the efficacy of which shows a strong correlation with the levels of Cyd deaminase activity in tumors.

MATERIALS AND METHODS

Chemicals and Enzymes. Gemcitabine was synthesized by the methods described elsewhere (16), whereas DMDC was obtained from Yoshitomi Pharmaceutical Industries, Ltd., Osaka, Japan. 2'-Deoxy-2'-methylideneuridine used as an HPLC standard was obtained from Yoshitomi Pharmaceutical Industries, Ltd. The other HPLC standard, 2',2'-difluorodeoxyuridine, used was prepared by deaminating gemcitabine by the recombinant human Cyd deaminase, which was prepared in our laboratory as described elsewhere (17). Pyruvate kinase was purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture serum and media were purchased from the following

Received 9/5/97; revised 11/24/97; accepted 11/24/97.

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2 The abbreviations used are: dCyd, 2'-deoxycytidine; Ara-C, 1-β-n-\arabinofuranosyl cytosine; NSCLC, non-small-cell lung cancer; DMDC, 2'-deoxy-2'-methylidenecytidine; Cyd, cytidine; HPLC, high-performance liquid chromatography; 5'-dFUr, 5'-deoxy-5-fluorouridine; 5'-dCyd, 5'-deoxy-5-fluorocytidine; MTD, maximum tolerated dose.
Activity of DMDC and Level of Cyd Deaminase in Tumors

companies: fetal bovine serum from BioWhittaker, Inc.; McCoy's 5A from Nipro K.K. (Osaka, Japan); MEM, DMEM, and RPMI 1640 from Nippon Bio Medical Research Laboratory (Kyoto, Japan). 3,4,5,6-Tetrahydrouridine was purchased from Calbiochem-Novabiochem International (La Jolla, CA). [2-14C]Deoxycytidine was purchased from Moravek Biochemicals, Inc. (Brea, CA). Aquasol and DE81 paper were purchased from Packard Instrument Co. (Meriden, CT) and Whatman International, Ltd. (Maidstone, England), respectively. 5'-dFUrd and 5'-dFCyd were obtained from Hoffmann-La Roche, Basel, Switzerland.

Animals. Male and female BALB/c-nu/nu mice were obtained from SLC Inc. (Hamamatsu, Japan). After at least 1 week of observation, the mice were used at the age of 6 weeks.

Tumors. The human tumor lines used were obtained from the following institutions: human colon cancer HCT116, COLO205, HT29, DLD-1, human pancreatic cancer AsPC-1, and BxPC-3 from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan); human NSCLC A549 from the American Type Culture Collection (Rockville, MD); human gastric cancer MKN45 and human NSCLC PC-13 from Immunobiological Laboratories (Fujioika, Japan); human colon cancer CXF280 and human breast cancer MAXF401 from Prof. H. H. Fiebig (Freiburg University, Freiburg, Germany); human small cell lung cancer LCX-1 from Dr. Tashiro (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 (University of Tsukuba, Institute of Clinical Medicine, Tsukuba, Japan). Human cervical cancer Yumoto clone 17 was established in this laboratory (18) from an original Yumoto line obtained from Dr. Tokita (Chiba Cancer Center, Chiba, Japan). Among them, MKN45, CXF280, MAXF401, and LCX-1 were maintained by continuous passage in BALB/c nu/nu mice. The others were maintained in in vitro culture with the following media containing 10–15% fetal bovine serum: HCT116 and HT29 in McCoy's 5A; Nakajima in MEM; A549 in DMEM; AsPC-1, BxPC-3, COLO205, DLD-1, PC-13, Yumoto clone 17, and T24 in RPMI 1640.

Human Cancer Xenograft Models. Small pieces of MKN45, CXF280, MAXF401, and LX-1 tumor tissues were inoculated s.c. into the right flank of mice with a trocar. A single-cell suspension of AsPC-1, BxPC-3, COLO205, HCT116, HT29, DLD-1, Yumoto clone 17, Nakajima, PC-13, and T24 (1–10 x 10^6 cells/mouse) was inoculated s.c. into the right flank of mice. To evaluate the antitumor effect of test compounds, tumor size was measured twice a week. The tumor volume was estimated by the following equation, \( V = \frac{1}{2} \cdot a \cdot b^2 \), where \( a \) and \( b \) are tumor length and width, respectively. The mice were also weighed twice a week. Drug administration was started on the day when the tumor volumes reached approximately 200–300 mm³. All of the compounds were dissolved or suspended in 5% gum arabic as the vehicle and administered p.o., except for i.v. gemcitabine, which was dissolved in a PBS solution (pH 7.0).

Deaminase Kinetics Assay. The susceptibility of test compounds to Cyd deaminase was determined by measuring the amounts of deaminated products from the test compounds. A reaction mixture (25 μl) for the enzyme reaction contained 50 mM Tris-acetate buffer (pH 7.4), 6.25 μM to 16 mM dCyd analogues (0.57 ng - 2.85 μg protein), 100 μg/ml of BSA, and the recombinant human Cyd deaminase. The reaction was carried out at 37°C for 30 min and then terminated by the addition of 25 μl of 4% trichloroacetic acid. After removal of the precipitate by centrifugation (7000 x g for 3 min), a portion of the reaction mixture (40 μl) was added to 120 μl of 50 mM K₂HPO₄ and then applied onto an HPLC column (ERC-ODS-1171). The solvent system used was: 4 mM KH₂PO₄:methanol = 97:5.2; for uridine; 3 mM KH₂PO₄:methanol = 95:5 for 2'-deoxyuridine; and 4 mM KH₂PO₄:methanol = 90:10 for 2'-deoxy-2'-methyldeoxuridine and 2',2'-difluoro-2'-deoxyuridine. The amount of product was measured by a UV monitor (265 nm). The kinetics parameters were determined from double reciprocal plots of enzyme activity as a function of changing substrate concentration.

Cyd Deaminase in Human Cancer Tissues. Various human tumor tissues, which were resected surgically, were obtained from many Japanese hospitals and stored at −80°C until used. These tissues were added in 5–10 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl₂, and 50 μM potassium phosphate and then homogenized with a glass homogenizer. This homogenate was then centrifuged at 105,000 x 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 performed at 4°C. The protein concentration was determined by the method of Lowry et al. (19). The enzyme activity was determined by measuring 5'-dFUrd and 5-fluorouracil generated from 5'-dF-Cyd, an enzyme substrate. A reaction mixture (100 μl) for the enzyme activity contained 50 mM Tris-aceble 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 x 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 mM 5-chlorouracil as the internal standard and then applied onto an HPLC column (ERC-ODS-1171). The solvent system used was as follows: 10 mM sodium phosphate buffer (pH 6.8) containing 5 mM 1-decanesulfonic acid:methanol = 85:15 (v/v). The amount of 5'-dFUrd and 5-fluorouracil produced was measured by a UV monitor (265 nm). Cyd deaminase activity was expressed as nmol 5'-dFCyd deaminated/mg protein/min.

dCyd Kinase Assay. The enzyme assay was carried out as described elsewhere (20). Enzyme sources were prepared the same method as Cyd deaminase assay. Briefly, the standard reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 7.6), 10 μl of tissue supernatant fraction, 7 mM MgCl₂, 2 mM DTT, 7 mM ATP, 1 mM phosphoenol pyruvate, 10 IU of pyruvate kinase [EC 2.7.1.40], 10 mM NaF, 0.4 mM 3,4,5,6-tetrahydrodine (inhibitor of Cyd deaminase), and 40 nmol of [2,14C]-dCyd (125 MBq/mmol). This mixture was incubated for 60 min at 37°C. The reaction mixture was stopped by adding an equal volume of cold methanol, and precipitated protein was sedimented by centrifugation. An aliquot of the supernatant (50 μl) was applied to DE81 DEAE paper. Unreacted dCyd was removed by three successive washes in 5 mM ammonium formate, followed by a 5-min wash in distilled water and 95% ethanol.

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Table 1  Deamination kinetics of 2'-deoxycytidine analogue

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (nmol/mg protein/min)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCyd</td>
<td>27.4</td>
<td>14500</td>
<td>529</td>
</tr>
<tr>
<td>Cyd</td>
<td>18.9</td>
<td>11500</td>
<td>609</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>264</td>
<td>30900</td>
<td>117</td>
</tr>
<tr>
<td>DMDC</td>
<td>2070</td>
<td>329</td>
<td>0.159</td>
</tr>
</tbody>
</table>

Fig. 1  Antitumor activity of DMDC and gemcitabine against AsPC-1 human pancreatic cancer xenograft. DMDC was given p.o. five times a week for 2 weeks, whereas gemcitabine was given i.v. twice a week for 2 weeks. Details were described in “Materials and Methods.” ☐, DMDC; ☐, gemcitabine. * P < 0.05 versus corresponding vehicle control group. Bars, SD.

After drying, the discs were placed in scintillation vials and counted in 5 ml of Aquasol.

Statistical Analysis. Statistical analyses were performed using the Bonferroni/Dunn test and the Mann-Whitney $U$ test. Differences were considered to be significant when the probability ($P$) was <0.05.

RESULTS

Susceptibility of dCyd Analogues to Cyd Deaminase. Table 1 compares $K_m$, $V_{max}$, and $V_{max}/K_m$ of human recombinant Cyd deaminase when the 2'-deoxycytidine analogues, dCyd, and Cyd were used as substrates. DMDC was highly resistant to Cyd deaminase, whereas gemcitabine was highly susceptible to the enzyme. The efficiency in catalyst ($V_{max}/K_m$) of DMDC was 736 times lower than that of gemcitabine.

Antitumor Activity in Vivo. The antitumor activities of DMDC and gemcitabine were compared by using 15 human cancer xenograft models with various levels of Cyd deaminase and dCyd kinase activities. Figs. 1 and 2 show examples of the efficacy of these dCyd analogues. DMDC was given p.o. daily 5 days per week for 2 or 3 weeks, whereas gemcitabine was given i.v. twice a week for 2 or 3 weeks, because it becomes highly toxic when given daily. DMDC was much more effective in the human pancreatic cancer xenograft AsPC-1 than gemcitabine in terms of antitumor potency and therapeutic margins (Fig. 1). In the human mammary cancer xenograft model MAXF401, gemcitabine was, however, more effective than DMDC (Fig. 2). Table 2 summarizes the efficacy of DMDC and gemcitabine in all of the 15 human cancer xenograft models with various levels of Cyd deaminase and dCyd kinase activities. The efficacy was expressed in terms of the degree of their therapeutic indices; the ratio of maximum tolerated doses to ED$_{50}$. DMDC and gemcitabine showed high antitumor activity with therapeutic index of more than 10 in 47% (7 of 15) and 27% (4 of 15) of human cancer xenograft lines used, respectively.

The antitumor spectra of DMDC and gemcitabine were quite different (Table 2). Figs. 3 and 4 compare the susceptibility of the 15 human cancer xenograft lines to the two dCyd analogues with levels of Cyd deaminase and dCyd kinase in tumors. Human cancer xenograft lines are defined to be susceptible when the therapeutic index for the analogues against them was more than 2. DMDC was effective in tumors with higher levels of Cyd deaminase ($P = 0.0071$), whereas gemcitabine was effective in many xenograft lines with lower levels of the enzyme activity, although the correlation between the susceptibility and the enzyme levels was not significant (Fig. 3). On the other hand, there was no correlation between the efficacy of either compound and dCyd kinase activity in tumors (Fig. 4).

Cyd Deaminase Activity in Human Cancer Tissues. Thus, the activity of DMDC correlates well with the levels of Cyd deaminase. To obtain an insight into the target diseases for DMDC, we investigated what types of human cancer show high levels of this enzyme activity. Fig. 5 compares the enzyme activity of various types of cancer tissues from individual patients. The enzyme activity is higher in colorectal cancer, hepatoma, NSCLC, gastric cancer, and cervical cancer, whereas breast cancer and prostate cancer do not express this enzyme as much. Cancer types with high Cyd deaminase activity or individual patients who have tumors with high levels of this enzyme would be targets for DMDC therapy.
DISCUSSION

The antitumor activities of the two dCyd analogues DMDC and gemcitabine, with similar mechanisms of action but with different susceptibility to Cyd deaminase (4-6, 10, 11), were investigated in human cancer xenograft models. DMDC is highly resistant to Cyd deaminase, whereas gemcitabine is easily deaminated to an inactive molecule by this enzyme. These studies showed that they have a different antitumor spectrum in xenograft models. DMDC was highly effective in tumors with higher levels of Cyd deaminase, whereas gemcitabine was not so effective in such tumors. In a preliminary experiment, we also observed that other dCyd analogue, 2'-deoxy-2'-fluoromethylenecytidine, with moderately resistant to Cyd deaminase, has different antitumor spectrum from those of the other two compounds. There must be a mechanistic explanation for the different antitumor activities shown by these compounds.

One possible mechanism for tumor resistance to gemcitabine is reported to be a lack of dCyd kinase activity, the enzyme that converts it to gemcitabine monophosphate (13, 21). Another mechanism might be the deamination of gemcitabine by Cyd deaminase (13). Peters et al. (22), however, reported that the efficacy was not well correlated with levels of either enzyme activity. The present study also showed that the in vivo susceptibility of human cancer xenografts did not correlate with levels of dCyd kinase. However, it is likely that deamination by Cyd deaminase is one resistant mechanism for gemcitabine, and it is possible that the antiproliferative activity of gemcitabine was enhanced by the Cyd deaminase inhibitor tetrahydrouridine in human tumor cell cultures (23).

The efficacy of DMDC correlated with levels of Cyd deaminase in tumors, although DMDC is highly resistant to the enzyme. It was highly effective against tumors with higher levels of Cyd deaminase, whereas it showed only slight activity against tumors with lower levels of the enzyme. The favorable

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Table 2  Antitumor activity of dCyd derivatives in human cancer xenograft models

<table>
<thead>
<tr>
<th>Xenograft lines</th>
<th>Cyd deaminase activity (nmol/mg protein/min)</th>
<th>dCyd kinase activity (nmol/mg protein/h)</th>
<th>Antitumor activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>AxPC-1 (pancreas)</td>
<td>15.8 ± 2.3</td>
<td>2.2 ± 0.27</td>
<td>++</td>
</tr>
<tr>
<td>A549 (NSCLC)</td>
<td>6.9 ± 1.4</td>
<td>1.0 ± 0.14</td>
<td>++</td>
</tr>
<tr>
<td>COLO205 (colon)</td>
<td>5.2 ± 0.8</td>
<td>1.6 ± 0.28</td>
<td>++</td>
</tr>
<tr>
<td>MKN45 (gastric)</td>
<td>3.2 ± 0.9</td>
<td>0.66 ± 0.12</td>
<td>++</td>
</tr>
<tr>
<td>CXXF280 (colon)</td>
<td>3.0 ± 1.0</td>
<td>5.8 ± 1.1</td>
<td>++</td>
</tr>
<tr>
<td>HCT116 (colon)</td>
<td>2.9 ± 0.6</td>
<td>1.3 ± 0.32</td>
<td>++</td>
</tr>
<tr>
<td>HT29 (colon)</td>
<td>2.8 ± 1.6</td>
<td>1.2 ± 0.15</td>
<td>++</td>
</tr>
<tr>
<td>LX-1 (SCLC)</td>
<td>1.9 ± 0.5</td>
<td>3.2 ± 1.5</td>
<td>++</td>
</tr>
<tr>
<td>BxPC-3 (pancreas)</td>
<td>1.8 ± 0.7</td>
<td>1.5 ± 0.19</td>
<td>+</td>
</tr>
<tr>
<td>DLD-1 (colon)</td>
<td>0.34 ± 0.03</td>
<td>1.4 ± 0.31</td>
<td>+</td>
</tr>
<tr>
<td>Yumoto (cervix)</td>
<td>0.20 ± 0.06</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Nakajima (ovary)</td>
<td>0.022 ± 0.002</td>
<td>3.3 ± 0.47</td>
<td>+</td>
</tr>
<tr>
<td>MAXF-401 (breast)</td>
<td>0.015 ± 0.002</td>
<td>1.4 ± 0.75</td>
<td>+</td>
</tr>
<tr>
<td>PC-13 (NSCLC)</td>
<td>0.014 ± 0.001</td>
<td>0.76 ± 0.064</td>
<td>+</td>
</tr>
<tr>
<td>T24 (bladder)</td>
<td>&lt;0.013</td>
<td>2.5 ± 0.57</td>
<td>-</td>
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</tbody>
</table>

*Therapeutic index (TI, MTD/ED50); TI < 2, NS; 2 < TI < 10, +; 10 ≤ TI, ++.

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Fig. 3 Correlation between the in vivo susceptibility of human cancer xenograft lines to dCyd analogues and Cyd deaminase activity in tumors. "Cyd deaminase susceptibility" represents Cyd deaminase activity in human cancer xenograft lines. TI, therapeutic index (MTD/ED50); NS, not significant.

Fig. 4 Correlation between the in vivo susceptibility of human cancer xenograft lines to dCyd analogues and dCyd kinase activity in tumors. "Cyd kinase susceptibility" represents dCyd kinase activity in each human cancer xenograft line. TI, therapeutic index (MTD/ED50); NS, not significant.
activity of DMDC in tumors with higher levels of Cyd deaminase was also demonstrated in the subsequent study, where the antiproliferative activity was enhanced by Cyd deaminase gene transfection but reduced by the enzyme inhibitor tetrahydrodoruridine (23). It is, therefore, of interest to investigate why Cyd deaminase activity is essential for the efficacy of DMDC. A positive correlation between the susceptibility to DMDC and Cyd deaminase levels should also allow us to design a more rational approach toward cancer therapy with DMDC. Briefly, either the cancer with higher levels of Cyd deaminase, such as colorectal cancer, NSCLC, hepatoma, gastric cancer, and cervical cancer, or individual patients who have a tumor with high levels of the enzyme activity would be rational targets for therapy with DMDC. In other words, the Cyd deaminase assay could be useful for selecting patients who are likely to respond to DMDC therapy. Preclinical studies using this approach for optimizing the efficacy of DMDC should be pursued.

REFERENCES


High susceptibility of human cancer xenografts with higher levels of cytidine deaminase to a 2'-deoxycytidine antimetabolite, 2'-deoxy-2'-methylidene-2'-cytididine.
