Sensitivity to Gemcitabine and Its Metabolizing Enzymes in Neuroblastoma

Masahiro Ogawa, Hiroki Hori, Takuya Ohta, Kaori Onozato, Masazumi Miyahara, and Yoshihiro Komada

Abstract

Purpose: We examined the activity of gemcitabine against neuroblastoma in vitro and in vivo. In addition, we investigated the cellular mechanisms of high sensitivity to the agent in neuroblastoma cells.

Experimental Design: We examined 11 neuroblastoma cell lines for sensitivity to gemcitabine and other chemotherapeutic agents used clinically for neuroblastoma. The in vivo sensitivity of neuroblastoma to gemcitabine was determined in xenograft models. Furthermore, the major metabolite of gemcitabine was assessed and compared in leukemia and carcinoma cells. Apoptosis and mitochondrial membrane potentials were also evaluated.

Results: The IC50s for gemcitabine in 11 neuroblastoma lines ranged between 3 nmol/L and 4 μmol/L. The high activity of gemcitabine against neuroblastoma was confirmed in animal models. Interestingly, enzymes in neuroblastoma cells involved in the metabolism of deoxycytidine analogue have unique characteristics among solid tumors. The median of deoxycytidine kinase activity in neuroblastoma lines was similar to that in leukemia lines, which have low IC50s for cytarabine. Cytidine deaminase (CDA) activity in neuroblastoma was hardly detectable and significantly lower than that in carcinoma. The defect of CDA activity was associated with negative expression of mRNA. Furthermore, gemcitabine-induced apoptosis was observed irrespective of the caspase-8 status of neuroblastoma cells, which indicates that apoptosis depends on the mitochondrial pathway.

Conclusions: Neuroblastoma is highly sensitive to gemcitabine. Although the cellular mechanism involved in sensitivity to gemcitabine is multifactorial, low CDA activity may contribute high sensitivity in neuroblastoma cells. These results suggest that clinical application of gemcitabine to the treatment of neuroblastoma is warranted.

Neuroblastoma, a neoplasm of neural crest cells, is the most common extracranial solid tumor in children (1). As well as disease stage and age at onset, MYCN amplification or histology has been reported as a prognostic factor (2). The outcome of patients >1 year of age with distant metastasis is still poor even after the introduction of some multimodal approaches, such as high-dose chemotherapy followed by hematopoietic stem cell transplantation (3). Although neuroblastoma is usually treated with combination chemotherapy including cisplatin, doxorubicin, vincristine, and cyclophosphamide, the disease tends to quickly exhibit resistance to chemotherapeutic agents through overexpression of multidrug resistance–associated protein (4). Therefore, there is an urgent need to introduce novel agents active against neuroblastoma, which can overcome chemoresistance.

Gemcitabine is a novel deoxycytidine analogue that has shown potent anticancer activity to a broad range of human solid tumors, including pancreatic and non–small cell lung cancers (5, 6). Gemcitabine, which bears metabolic resemblance to cytarabine, is phosphorylated to the monophosphate nucleotide by deoxycytidine kinase (dCK), then further phosphorylated to the active forms, difluorodeoxycytidine diphasphate and difluorodeoxycytidine triphosphate, by nucleotide kinases. dCK is the rate-limiting enzyme in the phosphorylation pathway. The agent is also converted to its deaminated metabolite by cytidine deaminase (CDA). The major effect of the triphosphate form is directed against DNA synthesis through masked chain termination (7). Furthermore, gemcitabine is known to cause cell death by apoptosis in various cancer cell lines (8). Mechanisms of resistance to this drug have been described in various cell lines and primary samples (9, 10). Insufficient intracellular accumulation of the triphosphate metabolite is the major cause of resistance. This may result from insufficient cellular uptake of the drug, reduced levels of dCK, and increased degradation by CDA or 5′ nucleotidase (11–14).

To summarize, gemcitabine may have an advantage in the treatment of neuroblastoma. First, deoxycytidine analogue has a unique mechanism of antineoplastic action that differs from those of drugs commonly used against neuroblastoma (15).
Second, gemcitabine is not a substrate for plasma membrane drug efflux pumps, such as P-glycoprotein or multidrug resistance–associated protein, which are closely related to multidrug resistance in neuroblastoma. Furthermore, recent studies suggest that P-glycoprotein or multidrug resistance–associated protein overexpression induces an up-regulation of dCK, resulting in increase in an intracellular active metabolite of gemcitabine (16).

Recently, clinical application of gemcitabine has been expanded but it has not been used in pediatric cancers. In addition, a recent phase I trial of gemcitabine in children with solid tumors did not include neuroblastoma patients (17). In this paper, we show that neuroblastoma cells are highly sensitive to gemcitabine in in vitro and in vivo experiments. These results suggest that gemcitabine may be a good candidate for chemotherapy against neuroblastoma.

### Materials and Methods

**Chemicals.** Gemcitabine and vincristine were obtained from Eli-Lilly, Co. (Nagoya, Japan), cytarabine from Nippon Shinyaku, Co. (Kyoto, Japan), doxorubicin from Kyowa Hakko Kogyo, Co. (Nagoya, Japan), and G418 from Bioworld (St. Louis Park, MN).

### Materials and Methods

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Annealing</td>
</tr>
<tr>
<td>DCK</td>
<td>Sense: 5′-GAATTCTAGAGCAATGGGCCACCCCGCCC-3′&lt;br&gt;Antisense: 5′-TTGAGTACTTTGAGATCGACGCCG-3′</td>
<td>55</td>
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<tr>
<td>CDA</td>
<td>Sense: 5′-ATGGCCCAGAAGCTCTGCCTGCA-3′&lt;br&gt;Antisense: 5′-TCACTGGGTCTTGCAGGTCCCTA-3′</td>
<td>63</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense: 5′-AAGAAGATGGGGCTGACTGAGGCCACAT-3′&lt;br&gt;Antisense: 5′-TCTCATGTTCAACCCATGACGAACT-3′</td>
<td>59</td>
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</table>

**Table 1. Summary of primer sequences and PCR conditions for dCK and CDA**

**Fig. 1. Sensitivity to gemcitabine.**

A. Neuroblastoma cell lines: Cell viability was measured by WST-1 assay after 3 days incubation with indicated concentrations at an initial cell density of 1 \times 10^5 cells/mL (100 µL/well). The experiment shown is representative of three independent experiments. Points, mean of three determinations. SD was <15% of the mean. B. Carcinoma cell lines: Cell viability was determined as described in (A). ○, PaCa2; ■, H460.
High Sensitivity of Neuroblastoma Cells to Gemcitabine

Japan), etoposide (VP16) and cisplatin from Bristol Myers Squibb, Co. (Nagoya, Japan), cyclophosphamide and 4-hydroxy-cyclophosphamide (4OHC-CTX) from Shionogi Co. (Osaka, Japan), and topotecan from Glaxo-Smith-Kline Co. (Nagoya, Japan). [3H]Deoxyribidine was purchased from Amersham (Tokyo, Japan). Propidium iodide and Triton-X were purchased from Sigma (Tokyo, Japan).

**Cell lines.** Eleven human neuroblastoma cell lines (IMR32, SK-N-SH, SJ-N-SD, SJ-N-JF, SJ-N-KP, KP-N-SI, KP-N-SFA, TGW, SCMC-N4, NB69, and LAN-1) were used in this study. The profiles of these cell lines were described in previous reports (18, 19). Seven human B precursor acute leukemia cell lines (MB-IK, MB-IT, MB-KG, MB-KS, MB-SY, MB-YD, and MB-YU) were established from children with leukemia at our laboratory (20). Carcinoma cell lines (DU145, PC-3, Sk col, SW-48, HLE, PLC/PRF/5, HepG2, and HeLa) including H460 and PaCa2 cells were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were incubated in plastic culture plates in RPMI 1640 supplemented with 10% fetal bovine serum in 5% CO₂ at 37°C.

**In vitro drug cytotoxicity assay.** The in vitro cytotoxicity of each cell line was measured using the cell proliferation reagent, WST-1 as previously described (21). Cells in the logarithmic growth phase were plated at a density of 1 × 10⁴ cells/well in a 96-well plate overnight before each chemotherapeutic agent was added. They were then cultured in the presence of varying concentrations of each cytotoxic agent for 72 hours. For the last 4 hours of drug exposure, cells were incubated with 10 μL of WST-1 solution and then viability was determined by measuring absorbance at 450 nm. IC₅₀ was determined from semilogarithmic dose-response plots. The experiments were done in triplicate.

**Drug washout experiments.** Drug washout experiments were conducted to assess the impact of drug exposure time on cell proliferation according to the method shown by Keshelava et al. (22). KP-N-SIFA cells established from a patient with neuroblastoma refractory to multiagent chemotherapy were used in the experiments. Cells were treated with various concentrations of gemcitabine, cisplatin, or 4OH₂-CTX for 1, 3, 6, or 24 hours, and then washed with drug-free medium twice and incubated in drug-free medium. Cell proliferation assay using WST-1 was done 72 hours after the initiation of treatment.

**Apoptosis assay.** Apoptosis was analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, CA) using an annexin V–FITC Apoptosis Detection kit (Medical & Biological Laboratories, Nagoya, Japan). Cell cycle was also determined to detect the sub-G₁ fraction as previously described (23). Briefly, neuroblastoma cells were treated with 100 nmol/L gemcitabine for the indicated duration. Cells (5 × 10⁶) were harvested after trypsinization and washing with PBS, and then subjected to flow cytometric analysis according to the manufacturer’s instructions. Cell Quest computer software was used to calculate cell death or analyze cell cycle. The results are shown as the mean of three experiments.

**Mitochondrial membrane potential assay.** Cells were incubated with 1 μmol/L gemcitabine and harvested at 0, 12, 24, and 48 hours after treatment with gemcitabine. Disruption of the mitochondrial membrane potential was analyzed by flow cytometry using a MitoCapture Mitochondrial Apoptosis Detection kit (BioVision, Tokyo, Japan) as previously described (24). Briefly, cells were resuspended in 1 mL incubation buffer containing 1 μL MitoCapture for 15 minutes at 37°C in a 5% CO₂ incubator. MitoCapture is a cationic dye that aggregates in the mitochondria and gives off a bright red fluorescence in healthy cells. In apoptotic cells, MitoCapture cannot aggregate in the mitochondria because of the altered mitochondrial transmembrane potential, and remains in the cytoplasm in its monomer form, which exhibits green fluorescence. After staining, the fluorescent signals were analyzed by flow cytometry using the FITC channel for green monomers.

**Enzyme assay.** Enzyme activity of dCK was assayed by the method of Ives and Durham (25). Cells were harvested and disrupted by freezing and thawing. After centrifugation, the supernatants were stored at -80°C until the time of measurement. One hundred and fifty microliters of a substrate mixture were added to 100 μL of supernatant (final concentration in the mixture: 10 mmol/L ATP, 10 mmol/L MgCl₂, 50 mmol/L Tris-HCl, 15 mmol/L NaF, 10 mmol/L DTT, 1 mmol/L deoxyuridine, 1 mmol/L cytidine, and 10 μmol/L [3H]deoxyribidine with specific activity of 34 μCi/μmol). Thymidine was also added to the mixture at a final concentration of 1 mmol/L to inhibit thymidine kinase-2–mediated phosphorylation of deoxyribidine (26). The assay mixture was incubated at 37°C for 30 minutes. The reaction was terminated by heating at 95°C for 2 minutes. Fifty microliters of reaction mixture were spotted on a Whatman DE81 paper disk, then dried immediately and washed thrice with distilled water for 90 minutes. The disk was dried and inserted into a vial containing 3 mL scintillation cocktail. The radioactivity was counted with a liquid scintillation counter. Each experiment was done in triplicate.

The enzyme activity of CDA was determined by the method of Chabner et al. (27) with minor modifications. In brief, the supernatant obtained by the methods described above was resuspended in 50 μmol/L [3H]deoxyribidine (specific activity 17 μCi/μmol) and 20 mmol/L Tris-HCl (pH 8.0) buffer at 37°C for 30 minutes. The reaction was terminated by adding cold 0.1 N HCl. The mixture was then applied to a column containing 0.5 g cation exchange AG 50W-X4 resin (Bio-Rad

**Table 2.** IC₅₀ to gemcitabine, dCK and CDA activities in each cell line

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC₅₀ (nmol/L)</th>
<th>dCK activity (pmol/min × mg total protein)</th>
<th>CDA activity (pmol/min × mg total protein)</th>
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<tbody>
<tr>
<td>NB69</td>
<td>3.2</td>
<td>637</td>
<td>1.2</td>
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<td>LAN-1</td>
<td>6.3</td>
<td>1,003</td>
<td>N.D.</td>
</tr>
<tr>
<td>KP-N-SIFA</td>
<td>8.2</td>
<td>792</td>
<td>0.24</td>
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<tr>
<td>IMR32</td>
<td>2.5 × 10⁻²</td>
<td>587</td>
<td>0.033</td>
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<td>SCMC-N4</td>
<td>3.5 × 10⁻²</td>
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<td>1.0</td>
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<tr>
<td>SK-N-SH</td>
<td>3.8 × 10⁻²</td>
<td>679</td>
<td>N.D.</td>
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<tr>
<td>TGW</td>
<td>8.4 × 10⁻²</td>
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<td>SJ-N-JF</td>
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<td>0.50</td>
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<td>SJ-N-KP</td>
<td>2.0 × 10⁻³</td>
<td>436</td>
<td>N.D.</td>
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<tr>
<td>SJ-N-SD</td>
<td>1.9 × 10⁻³</td>
<td>268</td>
<td>N.D.</td>
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<td>KP-N-SI</td>
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<td>MB-YU</td>
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<td>SW-48</td>
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<tr>
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<td>3.6</td>
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<td>HepG2</td>
<td>8.4 × 10⁻⁵</td>
<td>518</td>
<td>3.3</td>
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</table>

NOTE: Each experiment was done in triplicate. Values are presented as mean. Abbreviation: N.D., not detectable.

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Laboratories, Hercules, CA) to elute $[^{3}H]$deoxyuridine. The eluted $[^{3}H]$deoxyuridine was mixed with the scintillation cocktail and examined for radioactivity using a liquid scintillation counter. Each experiment was done in triplicate.

**Messenger RNA analysis.** The mRNA expression levels of dCK and CDA were quantified using real-time PCR. Five micrograms total RNA extracted with RNAzol B (TEL-TEST, Inc., Friendswood, TX) were transcribed to first-strand cDNA in 10 $\mu$L reaction mixture using a Superscript first-strand synthesis kit (Life Technologies, Grand Island, NY). PCR amplification was carried out in 20 $\mu$L reaction mixture containing 2 $\mu$L LightCycler-DNA Master SYBR Green 1 (Roche Diagnostics, Indianapolis, IN), 2.4 $\mu$L MgCl$_2$ (final concentration: 4 mmol/L), 2 $\mu$L transcribed cDNA from the reaction mixture of cDNA synthesis, 1 $\mu$L sense and antisense primers (final concentration: 0.5 $\mu$mol/L), and 11.6 $\mu$L PCR-grade distilled water. The template quantity was equivalent to the cDNA converted from 0.5 $\mu$g total RNA. Primer sequences and PCR profiles are shown in Table 1. Forty cycles were done using the LightCycler (Roche Diagnostics). Each cycle consists of denaturation at 94°C for 30 seconds, annealing for 10 seconds, extension at 72°C for 18 seconds, and denaturation of the primer dimers for 3 seconds. Denaturation of the primer dimers was added to exclude the effect of signals from primer dimers. The reaction temperature was determined according to the difference in the melting temperature ($T_m$) of double-stranded DNA between the target product and the primer dimers. $T_m$ of the target product was 89.5°C, whereas that of the primer dimers was 84.5°C. A series dilution of CDA cDNA or

<table>
<thead>
<tr>
<th>Chemotherapeutic agent</th>
<th>IC$_{50}$ (nmol/L)</th>
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<tr>
<td>Gemcitabine</td>
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</tr>
<tr>
<td>Cisplatin</td>
<td>$1.4 \times 10^8$</td>
</tr>
<tr>
<td>Topotecan</td>
<td>7.7</td>
</tr>
<tr>
<td>Etoposide</td>
<td>$1.3 \times 10^2$</td>
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<tr>
<td>Doxorubicin</td>
<td>3.0</td>
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<tr>
<td>Vincristine</td>
<td>3.6</td>
</tr>
<tr>
<td>4-hydroperoxycyclophosphamide</td>
<td>$1.4 \times 10^2$</td>
</tr>
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</table>

**NOTE:** Each experiment was done in triplicate. Values are presented as mean.
dCK cDNA derived from plasmid containing human CDA or dCK was used for an external standard. CDA cDNA was a gift from Dr. Kuhn (Max-Planck Society, Institute for Immunology, University of Erlangen-Nurnberg, Germany) (28). dCK cDNA was cloned by a co-worker (29).

A sample containing distilled water instead of template cDNA was also measured as a negative control. The reverse transcription-PCR analysis of an endogenous housekeeping gene encoding for glyceraldehyde 3-phosphate dehydrogenase as a reference was carried out using

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**Fig. 3.** Induction of apoptosis in KP-N-SI and KP-N-SIFA cells by gemcitabine.

**A,** apoptosis after exposure to 100 nmol/L gemcitabine in KP-N-SI and KP-N-SIFA cells: Apoptotic cells were measured by annexin V/propidium iodide on a FACScan flow cytometer. Percentages of annexin V+/propidium iodide− and annexin V+/propidium iodide+ cells are shown in dot-plot graphs. Each experiment was done in triplicate. Points, means. %, cell cycle analysis: After exposure to 100 nmol/L gemcitabine in KP-N-SI and KP-N-SIFA cells, apoptosis was assessed by propidium iodide staining of lysed cell nuclei. The DNA content of 20,000 events was analyzed by flow cytometry. Each experiment was done in triplicate. Points, means. C, change in mitochondrial membrane potential following gemcitabine treatment: KP-N-SI and KP-N-SIFA cells were exposed to 1 μm/L gemcitabine and examined for disruption of mitochondrial membrane potential by flow cytometry using the fluorescent probe MitoCapture (x-axis, log10 fluorescent intensity using FITC channel for green monomers; y-axis, cell count). Solid lines, staining with MitoCapture 1, 2, 24, and 48 hours after treatment with gemcitabine, respectively. Black-shaded histogram, 0 hours.
Takara Taq polymerase (Takara Biomedicals, Siga, Japan). The PCR products were separated by electrophoresis on 2% agarose gel and stained with ethidium bromide.

Animal experiments. The in vivo efficacy of gemcitabine was assessed in athymic mice transplanted with two different types of cell lines, KP-N-SIFA and LAN-1 cells. KP-N-SIFA cells were established from a patient who had relapsed after chemotherapy. The cells had high expression of antiapoptotic protein, bcl-2 (data not shown). LAN-1 cells were also established from a patient after chemotherapy and was proven to have mutated p53 (30). Male athymic nude mice with BALB/c genetic background were supplied from Charles River, Co. (Tokyo, Japan). They were housed under specific pathogen-free conditions. The animal experiments were done in accordance with the institutional guidelines. Neuroblastoma cells (10⁶/C2) were inoculated into the right flank of mice. When the inoculated tumor reached 100 mm³, the mice were randomized into three groups: treatment with gemcitabine (G) or cyclophosphamide (C) and control groups (N). Gemcitabine was given i.p. at 100 mg/kg on days 0, 3, 6, and 9 and cyclophosphamide at 20 mg/kg on days 0, 1, 2, and 3. Points, mean of eight independent experiments; bars, SD.

Statistics. Comparison between paired groups was done using Student's t test or the Wilcoxon single-rank test. Comparison between unpaired groups was done using Mann-Whitney's U test. The Spearman correlation test and regression analysis were used to calculate and test the correlation coefficients between the two groups. The difference was considered to be statistically significant when P < 0.05. These analyses were conducted using the StatView J 4.5.1.2 for Macintosh software program.

Results

In vitro sensitivity to gemcitabine. The antiproliferative activity of gemcitabine was determined by WST-1 assay after a 72-hour exposure to neuroblastoma or other cell lines. The IC₅₀s of 8 of 11 neuroblastoma cell lines evaluated were <1 µmol/L (Fig. 1A; Table 2). Notably, three cell lines, NB69, LAN-1, and KP-N-SIFA, showed the highest sensitivities (IC₅₀s of 3–8 nmol/L). Another three lines, SJ-N-KP, SJ-N-SD, and KP-N-SI, were relatively resistant but their IC₅₀s were 1 to 4 µmol/L. Whereas neuroblastoma cell lines had different caspase-8 status (18), the sensitivity to gemcitabine did not correlate with caspase-8 expression (P = 0.52; Fig. 1A).

To compare sensitivity to gemcitabine in neuroblastoma cells with that in carcinoma cells established from two types of cancer for which gemcitabine is used clinically, we examined the growth inhibitory effect of gemcitabine in H460 non–small cell lung cancer and PaCa2 pancreatic carcinoma cell lines. The IC₅₀s of these lines were 39 nmol/L and 0.84 µmol/L, respectively (Fig. 1B; Table 2). These results were comparable with those in neuroblastoma cells.

In addition, we evaluated the IC₅₀s of various chemotherapeutic agents in neuroblastoma cells and compared them with that of gemcitabine. In this experiment, the NB69 line, one of the most sensitive lines to gemcitabine, was incubated with each chemotherapeutic agent for 72 hours. As shown in Fig. 2A, NB69 cells showed high sensitivity to doxorubicin, vincristine, and topotecan. The IC₅₀s of these agents were <10 nmol/L (Table 3).
These IC_{50}s were comparable with that of gemcitabine. The cells were less sensitive to cisplatin, VP16, and 4OH_2-CTX. A similar trend of drug sensitivity was also observed in LAN-1 and IMR32 cells (data not shown).

**Drug washout assays.** To simulate the activity of gemcitabine in vivo, we tested the dose-response to gemcitabine after 1-, 3-, 6-, and 24-hour exposures in the KP-N-SIFA cell line (Fig. 2B). Exposure to gemcitabine for 3 hours or more seemed to increase activity relative to a 1-hour exposure. Furthermore, we compared the IC_{50} of gemcitabine with those of 4OH_2-CTX or cisplatin. As shown in Fig. 2B, gemcitabine was more active than 4OH_2-CTX or cisplatin.

**Induction of apoptosis.** We investigated gemcitabine-induced apoptosis in neuroblastoma cells and compared induction of apoptosis between the caspase-8 expressed (KP-N-SI) and unexpressed (KP-N-SIFA) cell lines. Induction of apoptosis was observed in both cell lines 24 hours after incubation with 100 nmol/L gemcitabine as evidenced by an increase in annexin V binding cells or an increase in the sub-G_1 fraction of the cell cycle (Fig. 3A and B). Figure 3C shows disruption of the mitochondrial membrane potential 12 hours after treatment with 1 μmol/L gemcitabine in both KP-N-SI and KP-N-SIFA cells irrespective of caspase-8 status.

**In vivo effect of gemcitabine in animal models.** To assess the in vivo effect of gemcitabine against neuroblastoma cells, we generated xenograft models using nude mice. The KP-N-SIFA and LAN-1 cell lines were chosen because we considered these cell lines to be models of chemoresistant tumors. We also compared the effectiveness of gemcitabine to that of cyclophosphamide. As shown in Fig. 4, tumor growth was significantly inhibited in the treatment groups and gemcitabine was more active than cyclophosphamide. There was no significant difference in loss of body weight between the treatment and control groups.

**Deoxycytidine kinase and cytidine deaminase activities.** To elucidate the mechanism of high sensitivity to gemcitabine in neuroblastoma cells, we examined the activities of the two major metabolic enzymes, dCK and CDA (Table 2). Seven leukemia and eight carcinoma cell lines (DU145, PC-3, Sk col, SW-48, HLE, PLC/PRF/5, HepG2, and HeLa) were used for comparison with 11 neuroblastoma lines. The median of IC_{50} for cytarabine was 69 nmol/L in leukemia, 4.7 × 10^3 nmol/L in neuroblastoma, and 4.7 × 10^2 nmol/L in carcinoma, whereas that for gemcitabine was 16 nmol/L in leukemia, 3.8 × 10^2 nmol/L in neuroblastoma, and 2.6 × 10^2 nmol/L in carcinoma (Fig. 5).

The median dCK activity in neuroblastoma lines was not significantly different from that in the leukemia or carcinoma lines (Fig. 6A). The median CDA activity in neuroblastoma was similar to that in leukemia and significantly lower than in carcinoma (Fig. 6B). Because a previous report showed that gemcitabine sensitivity correlated with dCK activity in various types of cancers (32), we also examined the correlation in neuroblastoma cells. However, we failed to show a correlation between gemcitabine sensitivity and dCK activity among neuroblastoma cells (r^2 = 0.20, P = 0.079).

**Messenger RNA expression of deoxycytidine kinase and cytidine deaminase.** dCK mRNA expression was detected by real-time PCR in both the leukemia and neuroblastoma lines (Fig. 7A). No statistical significance was found in dCK mRNA expression between the two types of neoplasms (P = 0.95; Fig. 7B). CDA mRNA was not detected by real-time PCR in either type of neoplasm (Fig. 7A). The result suggests that low CDA activity in neuroblastoma was caused by defective expression of CDA mRNA.

**Discussion**

Whereas gemcitabine has been used mainly for treatment of carcinomas since the 1990s, this agent has potent anticancer activity against other types of neoplasms, such as leukemia (5, 6, 33). In the present study, we examined the efficacy of gemcitabine for neuroblastoma. Surprisingly, the results showed that the median IC_{50} for gemcitabine was 123-fold lower than that for another deoxycytidine analogue, cytarabine, in neuroblastoma cells in vitro. Furthermore, gemcitabine showed a lower IC_{50} in neuroblastoma cells established from a relapsed patient (KP-N-SIFA) than cisplatin or 4OH_2-CTX even after a short-term exposure. This cytotoxic effect of
gemcitabine was confirmed in animal models transplanted with cells showing high expression of the antiapoptotic protein bcl-2 (KP-N-SIFA) and mutated p53 (LAN-1). Until now, nucleoside analogues have not been used in the treatment of neuroblastoma. The results for cyclocytidine, a slow release form of cytarabine, were disappointing (34). Recently, we found that a human myelomonocytic leukemia cell line that has acquired high resistance to cytarabine had no cross-resistance to gemcitabine. A similar observation shows that gemcitabine is more active than cytarabine in cytarabine-sensitive and cytarabine-resistant murine leukemia cell lines through its high substrate specificity to intracellular dCK and mitochondrial thymidine kinase-2 (35). From these observations, we speculated that gemcitabine might be active in neuroblastoma. Recently, a phase I study of gemcitabine was conducted in children with solid tumors (17), but the antineoplastic activity of gemcitabine was not sufficiently investigated in neuroblastoma. In this report, we have proved through in vitro and in vivo experiments that gemcitabine is a good candidate for chemotherapy against neuroblastoma.

Our paper focused on the efficacy of gemcitabine, as a single agent, against neuroblastoma. We also recognize the importance of combination chemotherapy with other agents. Our preliminary data indicate that combination with cisplatin or topotecan produced a synergistic effect in some cell lines (data not shown). We are planning to study this important aspect of chemotherapy as the next step.

To investigate the cellular mechanisms of high sensitivity to the agent in neuroblastoma cells, we examined enzymes involved in the first step of its metabolism. The results showed that, in general, neuroblastoma cell lines had high dCK and low CDA activities similarly to cytarabine-sensitive leukemia lines. Also, neuroblastoma cells had significantly lower CDA activity than cytarabine-resistant carcinoma cells. High dCK activity in neuroblastoma cells may explain high sensitivity to gemcitabine as reported in various solid tumors (32). Phosphorylation by dCK is essential for intracellular accumulation of difluorodeoxycytidine triphosphate, followed by incorporation of the metabolite into DNA (36). Although we failed to find a linear correlation between sensitivity and dCK activity in neuroblastoma cells, sensitivity may be affected by multiple factors. High activity of CDA, the degrading enzyme of deoxycytidine analogues, plays an important role in the development of resistance to these agents (37). In this study, we showed that most solid tumor cell lines had increased CDA activities, whereas 10 neuroblastoma lines showed extremely low activity. The observations of Bierau et al. (38) that no deaminated metabolites were detected after treatment with cytarabine in neuroblastoma cells support our finding of low or defective CDA activity in neuroblastoma cells. Furthermore, we showed that the defective enzyme activity of CDA was associated with negative expression of CDA mRNA. This property distinctly characterizes neuroblastoma as a subgroup of solid tumors. Also, this finding affords the opportunity to develop a novel treatment strategy targeting the enzyme deficiency. The CDA gene localizes to 1p35-36.2, in which loss of heterozygosity is frequently detected in neuroblastomas (39). Further molecular approaches to elucidate the mechanism of negative expression of CDA mRNA in neuroblastoma should be expected.

Furthermore, we suggested that gemcitabine-induced apoptosis occurs via the mitochondrial pathway, irrespective of the status of caspase-8 for which the gene is frequently inactivated in neuroblastoma (40). Recent studies have shown the
relevance of caspase-9, but not caspase-8, to apoptosis induced by anticancer agents in neuroblastoma cells (41, 42). Activation of the mitochondrial pathway is characterized by loss of mitochondrial transmembrane potential and release of cytochrome c from the mitochondria. Cytosolic cytochrome c triggers the assembly of apoptosome, which recruits and activates caspase-9 and caspase-3. Antiapoptotic bcl-2 family members, such as bcl-2 and bcl-xl, inhibit cytochrome c release by modulating the ability of Bax and Bak to facilitate the opening of pores in the outer mitochondrial membrane. It has been reported that the extent of gemcitabine-induced apoptosis is independent of the levels of bcl-2 expression in multiple myeloma cells (43). We are now evaluating the contribution of the bcl-2 family to the sensitivity of gemcitabine in neuroblastoma cells.

In conclusion, both in vitro and in vivo studies show that gemcitabine has high potential in the treatment of neuroblasta om even in patients refractory to chemotherapy. Although the cellular mechanism involved in sensitivity to gemcitabine in neuroblastoma is multifactorial, low CDA activity may contribute high sensitivity to gemcitabine. In addition, our results have shown that gemcitabine induced apoptosis via the mitochondrial pathway, irrespective of caspase-8 status. These results may warrant clinical application of gemcitabine in the treatment of neuroblastoma.

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Sensitivity to Gemcitabine and Its Metabolizing Enzymes in Neuroblastoma

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