Characterization of a Gemcitabine-Resistant Murine Leukemic Cell Line: Reversion of In vitro Resistance by a Mononucleotide Prodrug

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ABSTRACT
Resistance to cytotoxic nucleoside analogues is a major problem in cancer treatment. The cellular mechanisms involved in this phenomenon have been studied for several years, and some factors have been identified. Various strategies to overcome resistance have been suggested, but none has yet shown efficacy in vivo. We developed a gemcitabine-resistant cell line (L1210 10K) from the murine leukemic L1210 strain (L1210 wt) by continuous exposure to increasing concentrations of gemcitabine. L1210 10K is highly resistant to gemcitabine (14,833-fold), 1-β-D-arabinofuranosylcytosine (ara-C; 2,100-fold), and cladribine (160-fold) and slightly resistant to trimidoxylcytosine (ara-C; 2,100-fold), troxacitabine (160-fold), and can be circumvented by a prodrug approach.

INTRODUCTION
Gemcitabine (2′,2′-difluorodeoxycytidine) is a nucleoside analogue that shows activity against hematologic malignancies and solid tumors alone (1–4) or in combination with other chemotherapeutic agents (5–7). The cellular metabolism of gemcitabine is similar to that of physiologic nucleosides (reviewed in Ref. 8). Once inside the cell, it is phosphorylated by deoxycytidine kinase (dCK) to its monophosphorylated form difluoro-dCMP, and is phosphorylated further by other intracellular kinases to the metabolically active forms difluoro-dCDP and difluoro-dCTP (9–11). Diphosphorylated gemcitabine is an inhibitor of ribonucleotide reductase (12), thereby causing a decrease in dCTP pools and decreased feedback inhibition of dCK, leading to enhanced phosphorylation of gemcitabine (13, 14), a process designated as “self-potentiation.” The triphosphorylated form is cytotoxic by incorporation into DNA, causing masked chain termination after the incorporation of an additional nucleotide after gemcitabine (15, 16). The cytotoxicity of gemcitabine in nondividing cells has been attributed to the fact that difluoro-dCTP can disturb RNA metabolism by inhibiting CTP synthetase (17) or by direct incorporation into newly synthesized RNA (18).

Resistance to gemcitabine may involve a variety of mechanisms. In humans, cellular influx of gemcitabine involves different membrane nucleoside transporters, such as hENT1, hENT2, hCNT1, and hCNT3. It has been shown that nucleoside-transporter-deficient cells display resistance to gemcitabine (19). Various authors, including ourselves, have shown that dCK-deficient lines display a gemcitabine-resistant phenotype (20). Gemcitabine and its monophosphorylated form can be inactivated by intracellular enzymes such as cytidine deaminase and dCMP deaminase, producing difluorodeoxyuridine from 2′,2′-difluorodeoxycytidine and difluoro-dUMP from difluoro-dCTP, respectively (10, 21). Intra-cellular 5′-nucleotidases are also likely to be involved in the inactivation of 2′,2′-difluorodeoxycytidine by dephosphorylating difluoro-dCMP or by modifying pools of endogenous nucleotides.

To develop a model of gemcitabine resistance suited for in vivo strategies of reversion of drug resistance, we have developed the L1210 10K model, which displays resistance both in vitro and in vivo.

MATERIALS AND METHODS
Reagents. Compounds used for in vitro and in vivo experiments were 1-β-D-arabinofuranosylcytosine (ara-C; Aracytine; Pharmacia, Puurs, Belgium), cisplatin (Merck, Lyon,
Table 1 Primes, probes, and PCR conditions used in reverse transcription-PCR studies

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
<th>Probe (5′-FAM-3′-TAMRA)</th>
<th>MgCl₂ (mm)</th>
<th>Denaturation</th>
<th>Annealing/extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENT-1</td>
<td>AACTCTCCAACCCACACACACG</td>
<td>TCCACCTCTACAGCATTCAG</td>
<td>2 95°C/15 min 58°C/5 min; 72°C/10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRP5</td>
<td>CAGCGACCAAGCCAGTCTAACC</td>
<td>GGCTATTCACTGCCCAGATCT</td>
<td>95°C/15 min 55°C/5 min; 72°C/9 min</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>dCK</td>
<td>TGAGATTGGAATGTTGTCG</td>
<td>CACCGTGCTAGTTCTCAT</td>
<td>2 95°C/5 min 55°C/5 min; 72°C/8 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dAK</td>
<td>AACGGCTCTTGGTATCAAGC</td>
<td>GGCCTATTACGAGGAGTAA</td>
<td>2 95°C/5 min 60°C/10 min; 72°C/12 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TK1</td>
<td>CAGAGCTAGATCAAGTAATG</td>
<td>TTGACCATTATTCACAGAA</td>
<td>2 95°C/5 min 60°C/10 min; 72°C/12 min</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TK2</td>
<td>TACCTCTGATGGTATGATGA</td>
<td>GAGGATTGCGCAGATATAA</td>
<td>2 95°C/5 min 55°C/5 min; 72°C/6 min</td>
<td></td>
<td></td>
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<tr>
<td>cN-II</td>
<td>AGGTATGCGTACACAG</td>
<td>TCGTCATCTTAGAGCAATA</td>
<td>2 95°C/15 min 58°C/5 min; 72°C/10 min</td>
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<td></td>
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</tr>
<tr>
<td>cN-III</td>
<td>CTGCAATCTGCTGCAG</td>
<td>AGGGCTCTTTAAGGAGGA</td>
<td>2 95°C/15 min 58°C/5 min; 72°C/10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdN</td>
<td>GGAGAAGTAGAAGACATGA</td>
<td>ATGAGGACGTTCTCCATGAC</td>
<td>2 95°C/5 min 58°C/5 min; 72°C/10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mdN</td>
<td>CCTTCTTACACCTCCGACCA</td>
<td>AACTGGAATCCTCAGGAA</td>
<td>3 95°C/5 min 57°C/10 min; 72°C/8 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNR R1</td>
<td>CCCAAATCTACGCTTGCTGTTTC</td>
<td>GGTCTCTGTTCTGCTTCC</td>
<td>95°C/5 min 58°C/5 min; 72°C/10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNR R2</td>
<td>CCTACTAAACCCAGCTTGTA</td>
<td>GTTCCAGAGTCTGCTCCAGTG</td>
<td>95°C/15 min 58°C/5 min; 72°C/10 min</td>
<td></td>
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<td></td>
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</tbody>
</table>

Abbreviations: FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; ENT-1, murine equilibrative nucleoside transporter; MRP5, multidrug resistance protein 5; dCK, deoxycytidine kinase; dAK, deoxyguanosine kinase; TK1, thymidine kinase 1; TK2, thymidine kinase 2; cN-III, cytoplasmic 5′-nucleotidase II; cN-III, cytoplasmic 5′-nucleotidase III; cdN, cytoplasmic 5′-deoxynucleotidase 1; mdN, mitochondrial 5′-deoxynucleotidase 2; RNR R1, large subunit of ribonucleotide reductase; RNR R2, small subunit of ribonucleotide reductase.

France), gemcitabine (Gemzar; Lilly, Indianapolis, IN), Triapine (3-aminopryridine-2-carboxaldehyde thiosemicarbazide; gift from Vion Pharmaceuticals, Inc., New Haven, CT), Didox (N-3,4-trihydroxybenzamide) and Trimidox (N-3,4,5-trihydroxybenzoxcarboximide; gifts from Medicines for Health, Inc., Richmond, VA), 9-β-D-arabinofuranosyl-2-fluoro-AMP (Fludara; Schering Laboratories, Lys-les-Lannoy, France), cladribine (Leustatine; Janssen-Cilag, Boulogne-Billancourt, France), trioxatribine (Troxytal; Shire, Hampshire, UK), etoposide (Vépeside-Sandoz; Laboratoire Sandoz, Rueil-Malmaison, France), and doxorubicin (Adriablastine; Pharmacia & Upjohn, Saint-Quentin-en-Yvelines, France). 9-β-D-arabinofuranosyl-2-fluorodeoxyuridine, hydroxyurea, methylthiozoletriazolium, isopropanolol, and NaCl were purchased from Sigma Aldrich (Saint-Quentin, France), and HCl was from Merck. The TRizol RNA extraction kit, murine Moloney leukemia virus reverse transcriptase, TaqDNA polymerase, and RPMI 1640 cell culture medium were purchased from Invitrogen (Cergy Pontoise, France); l-glutamine and penicillin-streptomycin were from Gibco (Cergy Pontoise, France); and fetal bovine serum was from PAN Biotech GmbH (Aidenbach, Germany). UA911, the bis-(S-pivaloyl-2-thioethyl)phosphothioterivative derivative of ara-C, was obtained by adaptation of a previously published procedure and dissolved in dimethyl sulfoxide (100%) to a final concentration of 0.4% (22).

**Cell Culture.** L1210 murine leukemic cells were grown in RPMI 1640 containing L-glutamine, penicillin (200 IU/ml), streptomycin (200 μg/ml), and fetal bovine serum (10%) at 37°C in the presence of 5% CO₂.

**Cytotoxicity Studies.** Drug dilutions were prepared in RPMI 1640 medium at increasing concentrations (10⁻³) and 100 μl were added to each well in a 24-well plate (Becton Dickinson, Franklin Lakes, NJ). L1210 cells (900 μl containing 20,000 cells) were added to the wells and incubated at 37°C for 72 h. Methylthiozoletriazolium (500 μM) was added, and after 2 h of incubation at 37°C, the supernatant was removed and 300 μl of isopropanol–HCl–H₂O (90:9:1, v/v/v) were added to solubilize the formazan crystals. Absorbance was measured spectrophotometrically with a microplate reader (Labsystem Multiskanner RC). The IC₅₀ was defined as the concentration inhibiting proliferation to a level equal to 50% that of controls, and the resistance ratio was the ratio between the IC₅₀ of the gemcitabine-resistant L1210 10K cell line and the IC₅₀ of the sensitive parental cell line L1210 wt. IC₅₀ values were determined from concentration–effect curves generated using Microsoft Excel. Statistical significance was determined using Student’s t test.

**In vivo Studies.** On day 0, groups of 4- to 6-week-old female B6D2F₁ mice (IFFA CREDO, Arbeselle, France) received intraperitoneal injections containing 10⁵ L1210 wt or 10K cells in exponential growth phase. Gemcitabine-treated mice were treated on days 1, 4, 7, and 10 at similar doses. Three dose levels were tested: 360, 240, and 120 mg/kg. For ara-C studies, several schedules were tested: administration of 1000 mg/kg on days 1 and 2 or 300, 150, or 75 mg/kg on days 1–4 and 7–10, for a total of eight injections. The survival analysis was performed by constructing Kaplan–Meier survival curves. Drug solutions were prepared in distilled water at 24, 16, and 8 mg/ml for gemcitabine and 66.6, 20, 10, and 5 mg/ml for ara-C.

**Reverse Transcription-PCR.** Total mRNA was extracted by TRIzol as described previously (23). mRNA was converted to cDNA by reverse transcription at 37°C for 1 h using Moloney leukemia virus reverse transcriptase as described in the manufacturer’s manual. cDNA levels were standardized by use of primers for ribosomal 1S (Applied Biosystems, Foster City, CA), and specific mRNA levels were quantified by real-time reverse transcription-PCR in a LightCycler thermal cycler (Roche, Mannheim, Germany) in a final reaction volume of 6.67 μl containing forward and reverse primers (300 nM each), MgCl₂ (variable concentrations), deoxynucleotide triphosphates (500 μM) LC-FastStart DNA Master SYBRGreen or LC-FastStart DNA Master Hybridization Probes (Roche, Mannheim, Germany), and probes (130 nM). Cycling conditions and primer sequences are presented in Table 1.

Results were analyzed with RelQuant software (Roche) as indicated in the user’s manual, and results are given as mean values of three experiments expressed in arbitrary units using the parental L1210 wt cell line as reference. The statistical significance was determined using Student’s t test.
Sequencing of dCK Gene. Genomic DNA was prepared from L1210 wt and L1210 10K cells with a phenol–chloroform extraction method. dCK exons were amplified by PCR from 250 ng of genomic DNA using TaqDNA polymerase in a final volume of 25 μl containing forward and reverse primers (1.5 μM each; Table 2), deoxynucleotide triphosphates (200 μM) and MgCl₂ (1.5 mM). PCR conditions included initial denaturation for 15 min at 94°C followed by 50 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

Statistical Analysis. Statistical analyses were performed with STATISTICA version 6 (StatSoft, Inc., Tulsa, OK).

RESULTS

Development and Characterization of a Gemcitabine-Resistant Cell Line. Gemcitabine-resistant L1210 cells were developed by continuous exposure to increasing concentrations of gemcitabine over a period of 3 months, starting with an initial concentration of 1 nM, with 2–5-fold increases every 5 to 15 days. The cells obtained (L1210 10K) were viable in a medium containing 10 μM gemcitabine. L1210 10K cells showed no apparent morphologic differences or difference in growth rate compared with the parental L1210 wt cells.

The IC₅₀ values and resistance ratios for the gemcitabine-resistant L1210 10K cell line and the sensitive parental cell line L1210 wt are listed in Table 3. L1210 10K cells were 15,000-fold less sensitive to gemcitabine than the parental cell line. Cross-resistance was observed for other nucleoside analogues such as ara-C, cladribine, and troxacitabine, for which the resistance ratios were 2100, 160, and >200, respectively. Interestingly, no cross-resistance was found for the purine nucleoside analogue fludarabine, regardless of whether cells were exposed to the clinical formulation (9-β-D-arabinofuranosyl-2-fluoro-AMP; Schering) or unphosphorylated fludarabine (Sigma). Among the ribonucleotide reductase inhibitors tested, Trimidox displayed stronger cytotoxic activity on L1210 wt than on L1210 10K cells (resistance ratio = 7.22 ± 2.55), whereas Didox, hydroxyurea, and Triapine gave resistance ratios close to 1. Furthermore, L1210 10K showed no resistance to cisplatin, etoposide, doxorubicin, or homoharringtonine, suggesting that the resistance mechanism was specific to nucleoside analogues.

In vivo Sensitivity to Nucleoside Analogues. The median survival of mice receiving injections of 1210 wt or L1210 10K were almost similar (17 and 14 days, respectively; P, not significant). Survival of L1210 wt-carrying mice was significantly enhanced by gemcitabine treatment, with median survivals of 28, 35, and 42 days for doses of 120, 240, and 360 mg/kg, respectively. Survival of L1210 10K-carrying mice was unaffected by gemcitabine treatment (Fig. 1). No major toxicity

### Table 2 PCR primers used for sequencing the dCK gene (Locus ID: NT_039307)

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>AGGAACACACAGACATGCAGA</td>
<td>ACAGTTCCCAACAAAGGTTG</td>
<td>458</td>
</tr>
<tr>
<td>Exon 2</td>
<td>CTGCGCATGTTTCTTCAGT</td>
<td>GAAGTTTGGTGTTTACTCCC</td>
<td>263</td>
</tr>
<tr>
<td>Exon 3</td>
<td>AGCTAGAGAACGCTAGTGGAG</td>
<td>CAGGCGAGCCTCTAATTTGA</td>
<td>402</td>
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<tr>
<td>Exon 4</td>
<td>TCATAAAGGCCTCAACAGGG</td>
<td>AAGCTGGTGCTCTACAAAGA</td>
<td>408</td>
</tr>
<tr>
<td>Exon 5</td>
<td>AAGCCGACAGCAGCACAACCTA</td>
<td>AAGTACAAGTGCAGCAACCC</td>
<td>207</td>
</tr>
<tr>
<td>Exon 6</td>
<td>GGGGTATCAGCCTCTCTGT</td>
<td>CGGAGAAGCACAAGTTTTTA</td>
<td>322</td>
</tr>
<tr>
<td>3’UT</td>
<td>CTGACTCTGCAAGCCTTTCC</td>
<td>CAAGGACTTGGAGCAGGAC</td>
<td>166</td>
</tr>
</tbody>
</table>

NOTE. 3’-Untranscribed region (3’UT) corresponds to a region situated 2456 bp after the STOP codon of the dCK gene.

### Table 3 IC₅₀ values and resistance ratios in L1210 wt and L1210 10K cells as determined by methylthiazoletetrazolium assay

<table>
<thead>
<tr>
<th></th>
<th>L1210 wt</th>
<th>L1210 10K</th>
<th>RR</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemcitabine (μM)</td>
<td>0.000217 ± 0.000176</td>
<td>23.7 ± 11.0</td>
<td>14,833 ± 72,514</td>
<td>0.020†</td>
</tr>
<tr>
<td>Ara-C (μM)</td>
<td>0.0266 ± 0.0208</td>
<td>53.3 ± 35.1</td>
<td>2,100 ± 360</td>
<td>0.058</td>
</tr>
<tr>
<td>Troxacitabine (μM)</td>
<td>0.500 ± 0.440</td>
<td>&gt;100</td>
<td>&gt;200</td>
<td>0.00†</td>
</tr>
<tr>
<td>Cldribine (μM)</td>
<td>0.083 ± 0.029</td>
<td>12.67 ± 6.43</td>
<td>160 ± 69.3</td>
<td>0.028†</td>
</tr>
<tr>
<td>Fara-AMP (μM)</td>
<td>2.50 ± 0.886</td>
<td>3.00 ± 0.00</td>
<td>1.33 ± 0.577</td>
<td>0.37</td>
</tr>
<tr>
<td>Fara-A (μM)</td>
<td>3.33 ± 0.577</td>
<td>3.67 ± 1.12</td>
<td>1.08 ± 0.144</td>
<td>0.68</td>
</tr>
<tr>
<td>Trimidox (μM)</td>
<td>4.67 ± 1.15</td>
<td>33.3 ± 11.5</td>
<td>7.22 ± 2.55</td>
<td>0.013†</td>
</tr>
<tr>
<td>Didox (μM)</td>
<td>43.3 ± 5.78</td>
<td>46.7 ± 5.78</td>
<td>1.08 ± 0.144</td>
<td>0.52</td>
</tr>
<tr>
<td>HU (μM)</td>
<td>66.7 ± 20.8</td>
<td>80.0 ± 17.3</td>
<td>1.23 ± 0.251</td>
<td>0.44</td>
</tr>
<tr>
<td>Triapine (μM)</td>
<td>0.466 ± 0.0577</td>
<td>0.566 ± 0.0577</td>
<td>1.23 ± 0.252</td>
<td>0.10</td>
</tr>
<tr>
<td>Cisplatin (μM)</td>
<td>18.7 ± 10.0</td>
<td>27.0 ± 14.7</td>
<td>1.56 ± 0.962</td>
<td>0.46</td>
</tr>
<tr>
<td>Etoposide (μM)</td>
<td>5.00 ± 4.24</td>
<td>10.0 ± 0.00</td>
<td>3.13 ± 2.65</td>
<td>0.34</td>
</tr>
<tr>
<td>Doxorubicin (ng/ml)</td>
<td>7.75 ± 3.89</td>
<td>10.0 ± 1.41</td>
<td>1.42 ± 0.532</td>
<td>0.20</td>
</tr>
<tr>
<td>Homoharringtonine (ng/ml)</td>
<td>0.400 ± 0.100</td>
<td>0.366 ± 0.152</td>
<td>0.944 ± 0.419</td>
<td>0.77</td>
</tr>
</tbody>
</table>

NOTE. Data are mean values of three independent single experiments.

Abbreviations: RR, resistance ratio; ara-C, 1-β-D-arabinofuranosylcytosine; Fara-AMP, 9-β-D-arabinofuranosyl-2-fluoro-AMP; Fara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; HU, hydroxyurea.

* Student’s t test.
† Statistically significant.
related to gemcitabine was observed during this experiment, and all mice died of progressive disease.

Similar results were observed in mice treated with ara-C, with a median survival of 25 days in mice carrying L1210 wt cells and treated with 1000 mg/kg ara-C (Fig. 2A). When we used different treatment schemes with a total of eight injections, median survival was 23, 21, and 20 days for animals receiving doses of 300, 150, and 75 mg/kg, respectively (Fig. 2, B–D). Survival of L1210 10K-carrying mice was unaffected by ara-C treatment in all cases (median survival = 14, 14, 13, and 13 days with the different treatment schemes). Lethal toxicity was observed in L1210 wt-carrying mice treated with 1000 mg/kg (2 of 10) and with 300 mg/kg ara-C (3 of 7). These mice died before developing tumors as a result of ara-C toxicity.

Identification of Genes Involved in Resistance by Quantitative Reverse Transcription-PCR. To identify cellular mechanisms responsible for the resistance observed in L1210 10K cells, we quantified the expression of genes known to be involved in the metabolism and cytotoxicity of nucleoside analogues by quantitative reverse transcription-PCR (Fig. 3). The main difference was the total absence of dCK mRNA in L1210 10K cells, whereas this gene was found to be expressed in L1210 wt cells. Conversely, no significant difference was found in the expression of equilibrative nucleoside transporter 1, which is the main transporter of nucleoside analogues at the cell membrane level (24), or of MRP5, which has been described as a potential gemcitabine-efflux pump (25). Furthermore, we observed no differences in the levels of deoxyguanosine kinase; thymidine kinase 2; the 5’ nucleotidases cN-II, cN-III, cdN, and mdN (see Ref. 26 for new nomenclature for 5’-nucleotidases); or of the large subunit of ribonucleotide reductase between L1210 10K and L1210 wt cells. A small difference in the expression of thymidine kinase 1 and in the small subunit of ribonucleotide reductase was observed, with a lower level of expression in L1210 10K cells.

Analysis of the dCK Gene. Because dCK mRNA could not be detected in L1210 10K cells, we sought to determine whether the dCK gene was altered in these cells. We thus attempted to amplify the six exons of the murine dCK gene in both L1210 wt and L1210 10K cells. As shown in Fig. 4, all exons were amplified in L1210 wt cells. However, only the first five exons were amplified in the L1210 10K cells. The sixth exon as well as a 3’-untranscribed fragment of 166 bp starting 2456 bp after the STOP codon of the dCK gene could not be amplified in this line. These results suggest a genetic modification of chromosome 5 in the 3’ region of the dCK gene. The fragments corresponding to exons 2–5 were sequenced, and no difference was observed between the two cell lines.

Reversion of In vitro Nucleoside Resistance by an Ara-C Prodrug. Different strategies are being studied to circumvent nucleoside analogue resistance, in particular that due to dCK deficiency. One possibility is to expose cells to compounds that are already phosphorylated. Because monophosphates are not transported by nucleoside transporters such as ENT1 and do not permeate freely through the plasma membranes, we have used in our experiments a mononucleotide prodrug (pronucleotide) of ara-C (UA911; Fig. 5). In such constructs, the phosphate negative charges were temporarily masked by two S-pivaloyl-2-thioethyl groups. We have previously demonstrated that such pronucleotides are able to selectively deliver their parent 5’-monophosphates inside cells through an esterase-mediated process, leading to the accumulation of active triphosphorylated forms (27).
To study whether the nucleoside resistance of L1210 10K cells could be circumvented by the bis-S-pivaloyl-2-thioethyl approach, we compared the cytotoxic effect of ara-C to that of UA911 in methylthiazoletetrazolium assays (Table 4). L1210 wt cells were less sensitive to UA911 cytotoxicity than to ara-C cytotoxicity (IC50 values of 0.413 μM versus 0.0550 μM, respectively), whereas L1210 10K cells were significantly more sensitive to UA911 than to ara-C (6.88 μM versus 78.8 μM, respectively). The resistance ratios were 25.8 for UA911 and 1629 for ara-C (P < 0.00023), showing that UA911 partially circumvented the resistance of L1210 10K cells to ara-C.

**DISCUSSION**

Gemcitabine is now widely used in the treatment of various solid tumors such as pancreatic cancer, bladder cancer, and lung

<table>
<thead>
<tr>
<th>IC50 (μM)</th>
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<tbody>
<tr>
<td></td>
<td>L1210 wt</td>
<td>L1210 10K</td>
<td>RR</td>
<td></td>
</tr>
<tr>
<td>Ara-C</td>
<td>0.0550 ± 0.0370</td>
<td>78.8 ± 69.7</td>
<td>1629 ± 410</td>
<td>0.0074†</td>
</tr>
<tr>
<td>UA911</td>
<td>0.413 ± 0.400</td>
<td>6.88 ± 4.09</td>
<td>25.8 ± 20.5</td>
<td>0.020†</td>
</tr>
</tbody>
</table>

*Student’s t test.*
†Statistically significant. P value for comparison of resistance ratios = 0.00023.
cancer. More recent indications include breast cancers and certain hematologic malignancies. Unfortunately, response to gemcitabine treatment is limited by resistance (28). To better understand the cellular mechanisms involved in this resistance and to construct circumvention strategies, we have developed a murine cellular model for gemcitabine resistance that can be studied both in vitro and in vivo. The L1210 10K cell line is highly resistant to gemcitabine and two other pyrimidine nucleoside analogues, ara-C and troxacitabine, as well as the purine derivative cladribine. This cross-resistance is in accordance with results from similar studies on nucleoside-analogue-resistant cell lines (20, 27, 29–35). Surprisingly, L1210 10K was not resistant to the purine analogue fludarabine. This result indicates that there exist differences in the metabolism and/or mechanism of action of gemcitabine and fludarabine.

We chose the L1210 line because it is easily transposed into a syngeneic murine model. Our animal studies on the gemcitabine-resistant cell line showed that the resistance to nucleoside analogues observed in vitro was transposable in vivo. Mice receiving injections of L1210 wt cells and treated with gemcitabine or ara-C had significantly longer survival than untreated controls, whereas mice receiving injections of L1210 10K cells and treated with gemcitabine or ara-C did not live longer than untreated littermates. These results show that we have developed a simple animal model to study resistance to nucleoside analogues and, particularly, to evaluate different strategies aiming to revert this resistance.

The major mechanism of resistance to nucleoside analogues in the L1210 10K line appears to involve drug activation. We observed that expression of the two cytoplasmic nucleoside kinases dCK and thymidine kinase 1 was altered in L1210 10K. Using quantitative reverse transcription-PCR we detected 2-fold less thymidine kinase 1 mRNA in L1210 10K than in L1210 wt cells, whereas dCK mRNA remained undetectable in this model. dCK is the kinase responsible for the phosphorylation of most of the nucleoside analogues, and its down-regulation is frequently observed in nucleoside-analogue-resistant cell lines (29, 32–41) or in nonresponding patients treated with nucleoside analogues (42, 43). An associated down-regulation of thymidine kinase 1 had also been observed earlier in ara-C-resistant MOLT-4/8 cells (36). These observations led us to conclude that dCK deficiency plays a significant role in the resistance observed in this model.

To identify combined mechanisms of resistance, which are
likely to be present because UA911 does not allow complete reversal of the resistant phenotype, we explored other parameters involved in transport, metabolism, or targeting. We observed no or small differences in the expression of the nucleoside transporter equilibrative nucleoside transporter 1, the efflux pump MRPS5, and the 5′-nucleotidases Cn-II, Cn-III, cdN, and mdN as well as for the mitochondrial nucleoside kinases deoxyguanosine kinase and thymidine kinase 2 and the ribonucleotide reductase R1 and R2 subunits. We determined the expression of these genes because of their potential role in gemcitabine resistance. It has been shown that equilibrative nucleoside transporter 1 is the major transporter of nucleoside analogues at the cell membrane (24), and MRPS5 has been shown to efflux gemcitabine out of cells (25). 5′-Nucleotidases may be involved in drug resistance because they are likely to cause dephosphorylation of monophosphorylated nucleoside analogues and there are clinical observations showing that these proteins could play a role in nucleoside analogue resistance (44). However, recent in vitro studies with recombinant enzymes have not shown direct degradation of monophosphates (45), suggesting that they induce this resistance by modifying intracellular levels of endogenous nucleotides. Finally, ribonucleotide reductase is a target for diphosphorylated gemcitabine, and a modification of its expression could be involved in gemcitabine resistance.

The mechanism leading to a down-regulation of the expression of the dCK gene in nucleoside-analogue-resistant cells remains under discussion. One hypothesis involves a disturbance in the machinery responsible for the post-translational modifications of dCK (46). Another possibility is induced hyperpermylation of the dCK promoter that would lead to down-regulated transcription. This phenomenon has been studied in other models resistant to nucleoside analogues and could be involved in this mechanism (47, 48). However, treatment of L1210 10K cells with 5-azacytidine did not induce dCK expression (data not shown). Finally, the direct effect of nucleoside analogues on genomic DNA might lead to mutations of the dCK gene (49). Because we were unable to detect any expression of dCK mRNA in L1210 10K cells, we tried to sequence the corresponding gene. As shown in Fig. 4, we identified a genomic modification on chromosome 5 in this cell line. In fact, prolonged exposure to gemcitabine seems to have led to a partial deletion of the 3′ portion of the dCK gene, as shown by the fact that exon 6 was not amplified in this cell line. This result was confirmed by the fact that a portion of the 3′-untranscribed region of the dCK gene was also absent in the L1210 10K line. We have obtained similar results in other dCK-deficient cell lines, confirming this effect of gemcitabine on genomic DNA (50).

Different strategies to circumvent nucleoside analogue resistance include dCK stimulation (51–54), inhibition or stimulation of DNA repair (55), modulation of membrane transport (56), or intracellular deoxyxynucleotide triphosphate pools. UA911, a pronucleotide of ara-C, is more efficient than ara-C against L1210 10K cells, thereby demonstrating that this approach allows at least partial sensitization of totally dCK-deficient tumor cells. The fact that we did not observe total sensitization of L1210 10K cells with UA911 might be due to differences in the mechanism of action between gemcitabine and ara-C. Future studies will exploit the in vitro model to explore whether prodrugs are also able to sensitize resistant cells in vivo.

REFERENCES


Characterization of a Gemcitabine-Resistant Murine Leukemic Cell Line: Reversion of In vitro Resistance by a Mononucleotide Prodrug

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