Mechanisms of Uptake and Resistance to Troxacitabine, a Novel Deoxycytidine Nucleoside Analogue, in Human Leukemic and Solid Tumor Cell Lines

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ABSTRACT

Troxacitabine (Troxatyl; BCH-4556; (−)-2′-deoxy-3′-oxacytidine), a deoxycytidine analogue with an unusual dioxolane structure and nonnatural L-configuration, has potent antitumor activity in animal models and is in clinical trials against human malignancies. The current work was undertaken to identify potential biochemical mechanisms of resistance to troxacitabine and to determine whether there are differences in resistance mechanisms between troxacitabine, gemcitabine, and cytarabine in human leukemic and solid tumor cell lines. The CCRF-CEM leukemia cell line was highly sensitive to the antiproliferative effects of troxacitabine, gemcitabine, and cytarabine with inhibition of proliferation by 50% observed at 160, 20, and 10 nM, respectively, whereas a deoxycytidine kinase (dCK)-deficient variant (CEM/dCK−) was resistant to all three drugs. In contrast, a nucleoside transport-deficient variant (CEM/ARAC8C) exhibited high levels of resistance to cytarabine (1150-fold) and gemcitabine (432-fold) but only minimal resistance to troxacitabine (7-fold). Analysis of troxacitabine transportability by the five molecularly characterized human nucleoside transporters [human equilibrative nucleoside transporters 1 and 2, human concentrative nucleoside transporter (hCNT) 1, hCNT2, and hCNT3] revealed that short- and long-term uptake of 10−10 M [3H]troxacitabine was low and unaffected by the presence of either nucleoside transport inhibitors or high concentrations of nonradioactive troxacitabine. These results, which suggested that the major route of cellular uptake of troxacitabine was passive diffusion, demonstrated that deficiencies in nucleoside transport were unlikely to impart resistance to troxacitabine. A troxacitabine-resistant prostate cancer subline (DU145R; 6300-fold) that exhibited reduced uptake of troxacitabine was cross-resistant to both gemcitabine (350-fold) and cytarabine (300-fold). dCK activity toward deoxycytidine in DU145 cell lysates was <20% of that in DU145 cell lysates, and no activity was detected toward troxacitabine. Sequence analysis of cDNAs encoding dCK revealed a mutation of a highly conserved amino acid (Trp92 <130 Leu) in DU145 R dCK, providing a possible explanation for the reduced phosphorylation of troxacitabine in DU145 R lysates. Reduced deamination of deoxyxycytidine was also observed in DU145 R relative to DU145 cells, and this may have contributed to the overall resistance phenotype. These results, which demonstrated a different resistance profile for troxacitabine, gemcitabine, and cytarabine, suggest that troxacitabine may have an advantage over gemcitabine and cytarabine in human malignancies that lack or have low nucleoside transport activities.

INTRODUCTION

Troxacitabine (Troxatyl; BCH-4556; (−)-2′-deoxy-3′-oxacytidine) has potent antitumor activity against human leukemic (1) and solid tumor (2–5) xenograft animal models and is in clinical trials against human malignancies. Unlike naturally occurring nucleosides and deoxycytidine analogues such as gemcitabine (2′,2′-difluorodeoxycytidine; dFdC) and cytarabine (1-β-D-arabinofuranosycytosine; araC), which are in the β-D configuration, troxacitabine has a nonnatural β-L configuration. Troxacitabine, which shares the same intracellular activation pathway as gemcitabine and cytarabine, undergoes a series of phosphorylation reactions through a first rate-limiting step catalyzed by dCK5 (EC 2.7.1.74) to form the active triphosphate nucleotide. Troxacitabine triphosphate is incorporated into DNA (2), which is believed to be the main mechanism of cytototoxicity of deoxycytidine analogues, although there are differences in their DNA incorporation patterns. The incorporation of troxacitabine causes immediate chain termination (6), whereas gemcitabine incorporation allows the addition of a single deoxynucleotide (7, 8), and cytarabine incorporation allows the addition of multiple deoxynucleotides before chain termination (9, 10). Troxacitabine also differs from gemcitabine and cytarabine in that it is resistant to CDA (EC 3.5.4.5; Ref. 2). Although cellular uptake of troxacitabine has been reported to occur via the equilibrative nucleoside transport processes (11), it is not known whether functional plasma membrane nucleoside transporters are required for troxacitabine cytotoxicity, as has been reported for gemcitabine (12, 13) and cytarabine (14, 15).

Understanding of nucleoside transport processes of human cells has advanced considerably during recent years (reviewed in Refs. 16–18). Seven distinct nucleoside transport processes have been demonstrated in human cells on the basis of permeant selectivities, inhibition by diagnostic agents, and mechanisms of transport. The proteins responsible for the five major nucleoside transport processes have been identified by molecular cloning. The human transporter proteins comprise two functionally and molecularly distinct groups: (a) the hENTs; and (b) the sodium-dependent hCNTs. The proteins and their functional activities are: (a) hENT1, broadly selective and mediates es (equilibrative NBMPR-sensitive) activity; (b) hENT2, broadly selective and mediates ei (equilibrative NBMPR-insensitive) activity; (c) hCNT1, pyrimidine nucleoside selective and mediates cif (concentrative, insensitive to NBMPR and thymidine-selective activity); (d) hCNT2, purine nucleoside and uridine selective and mediates cif (concentrative, insensitive to NBMPR and formycin B-selective activity); and (e) hCNT3, broadly selective and mediates esi (concentrative, insensitive to NBMPR and broadly selective activity). The proteins responsible for csg (concentrative, sensitive to NBMPR and guanosine-selective) and cs (concentrative, sensitive to NBMPR)-mediated transport activities, which have only been reported in a few cell types, have not been identified.

The current work was undertaken to identify potential biochemical mechanisms of resistance to troxacitabine. The antiproliferative effects of troxacitabine against human cell lines that either possessed or lacked the capacity for nucleoside transport or phosphorylation of deoxycytidine were compared to determine whether the loss of either
process gave rise to resistance to troxacitabine. The transportability of troxacitabine was then examined in human cell lines that either lacked nucleoside transport activity altogether or exhibited a single nucleoside transporter by measuring the rates of uptake of \(^{3}H\)troxacitabine in the presence and absence of transport inhibitors or high concentrations of nonradioactive troxacitabine. These studies indicated that troxacitabine, a poor permeant of nucleoside transporters, enters cells primarily by passive diffusion, suggesting that a lack of nucleoside transport capability was unlikely to be involved in the development of resistance. This was confirmed in a troxacitabine-resistant prostate cancer cell line (DU145\(^{38}\)) that was evaluated for cross-resistance to gemcitabine and cytarabine and for changes in cellular uptake of troxacitabine. Reduced quantities of troxacitabine metabolites were observed in the resistant cells that could not be explained by changes in troxacitabine permeation, and dCK and CDA activities were therefore compared in the parental and resistant cells. Because the resistant cells also exhibited reduced CDA activity (in addition to reduced dCK activity), the impact of inhibition of CDA on troxacitabine toxicity was assessed by evaluating the sensitivity of DU145 cells to troxacitabine in the presence and absence of THU, an inhibitor of CDA (19).

The results indicated that troxacitabine, a novel deoxycytidine analogue, has a different uptake and metabolism profile in cultured human leukemic and prostate cancer cell lines than either gemcitabine or cytarabine. Troxacitabine may thus have an advantage for treatment of human malignancies that lack or have low nucleoside transport activities and may also be useful in human leukemias or solid tumors refractory to cytarabine or gemcitabine, respectively.

**MATERIALS AND METHODS**

**Materials.** Troxacitabine was synthesized at Shire BioChem Inc. (20), and \(^{3}H\)troxacitabine (3.9 Ci/mmol) was prepared by Moravek Biochemicals, Inc. (Brea, CA) from material provided by Shire Biochem Inc. Cytarabine and gemcitabine were gifts, respectively, from Bristol-Myers Squibb (Montreal, Canada) and Eli Lilly (Toronto, Canada). Uridine, NBMPR, dipyridamole, dilazep, tubercidin, and phosphorob 12-myristate 13-acetate were purchased from Sigma Chemical Co.-Aldrich Canada Ltd. (Oakville, Canada); THU was from Calbiochem (San Diego, CA); [methyl-\(^{3}H\)]thymidine (2 Ci/mmol) and [\(^{3}H\)]deoxyctydine (18.4 Ci/mmol) were from Amersham Canada (Oakville, Canada); [\(^{3}H\)]uridine (40 Ci/mmol) was from Moravek Biochemicals, Inc.; \(^{[14}C\)]U-glucose (0.6 Ci/mmol) was from New England Nuclear Life Science Products, Inc. (Boston, MA); the CellTiter 96 proliferation kit was from Promega Corp. (Madison, WI); the pcDNA3 mammalian expression vector was from Invitrogen (Carlsbad, CA); and DEAE-dextran was from Pharmacia Biotech Inc. (Baie d’Urfé, Canada). All other reagents used were of analytical grade and were obtained from commercial sources.

**Cell Culture.** The human CCRF-CEM leukemia, DU145 prostate cancer, HeLa cervical carcinoma, and HL-60 promyelocytic leukemia cell lines were purchased from the American Type Culture Collection (Manassas, VA). CEM/ARAC8C, a nucleoside transport-deficient derivative of CCRF-CEM, was a gift from Dr. B. Ullman (14) and was routinely cultured with 0.5 \(\mu\)M cytarabine and 0.25 \(\mu\)M tubercidin to maintain the mutant phenotype. CEM/dCK\(^{-}\), a dCK-deficient derivative of CCRF-CEM, was a gift from Dr. A. Fridland (21). Cells were grown in RPMI 1640 (CCRF-CEM, CEM/ARAC8C, CEM/dCK\(^{-}\), HeLa, and HL-60) or Eagle’s MEM with 0.1 mM nonessential amino acids (DU145 and DU145\(^{8}\)) supplemented with 10% fetal bovine serum (Life Technologies, Inc., Burlington, Canada). Stock cell lines, which were demonstrated to be free of Mycoplasma, were maintained as suspension (CCRF-CEM, CEM/ARAC8C, CEM/dCK\(^{-}\), and HL-60) or adherent (DU145, DU145\(^{8}\), and HeLa) cultures in the absence of antibiotics and incubated at 37°C in a humidified atmosphere (5% CO\(_2\)).

Exponentially growing HL-60 cells (3 \(\times\) 10\(^6\) cells) were induced to differentiate by plating in 100-mm Falcon Primaria tissue culture dishes (Becton Dickinson, Mississauga, Canada) in the presence of phorbol myristate acetate (200 ng/ml) as described previously (22).

Resistance to troxacitabine was induced by exposing DU145 cells stepwise to 2-fold increments of troxacitabine (0.01–10 \(\mu\)M) that were increased only when the proliferation rates of drug-treated cultures were similar to those of untreated cultures. After 8 months of continuous exposures, the resistant variant (designated DU145\(^{8}\)) was maintained in 2 \(\mu\)M troxacitabine.

**Chemosensitivity Testing.** The relative cytotoxicities of troxacitabine, gemcitabine, and cytarabine against CCRF-CEM, CEM/ARAC8C, and CEM/dCK cells were assessed using the CellTiter 96 proliferation assay. This assay is based on the reduction of a tetrazolium compound to a soluble formazan derivative by the dehydrogenase enzymes of metabolically active cells. The absorbance (490 nm) is directly proportional to the number of living cells in culture. Cells were added to 96-well tissue culture plates (10\(^3\) cells/well; 8 replicates/condition) and exposed to graded concentrations (1–10 \(\mu\)M) of cytarabine, gemcitabine, or troxacitabine, after which the numbers of living cells remaining were determined according to the manufacturer’s instructions. Antiproliferative activity of drugs against DU145 and DU145\(^{8}\) cells was determined by seeding cells in 12-well tissue culture plates (10\(^3\) cells/well; 3 replicates/condition) and initiating drug exposures 24 h later by a complete change of medium. Cells were enumerated by trypsinization and electronic particle counting (Coulter Electronics Inc., Luton, United Kingdom) after exposure to drugs for 48 h. Chemosensitivity was expressed as the drug concentration that inhibited cell proliferation by 50% (IC\(_{50}\) values) and determined from concentration-effect relationships using GraphPad Prism 2.01 (GraphPad Software, San Diego, CA).

**Transient Expression of hCNT1 and hCNT2 in HeLa Cells.** The cDNAs encoding the hCNT1 and hCNT2 proteins (GenBank accession number U62966 and AF036109, respectively) were subcloned from plasmids pMHK2 (23) and pMH15 (24) into the mammalian expression vector, pcDNA3, to produce pcDNA3-hCNT1 (25) and pcDNA3-hCNT2.\(^6\) The plasmids were transfected separately into actively proliferating HeLa cells as described previously (12, 25, 26).

**Cellular Uptake Assays.** All assays were conducted at room temperature. For suspension cells, uptake assays were conducted in microfuge tubes (10\(^6\) cells/tube) as described previously (27, 28). For adherent cells, assays were conducted in either sodium-containing transport buffer [20 mM Tris-HCl, 3 mM K\(_2\)HPO\(_4\), 1 mM MgCl\(_2\), 5 mM glucose, and 10 mM NaCl (pH 7.4); 300 \(\times\) 15 mOsm] or a sodium-free transport buffer in which NaCl was replaced by \(N\)-methyl-d-glucamine as described previously (25, 26).

**Intracellular Metabolism of \(^{3}H\)Troxacitabine and \(^{14}C\)Deoxycytidine.** Subconfluent cultures of DU145 and DU145\(^{8}\) cells were exposed to \(^{3}H\)troxacitabine or \(^{14}C\)deoxycytidine for 4 and 24 h. Cells were then harvested by trypsinization and recovered by centrifugation, and the resulting cell pellets were resuspended in 60% ice-cold methanol. After overnight incubation at \(-20°C\), the methanol extracts were centrifuged, and the pellets were used for determination of protein levels with the Bradford protein assay (Bio-Rad Laboratories, Mississauga, Canada). The supernatants were collected, evaporated to dryness, and resuspended in 200 \(\mu\)l of \(H_2O\) for HPLC analysis. Samples were analyzed with a C\(_4\) reversed-phase column (YMC ODS-A: 5 \(\mu\m, 120 A, and 250 \times 6.6 \)(mm inside diameter; Waters Corp., Milford, MA) and a LC module 1 (486 detector and injector 715) connected to UV (254 nm) and radioactivity monitors (Canberra Packard Canada Ltd., Montréal, Canada).

A linear gradient elution was started with CH\(_3\)CN (buffer A) to reach 50% of buffer B \(0.05 \mM\) NaPO\(_4\) (pH 6.7) containing 5 mM tetrabutyl ammonium dihydrogen phosphate) in 25 min at a flow rate of 1 ml/min. Elution was continued for 15 min. Analysis of standards (15 mM each of troxacitabine, tropoxabutamide monophosphate, tropoxabutamide diphosphate, and tropoxabutamide triphosphate) gave retention times of 6.1, 10.4, 21.1, and 39.9 min, respectively. Peak areas were quantified using Millennium software (Waters Corp.).

**dCK and CDA Assays.** DU145 and DU145\(^{8}\) cells were harvested from actively proliferating cultures by trypsinization and centrifugation, and cell pellets were stored (\(\leq\)2 months) at \(-70°C\) until use. For analysis, the pellets were thawed on ice, mixed (2.5 \(\times\) 10\(^3\) cells/ml) with 0.3 mM Tris-HCl (pH 8.0) containing 50 \(\mu\m\) B-mercaptoethanol, sonicated, and centrifuged. The resulting extracts were used for enzyme assays (conducted at 37°C) and for determina-
tion of protein content by the Bio-Rad Bradford protein assay. dCK activity was determined as described elsewhere (30, 31). Experiments were performed in the presence and absence of 1 mM thymidine to assess the mitochondrial thymidine kinase contribution to dCK activity, and in some experiments, [3H]troxacitabine was used as a substrate instead of [3H]deoxycytidine. For CDA activity, 100–200 μl portions of extract were incubated for up to 60 min in the presence of 20 μl of 5 mM deoxycytidine and 80 μl of CDA buffer [0.1 M Tris-HCl (pH 8.0) containing 50 μM β-mercaptoethanol]. The deoxycytidine was separated from the product (uridine) by HPLC analysis. The mobile phases used were 0.1% trifluoroacetic acid (pH 3.27; buffer A) and 0.1% CH3CN in buffer A (buffer B). A linear gradient elution was used (2% A→20% B, 30 min, 1 ml/min). Retention times (UV detection at 270 nm) for deoxycytidine and uridine were 7.2 and 8.6 min, respectively.

Sequence Analysis of DU145R dCK Gene. Actively proliferating DU145 and DU145R cells were harvested by trypsinization and centrifugation as described above, and cytoplasmic RNA was isolated and incubated with reverse transcriptase to yield cDNA using the GeneAmp RNA PCR kit (Roche Molecular Systems Inc., Branchburg, NJ). A 701-bp fragment corresponding to the human dCK open reading frame (residues 218–917; GenBank accession number M60527) was amplified using sense (5′-CGCATAAGAAAATCTC-GCATCAAGAAAATCTC-3′) and antisense (5′-ACCTTCTACACAGCTTCT-3′) primers. PCR products from two independent reverse transcription-PCR reactions for both DU145 and DU145R cells were cloned into pCR2.1-TOPO (Invitrogen). Plasmid DNA was prepared using the Qiagen-tip 100 kit (Qiagen, Mississauga, Canada) and sequenced from M13R and T7 primers using dye primer cycle sequencing (Bio S&T Inc., Lachine, Canada). Sequences were aligned and analyzed using Losergene software (DNASTAR), and homology searches were conducted using the BLAST server from the National Center for Biotechnology Information (Bethesda, MD).

RESULTS

Antiproliferative Activity of Troxacitabine, Gemcitabine, and Cytarabine against CCRF-CEM Cells and Drug-resistant Variants That Lack dCK or Nucleoside Transport Activity. The toxicity of troxacitabine against CCRF-CEM and CEM/dCK− cells was compared with that of gemcitabine and cytarabine by assessing their relative abilities to inhibit proliferation during continuous 48-h exposures (Table 1). Proliferation of CCRF-CEM cells was potently inhibited, with IC50 values of 160, 20, and 10 nM, respectively, for troxacitabine, gemcitabine, and cytarabine. In contrast, the IC50 values for CEM/dCK− cells were substantially greater than the values observed for CCRF-CEM cells (Table 1). These results confirmed that impaired dCK activity greatly decreased cellular sensitivity to troxacitabine, as reported previously (2), and were consistent with the three drugs sharing the same initial activation pathway whereby phosphorylation is catalyzed by dCK.

CEM/ARAC8C cells, which exhibit high-level resistance to cytarabine because of the genetic loss of hENT1-mediated transport, are cross-resistant to other nucleoside analogues that are permeants for this transporter (32). When exposed to troxacitabine, gemcitabine, and cytarabine, the levels of resistance differed by 7-, 431-, and 1150-fold, respectively (Table 1). These results indicated that troxacitabine toxicity was less affected than that of gemcitabine or cytarabine by the absence of a functional nucleoside transporter.

Uptake of [3H]Troxacitabine and [3H]Uridine by CCRF-CEM and CEM/ARAC8C Cells. The contribution of permeation to cellular uptake of troxacitabine was examined by comparing time courses of uptake of [3H]troxacitabine by CCRF-CEM and CEM/ARAC8C cells (Fig. 1). Parallel measurements were also conducted with [3H]uridine, a physiological substrate of hENT1. CCRF-CEM cells exhibited a large difference in capacity for uptake of 30 μM [3H]troxacitabine and [3H]uridine, with initial rates of uptake (2–10 s) of 0.073 and 0.585 pmol/106 cells/s, respectively. Although uptake (2–60 s) of [3H]uridine by CCRF-CEM cells was reduced by >99% to 0.002 and 0.005 pmol/106 cells/s by the presence of either 5 mM nonradioactive uridine or 100 nM NBMPR, respectively, uptake of [3H]troxacitabine by CCRF-CEM cells was unaffected by 5 mM nonradioactive cytarabine or 100 nM NBMPR. Extended time courses of uptake of [3H]troxacitabine and [3H]uridine were also determined in CEM/ARAC8C cells. In these cells, the uptake of troxacitabine was comparable with that of uridine, with initial rates (2–60 s) of 0.057 and 0.030 pmol/106 cells/s, respectively. Because the initial uptake rates (2–10 s) observed for 30 μM troxacitabine were so low, uptake at higher concentrations (40, 50, and 60 μM) was also examined in CCRF-CEM cells (data not shown). The total amounts of troxacitabine accumulated for all four concentrations at 60 s increased only slightly, from 12.8 to 14.4 pmol/106 cells. These results were consistent with uptake by passive diffusion and/or a low-affinity transport process.

Table 1 A comparison of chemosensitivities of CCRF-CEM, CEM/dCK−, and CEM/ARAC8C cells to troxacitabine, gemcitabine, and cytarabine

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Troxacitabine IC50 (nM)</th>
<th>Gemcitabine IC50 (nM)</th>
<th>Cytarabine IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>160 ± 12</td>
<td>20 ± 0.4</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>CEM/dCK−</td>
<td>&gt;100,000</td>
<td>&gt;50,000</td>
<td>&gt;50,000</td>
</tr>
<tr>
<td>CEM/ARAC8C</td>
<td>1,180 ± 320</td>
<td>8,630 ± 880</td>
<td>11,500 ± 2,700</td>
</tr>
</tbody>
</table>

Fig. 1. A comparison of uptake of troxacitabine and uridine by nucleoside transport-competent CCRF-CEM and nucleoside transport-deficient CEM/ARAC8C cells. Uptake of 30 μM [3H]nucleoside was determined at the time intervals shown as described in “Materials and Methods” in the absence or presence of either 5 mM nonradioactive nucleoside or 100 nM NBMPR. Each data point represents the mean ± SD of three determinations; error bars are not shown where values were small and therefore obscured by data points. A (CCRF-CEM cells): [3H]troxacitabine alone, •; [3H]uridine alone, ■; and [3H]uridine plus 5 mM nonradioactive uridine (•) or 100 nM NBMPR (■). The values for [3H]troxacitabine in the presence of nonradioactive troxacitabine or NBMPR were the same as those obtained for troxacitabine alone and therefore are not shown. B (CEM/ARAC8C cells): [3H]troxacitabine alone, •; and [3H]uridine alone, ■. The values in the absence of nonradioactive nucleoside were the same as those obtained for [3H]nucleoside alone and therefore are not shown.

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The Influence of Enantiomeric Configuration of Troxacitabine on Its Ability to Inhibit Uridine Transport in CCRF-CEM Cells.

Equilibrative transporters are stereoselective (33), which could explain the lack of recognition of the L-enantiomer of troxacitabine by hENT1. The influence of enantiomeric configuration on troxacitabine transportability was examined in CCRF-CEM cells by comparing the effects of 10 mM D- or L-troxacitabine on initial uptake rates of 10 μM [3H]uridine (the natural D-enantiomer). The addition of 10 mM non-radioactive D-uridine to assay mixtures reduced transport of 10 μM [3H]uridine (2–8 s) from 1.02 to 0.09 pmol/10^6 cells/s. In contrast, the presence of D- or L-troxacitabine produced only partial inhibitions, reducing initial D-uridine transport rates from 1.02 to 0.56 (D-troxacitabine) and 0.64 (l-troxacitabine) pmol/10^6 cells/s. These results, which demonstrated that D- and L-troxacitabine enantiomers were not capable of effectively competing with D-uridine for interaction with hENT1 when present at 1000-fold greater concentrations than uridine, suggested that troxacitabine was poorly recognized by hENT1 because of its dioxolane structure rather than its enantiomeric configuration.


Uptake of troxacitabine by DU145 cells was reported previously to be inhibited by NBMPR and dipyrinamid, suggesting entry into DU145 cells via both hENT1- and hENT2-mediated transport processes (11). Because the nucleoside transport characteristics of DU145 cells have not been defined previously, the transportability of uridine, which is a permeant of both equilibrative and concentrative nucleoside transporters (16), was examined in experiments that assessed the effects of (a) excess nonradioactive uridine, (b) inhibitors of equilibrative nucleoside transport processes (NBMPR and dilazep), and (c) replacement of sodium with N-methyl-D-glucamine.

Addition of nonradioactive uridine (1 mM) to uptake assay mixtures reduced the initial rate of uptake (2–60 s) of 5 μM [3H]uridine by DU145 cells from 0.35 to 0.02 pmol/10^6 cells/s, indicating that >95% of uridine uptake was mediated. In separate experiments (Fig. 2A), the initial rate of uptake (2–60 s) of 10 μM [3H]uridine was reduced by 57% (from 0.60 to 0.26 pmol/10^6 cells/s) in the presence of 100 nM NBMPR and by >99% (from 0.60 to 0.01 pmol/10^6 cells/s) in the presence of 100 μM dilazep, indicating that the major routes of uridine entry were via hENT1- and hENT2-mediated transport processes, respectively.

The concentrative nucleoside transporters (hCNT1, hCNT2, and hCNT3), which are sodium/nucleoside symporters, can be detected by comparing initial rates of uptake in sodium-containing assay mixtures with those in which sodium has been replaced with N-methyl-D-glucamine; the latter manipulation eliminates the extracellular sodium gradient required for functionality of the concentrative nucleoside transporters. Rates of uptake of [3H]uridine by DU145 cells were similar in medium with or without sodium (Fig. 2A), indicating that DU145 cells did not possess sodium-dependent nucleoside transport activity.

Uptake of [3H]troxacitabine by DU145 cells was examined in the presence and absence of NBMPR at a concentration that is known to block hENT1-mediated but not hENT2-mediated transport processes (Fig. 2B). Uptake was barely detectable, even after 4-h exposures to [3H]troxaicitabine in the presence or absence of NBMPR. These results, which indicated that hENT1-mediated transport in DU145 cells was not responsible for troxacitabine permeation, were consistent with the low and primarily nonmediated uptake of troxacitabine observed in CCRF-CEM cells. High concentrations of nonradioactive troxaicitabine and dilazep also had no effect on uptake of [3H]troxacitabine by DU145 cells (data not shown).

The apparent absence of mediated permeation of troxacitabine in DU145 cells was confirmed in HeLa cells (Fig. 3), which possess high levels of both hENT1- and hENT2-mediated transport activities (34). Uptake of troxacitabine was low and unaffected by the addition of a 100-fold excess of nonradioactive troxacitabine and reduced only
slightly by NBMPR or dilazep (Fig. 3A). The extended time courses for uridine uptake shown in Fig. 3B, which were obtained in cells treated with 100 nM NBMPR to block hENT1, revealed a large difference in uptake of uridine and troxacitabine. At 4 h, HeLa cells had accumulated 1221 pmol/10^6 cells of uridine and only 9 pmol/10^6 cells of troxacitabine.

**Transportability of [3H]Troxacitabine by the Sodium-dependent Concentrative Nucleoside Transporters.** The transportability of troxacitabine by each of the three known human members of the CNT protein family was assessed in experiments that used either recombinant hCNT1 or hCNT2 that was introduced into HeLa cells by transient transfection or native hCNT3 that was activated by stimulation of HL-60 cells to differentiate. HeLa and HL-60 cells were treated with 100 μM dilazep to block the equilibrative transport processes, and the uptake of 10 μM [3H]troxacitabine was compared with that of 10 μM [3H]uridine during 4-h exposures. Sodium-dependent uptake of uridine was evident for each of the three transporters, whereas troxacitabine uptake was low and independent of sodium. In the experiments shown in Fig. 4, hCNT1- and hCNT2-mediated uptake of uridine at 4 h (1077 and 939 pmol, respectively) was much greater than uptake of troxacitabine (12 and 7 pmol, respectively). The uptake of uridine observed in the absence of sodium may represent uncoupled Na+/uridine fluxes, or “slippage,” a process that has been observed previously for recombinant CNT family members (26). A similar result was obtained for hCNT3 in experiments (data not shown) in which sodium-dependent, dilazep-insensitive uptake of 10 μM [3H]uridine and [3H]troxacitabine was compared in differentiated HL-60 cells. After 4-h exposures, uridine uptake was 54-fold greater than troxacitabine uptake (644 versus 12 pmol, respectively).

**Characterization of Troxacitabine Resistance and Metabolism in DU145R Cells.** To further examine mechanisms of troxacitabine resistance, a drug-resistant variant (DU145R) of cultured human prostate DU145 cells was developed by continuously growing the cells in increasing concentrations of drug over an 8-month period. The toxicity of troxacitabine against DU145 and DU145R cells was compared with that of gemcitabine and cytarabine by assessing their relative abilities to inhibit proliferation during continuous 48-h exposures. Proliferation of DU145 cells was inhibited, with IC_{50} values of 10, 20, and 100 nm, respectively, for troxacitabine, gemcitabine and cytarabine. In contrast, DU145R cells exhibited 6300-fold resistance to troxacitabine and 350- and 300-fold cross-resistance to gemcitabine and cytarabine, respectively (data not shown).

The metabolism of [3H]troxacitabine and [3H]deoxycytidine by DU145 and DU145R cells was compared after 4- and 24-h exposures (Fig. 5). Parental DU145 cells converted troxacitabine to its mono-, di-, and triphosphorylated forms (Fig. 5A), and the diphosphate was the major metabolite, which was consistent with the findings of a previous report (2). The ATP:ADP ratios in the DU145 cell extracts varied between 2 and 4, indicating that the higher levels of diphosphorylated troxacitabine relative to triphosphorylated troxacitabine were not due to degradation by phosphatase(s) during extraction procedures. Furthermore, when [3H]deoxycytidine metabolism was examined in DU145 cells, dCTP was the major phosphorylated metabolite (Fig. 5B). Troxacitabine-derived radioactivity in DU145R cells was low compared with that in DU145 cells (5 versus 254 pmol/mg protein, respectively, at 24 h) and was mostly present as unmetabolized troxacitabine (Fig. 5C). Whereas deoxycytidine-derived radioactivity in DU145R cells was also low compared with that in DU145 cells (3 versus 34 pmol/mg protein, respectively), all three phosphorylated metabolites (mono-, di-, and triphosphates) were detected (Fig. 5D). These results, which demonstrated reduced accumulation and an altered pattern of phosphorylation in the troxacitabine-resistant variant, suggested a change in dCK activity.

**Demonstration of Altered dCK Activity in DU145R Cells.** The activity of dCK toward troxacitabine and its natural substrate, deoxycytidine, was examined in extracts from DU145R and DU145 cells and, for comparison, in extracts from CCRF-CEM cells and its cytarabine-resistant variants (Table 2). As reported previously (2, 35), phosphorylation of deoxycytidine by dCK was greatly reduced in CEM/dCK− cells relative to either CCRF-CEM or CEM/ARAC8C cells, and the almost 2-fold difference observed in dCK activity between CCRF-CEM and CEM/ARAC8C cells was likely due to
formed/mg protein and are the mean/H11006

cells as described in “phan is conserved in all known sequences of dCK and is also present from tryptophan to leucine at position 92 in the protein. This tryptophan conversion to T in DU145R cells was not detected in either of the resistant variants (Table 2). A single base change was detected in which a G residue at position 434 in DU145 cells, was not observed in extracts from both CCRF-CEM and DU145 cells. Phosphorylation of troxacitabine, DU145R cells, whereas there was a 137-fold difference between

dCK activity between DU145 and DU145R cells.

Table 2 A comparison of the dCK and CDA activities of parental and drug-resistant
human leukemic and prostate cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>dCK</th>
<th>CDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>5.49 ± 1.55</td>
<td>ND*</td>
</tr>
<tr>
<td>CEM/ARAC8C</td>
<td>3.17 ± 0.67</td>
<td>0.10</td>
</tr>
<tr>
<td>CEM/dCK</td>
<td>0.04 ± 0.01</td>
<td>ND</td>
</tr>
<tr>
<td>DU145</td>
<td>0.93 ± 0.38</td>
<td>1.49 ± 0.11</td>
</tr>
<tr>
<td>DU145R</td>
<td>0.14 ± 0.03</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not detected.

metabolic differences that have arisen since the selection of the resistant variant in 1980 (21). Although phosphorylation of deoxycytidine was also reduced in DU145R cells relative to DU145 cells, there was only a 7-fold difference in dCK activity between DU145 and DU145R cells, whereas there was a 137-fold difference between CEM/dCK- and CCRF-CEM cells. Phosphorylation of troxacitabine, which was observed in extracts from both CCRF-CEM and DU145 cells, was not detected in either of the resistant variants (Table 2).

To determine whether a mutation in dCK of DU145R cells was present and could be responsible for the observed differences in activities, a partial cDNA encoding dCK was amplified from both DU145 and DU145R cells and sequenced. A single base change was detected in which a G residue at position 434 in DU145 dCK was converted to T in DU145R dCK, resulting in a nonconserved change from tryptophan to leucine at position 92 in the protein. This tryptophan is conserved in all known sequences of dCK and is also present in the homologous domain of deoxyguanosine and thymidine kinases (Fig. 6).

DU145R Cells Exhibit Reduced CDA Activity. Although CDA activity is normally low in human tissues, aside from liver and placenta (36), it is elevated in solid tumors (31). CCRF-CEM, CEM/ dCK−, and CEM/ARAC8C cells, consistent with their hematopoietic origin, lacked detectable CDA activity, whereas DU145 cells exhibited high CDA activity (Table 2). Unexpectedly, the level of CDA activity was significantly reduced in DU145R cells (Table 2). Because troxacitabine is resistant to deamination (2), the 11–12-fold reduction observed in DU145R cells suggested that reduced deamination of deoxycytidine might have contributed to troxacitabine resistance in DU145R cells.

To provide evidence that a difference in CDA activity might be involved in resistance to troxacitabine, DU145 cells were exposed to graded concentrations of troxacitabine (0–10 μM) in the absence or presence of 50 μM THU, a competitive inhibitor of CDA (37). The IC50 values for inhibition of proliferation of DU145 cells by troxacitabine in the presence of THU was increased in two separate experiments from 15 to 140 nM (9.3-fold) and from 16 to 104 nM (6.5-fold).

DISCUSSION

The purpose of this study was to identify potential mechanisms of resistance to troxacitabine. Troxacitabine cytotoxicity was first compared with that of cytarabine and gemcitabine in dCK-deficient (CEM/dCK−) and nucleoside transport-deficient (CEM/ARAC8) leukemia cell lines. Although previous research has shown that cells deficient in dCK are resistant to troxacitabine (2), gemcitabine (38, 39), and cytarabine (2, 40), a comparative analysis of all three deoxycytidine analogues has not been reported. Although CCRF-CEM cells deficient in dCK activity were cross-resistant to troxacitabine, gem-

Fig. 6. PCR amplification and sequence analysis of dCK cDNA from cytokrinal RNA of DU145 and DU145R cells. A, PCR products were analyzed on 1% agarose gels and stained with ethidium bromide. PCR amplification of GAPDH was used to control for mRNA levels. B, partial sequence of human and mouse dCK and other human deoxynucleoside kinases as well as the unrelated Drosophila melanogaster nucleoside kinase. Accession numbers in SwissProt database are as follows: human dCK, P27707; murine dCK, P43346; human dOK, Q16854; human TK2, Q00142. Accession number in the European Molecular Biology Laboratory database is as follows: Drosophila melano-
gaster dNK, Y10048.1.
citabine, and cytarabine, CCRF-CEM cells deficient in nucleoside transport activity were cross-resistant to gemcitabine and cytarabine but exhibited only low-level resistance to troxacitabine. These results suggested a different mechanism of cellular uptake for troxacitabine than that of cytarabine and gemcitabine and a common initial activation pathway whereby phosphorylation is catalyzed by dCK. Although troxacitabine was reported previously to be transported by hENT1 (11), the low level of troxacitabine resistance observed in CEM/ARAC8C cells as compared with CCRF-CEM cells, which possess only hENT1-mediated nucleoside transport activity (41, 42), suggested that a functional nucleoside transporter is not required for manifestation of troxacitabine toxicity.

The demonstration of very low rates of uptake of troxacitabine relative to uridine in transport-competent CCRF-CEM cells and similar rates of uptake of troxacitabine and uridine in transport-deficient cells suggested that the primary mode of uptake of troxacitabine in both CCRF-CEM cell lines was passive diffusion. In studies of uptake of radiolabeled troxacitabine by cell lines with defined nucleoside transport activities, troxacitabine was shown to be a poor permeant for the molecularly characterized equilibrative (hENT1 and hENT2) and sodium-dependent (hCNT1, hCNT2, and hCNT3) nucleoside transporters, and thus its uptake was probably not mediated by these nucleoside transporters. Although minor contributions from other uncharacterized transporters have not been ruled out, the low uptake observed for troxacitabine was consistent with a diffusion model and with results of previous studies with t-nucleosides, which indicated that the t-enantiomers of several natural nucleosides (thymidine, uridine, and adenosine) are poor permeants of hENT1 and nonpermeants of hENT2 and hCNT2-mediated processes (27, 33, 43–45). The basis for the lack of agreement between the results reported here for DU145 cells and those of a previous report (11), which suggested that the permeation of troxacitabine was mediated by equilibrative transporters, is unclear.

To further explore potential mechanisms of resistance to troxacitabine, a troxacitabine-resistant cell line was developed by growing DU145 cells in the presence of stepwise increasing concentrations of troxacitabine. Although DU145 and DU145R cells exhibited similar low and apparently nonmediated uptake of radiolabeled troxacitabine, metabolism studies using radiolabeled troxacitabine or deoxycytidine indicated that a drastic reduction in the dCK activity of resistant DU145R cells toward troxacitabine was the major mechanism of resistance. DU145R cells were unable to phosphorylate troxacitabine and had significantly reduced intracellular levels of phosphorylated deoxycytidine species. These results and the cross-resistance toward cytarabine and gemcitabine suggested a deficiency in and a change in specificity of dCK, the common rate-limiting enzyme for the activation of these three deoxycytidine analogues. A change in dCK specificity has been reported previously for a cytarabine-resistant rat leukemia cell line (38). PCR analysis confirmed that the amount of dCK mRNA isolated from DU145R cells was significantly reduced compared with that isolated from the parental cells, suggesting that only one allele of the dCK gene was expressed. It is also possible that the mutation, which resulted in a change from a highly conserved trypthphan residue to a leucine residue at position 92 in dCK, may have destabilized the dCK mRNA. These observations are similar to those obtained in a CCRF-CEM cell line resistant to dideoxycytidine (46).

Because DU145R cells retained the capacity to phosphorylate deoxycytidine, although at reduced levels compared with parental cells, the capacity of the resistant cells for deamination of deoxycytidine was examined. CDA activity in the troxacitabine-resistant DU145R cells was reduced to <10% of that observed in the sensitive parental DU145 cells. One possible explanation is that decreased deamination of deoxycytidine (the natural substrate for CDA and dCK) would confer resistance by increasing deoxycytidine levels, thereby increasing its capacity to compete with troxacitabine for phosphorylation by dCK. Interestingly, the antiproliferative activity of DMDC, an anti-tumor nucleoside analogue resistant to deamination, is also modulated by changes in CDA activity. Cells treated with THU, a competitive inhibitor of CDA (47), exhibited reduced sensitivity toward gemcitabine but increased sensitivity toward DMDC (19). The finding that THU protected DU145 cells from troxacitabine toxicity was consistent with the conclusion that the coincidental reduction in CDA activity observed in DU145R cells contributed to the overall resistance mechanism to troxacitabine, similar to what has been demonstrated recently for DMDC (48).

In conclusion, troxacitabine exhibited mechanisms of cellular uptake, metabolism, and resistance that differed from those of the other deoxycytidine analogues, cytarabine and gemcitabine. These differences give troxacitabine properties that may be beneficial to patients who are refractory to cytarabine and/or gemcitabine. Indeed, significant antileukemic activity has been observed with troxacitabine in a Phase I clinical trial in patients with primary refractory or relapsed acute myeloid leukemia after prior therapy with cytarabine (49). Troxacitabine is currently being evaluated in Phase II studies for acute myeloid leukemia, chronic myeloid leukemia (blast phase), and pancreatic cancer.

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Mechanisms of Uptake and Resistance to Troxacitabine, a Novel Deoxycytidine Nucleoside Analogue, in Human Leukemic and Solid Tumor Cell Lines

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