Two Distinct Molecular Mechanisms Underlying Cytarabine Resistance in Human Leukemic Cells

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Abstract
To understand the mechanism of cellular resistance to the nucleoside analogue cytarabine (1-β-D-arabinofuranosylcytosine, AraC), two resistant derivatives of the human leukemic line CCRF-CEM were obtained by stepwise selection in different concentrations of AraC. CEM/4 × AraC cells showed low AraC resistance, whereas CEM/20 × AraC cells showed high resistance. Both cell lines showed similar patterns of cross-resistance to multiple cytosine nucleoside analogues, with the exception that CEM/20 × AraC cells remained sensitive to 5-fluorouridine and 2-deoxy-5-fluorouridine. Both cell lines were sensitive to 5-fluorouracil and to a variety of natural nucleoside transporters. Two distinct families of human nucleoside transporters have been identified and characterized to date: the human equilibrative nucleoside transporters (hENT1–hENT4; also known as SLC29A1–SLC29A4) and the human concentrative nucleoside transporters (hCNT1–hCNT3; also known as SLC28A1–SLC28A3). hENT1/SLC29A1 has been shown to be the primary transporter responsible for cellular uptake of AraC (see refs. 13–17 for recent reviews). Inside cells, AraC is “activated” to form AraCTP through sequential phosphorylation by deoxycytidine kinase (DCK), DCTP kinase, and nucleoside diphosphate kinase. The cytotoxicity of AraC is subsequently accomplished by nonproductive incorporation of AraCTP into nascent DNA or RNA. Decreased expression of SLC29A1 mRNA and DCK mRNA is associated with reduced level of intracellular AraC uptake and reduced DCK activity, respectively; both alterations are associated with increased cellular resistance to AraC and gemcitabine in cancer cell lines (14–16, 18, 19). Although loss of SLC29A1 gene function has yet to be described in AraC-resistant cells, numerous sequence polymorphisms (silent or missense variants) have been detected in the SLC29A1 gene from different ethnic populations and have been speculated to contribute in part to the variable efficacy of AraC (20–22). On the other hand, overexpression of the gene encoding cytidine deaminase in transfected cells has been shown to cause increased AraC resistance, via deamination of AraC, the prodrug, to an inactive form (23–25). In addition, activation of 5′-nucleotidase and ribonucleotide reductase may affect conversion of AraC to the active inhibitor, possibly reducing its therapeutic efficacy (26–30). The overall efficacy of AraC as an antitumor agent may thus be modulated by a combination of factors, including variations in drug uptake, metabolic, and catabolic pathways, as well as efflux activity (13, 17, 31–33).

Introduction
Cytarabine (1-β-D-arabinofuranosylcytosine, AraC) is a deoxycytidine analogue that has been used either alone or in combination with other chemotherapeutic agents for the treatment of acute myeloid leukemia (AML; refs. 1–3), relapsed and refractory acute lymphoblastic leukemia (ALL; refs. 4–6), and other malignancies (7–10). The chemotherapeutic efficacy of cytarabine varies dramatically between individuals. In general, standard induction therapy with combination of anthracycline and cytarabine produced 50% to 75% of complete remission rate in adult AML patients (1–3). Higher remission rate has been observed in newly diagnosed pediatric AML patients treated with improved chemotherapeutic regimens (11, 12). However, ~30% to 50% of patients relapse with drug-resistant disease. Differences in genetic background, as well as efficiency of drug uptake, metabolism, and elimination may account for variable effectiveness of AraC-containing drug regimens.

Nucleoside analogue drugs, such as AraC and gemcitabine, have low rates of passive diffusion across membranes and enter cells primarily as fraudulent substrates for specialized nucleoside transporter proteins. Two distinct families of human nucleoside transporter proteins have been identified and characterized to date: the human equilibrative nucleoside transporters (hENT1–hENT4; also known as SLC29A1–SLC29A4) and the human concentrative nucleoside transporters (hCNT1–hCNT3; also known as SLC28A1–SLC28A3). hENT1/SLC29A1 has been shown to be the primary transporter responsible for cellular uptake of AraC (see refs. 13–17 for recent reviews). Inside cells, AraC is “activated” to form AraCTP through sequential phosphorylation by deoxycytidine kinase (DCK), DCTP kinase, and nucleoside diphosphate kinase. The cytotoxicity of AraC is subsequently accomplished by nonproductive incorporation of AraCTP into nascent DNA or RNA. Decreased expression of SLC29A1 mRNA and DCK mRNA is associated with reduced level of intracellular AraC uptake and reduced DCK activity, respectively; both alterations are associated with increased cellular resistance to AraC and gemcitabine in cancer cell lines (14–16, 18, 19). Although loss of SLC29A1 gene function has yet to be described in AraC-resistant cells, numerous sequence polymorphisms (silent or missense variants) have been detected in the SLC29A1 gene from different ethnic populations and have been speculated to contribute in part to the variable efficacy of AraC (20–22). On the other hand, overexpression of the gene encoding cytidine deaminase in transfected cells has been shown to cause increased AraC resistance, via deamination of AraC, the prodrug, to an inactive form (23–25). In addition, activation of 5′-nucleotidase and ribonucleotide reductase may affect conversion of AraC to the active inhibitor, possibly reducing its therapeutic efficacy (26–30). The overall efficacy of AraC as an antitumor agent may thus be modulated by a combination of factors, including variations in drug uptake, metabolic, and catabolic pathways, as well as efflux activity (13, 17, 31–33).

In the present study, we used CCRF-CEM, an ALL cell line, as a cellular model to investigate molecular mechanisms underlying cellular resistance to AraC. Two cytarabine-resistant cell lines, CEM/4 × AraC and CEM/20 × AraC, were developed by continuous exposures and stepwise selection in increasing concentrations of AraC. The resistance phenotypes, the drug uptake properties, and the molecular mechanisms responsible for the observed drug
resistance were analyzed in the two resistant cell lines. Although derived from the same parental cell line, the two cell lines exhibited two distinct mechanisms of resistance to nucleoside analogue drugs.

**Materials and Methods**

**Materials.** AraC, 5-fluorouracil (5-FU), 5-fluorouridine, 5-fluoro-2-deoxyuridine, nitrobenzylmercaptopurine ribonucleoside (NBMPR), and dideoxycytidine were purchased from Sigma-Aldrich, Genetuxin, Fludara, and Cladrilide were obtained from the Hematology Department of Royal Victoria Hospital. [5-3H]Uridine (193 Ci/mm), [3H]cytosine-β-D-arabinofuranoside (103H]AraC; 15-30 Ci/mm), [3H]NBMPR (15 Ci/mm), and [3H]dideoxyadenosine (27.7 Ci/mm) were from Moravek Biochemicals.

The anti-hENT1 monoclonal antibodies were developed and characterized previously (34) using synthetic peptides corresponding to amino acid residues 254 to 271 of hENT1/SLC29A1. 5'-[2(1-[fluorescein-5-sulfo-hexanamido]-hexanamido)ethyl]-6-N-([4-nitrobenzyl]-5'-thioadenosine (FTH-SAENTA) is a fluorescein-tagged structural analogue of NBMPR that binds tightly to the transport-inhibitory site of the hENT1/SLC29A1 protein (35) and, like SAENTA-FITC (36), can be used as a fluorescent probe to detect hENT1/SLC29A1 on cell surfaces. All other materials were of the highest available commercial grade.

**Cell lines and culture conditions.** Human CCRF-CEM (CEM, T-lymphoblastic leukemia) and HeLa cells were purchased from American Type Culture Collection. CEM/100-AraC and CEM/200-AraC were derived, respectively, by stage-wise selection of CEM cells in the presence of AraC at concentrations equivalent to 4- and 20- μM IC50 values for cytotoxicity against parental CEM cells. Clonal populations of CEM/100-AraC were isolated by limited dilution. RPMI 1640 and DMEM, supplemented with 10% fetal bovine serum, were used to grow CEM cells and HeLa cells, respectively. All cells were maintained at 37°C in a humidified air containing 5% CO2. For CEM/4-AraC and CEM/20×AraC cells, AraC was added at the final concentrations used for selection.

**Cytotoxicity assay.** Cytotoxic effects of various drugs on CEM-resistant and AraC-resistant cells were assessed using a Trypan Blue dye exclusion assay. Briefly, cell suspensions (starting densities, 2 × 10^5 cells/ml) were cultured in the presence of graded concentrations of a drug of interest for 72 h. The viable cell counts were determined at various times by Trypan blue dye exclusion, and the growth rate for each condition was represented by the slope of the growth curve at exponential phase. The relative growth rate was obtained as a percentage of the growth rate of each cell line at a given drug concentration over that seen in the absence of drug. IC50 values correspond to drug concentrations that led to 50% reductions in cell growth rates compared with that in the absence of drug.

**Preparation of total RNA and standard reverse transcription–PCR.** Total cellular RNA was prepared from exponentially growing CEM, CEM/4-AraC, and CEM/20×AraC cells used for the manufacturer's instructions. Reverse transcription reactions using total RNA were carried out with random hexamer oligos using M-MuLV reverse transcriptase (New England Biolabs) following the manufacturer’s instructions. Genes-specific PCR amplification (reverse transcription–PCR, RT-PCR) was performed for 25 to 30 cycles using Platinum Taq DNA polymerase high fidelity (Invitrogen) under experimental conditions suggested by the supplier. Gene-specific oligonucleotides used to amplify individual cDNA fragments are listed in Supplementary Table S1.

**Cloning and sequencing of human SLC29A1 and DCK1 cDNAs and genomic segments.** The oligonucleotides used in RT-PCR to generate a full-length cDNA for SLC29A1 were SLC29A1-F1, 5'-AGCTAGGACAGTCTCTACACCTGACAGAC-3', and SLC29A1-R2, 5'-CTCTAGAACAATTGGCCGACAGGG-3', which includes restriction enzyme sites used for subcloning (in italics). SLC29A1 cDNA products were cloned in a plasmid vector (TOPO cloning kit; Invitrogen). Similarly, full-length hDCK cDNAs were generated using oligonucleotides hDCK-F1 (5'-AGATCTATGGACACCCCGGCTTGA-3') and hDCK-R2 (5'-TCTAGA CAAAGACTACAAAAACTCTGGAG-3'). Twenty-four cDNA clones from each transformation were expanded, and their DNA sequences were determined. Sequencing data alignment analysis was performed using BioEdit software.

Altered clones detected in cDNA clones of SLC29A1 and DCK1 were validated at the genomic level by PCR amplification and sequencing of the corresponding gene exons (5') and introns (I). PCR fragments corresponding to 19-F and 12-I2-E13 of the SLC29A1 gene and 11-E2 and 12-E3 regions of DCK were obtained using gene-specific oligonucleotide primer pairs including SLC29A1-13I5/SLC29A1-13INIR (5'-TGGAGGACCTGACAGCGGACAG-3'/5'-TGCAAGACAGTGGGAGCATTGACT-3'), SLC29A1-11IF/SLC29A1-11IR3 (5'-TGGCTCTTGAGCGCTAAT-5'/5'-ACAGTACTGCTTGAT-3') and DCK-In1F/DCK-In2R (5'-GTAATACCGTCAAGAGCTGCTAGT-3'/5'-GAAGATATATACCTAAAGCGAGAT-3').

**Transfection of human SLC29A1 cDNA into HeLa cells.** A wild-type full-length hENT1/SLC29A1 cDNA (from CEM) cloned in TOPO vector was further subcloned into the mammalian expression vector pcDNA6-V5-HisA (Invitrogen) and transiently transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. The "empty" pcDNA6-V5-His vector was also transfected into HeLa cells as a negative control. For immunoblotting, cells were trypsinized, collected by centrifugation, and washed with PBS. Crude membrane fractions and whole-cell extracts were prepared and separated by SDS-PAGE. Immunodetection of the hENT1/SLC29A1 protein was carried out using a monoclonal anti-ENT1 antibody preparation.

**Cell surface staining using FTH-SAENTA.** The cell surface abundance of hENT1/SLC29A1 in different CEM cells was determined using FTH-SAENTA, a membrane-impermeable NBMPR analogue. Briefly, cells washed free of media were resuspended in PBS to ~10^6 cells/ml and incubated with either 0, 100 nmol/L FTH-SAENTA, or 100 nmol/L FTH-SAENTA + 10 μmol/L NBMPR (cells were preincubated with NBMPR for 10 min before addition of FTH-SAENTA) for 1 h at room temperature. Cells were then centrifuged, washed once quickly, and, in a small volume of PBS, placed on polylysine-coated dishes to attach for visualization by confocal microscopy.

For fluorescence-activated cell sorting (FACS) analysis of cell surface binding of FTH-SAENTA, cells were washed free of growth medium and resuspended in sodium buffer (144 mmol/L NaCl, 20 mmol/L Tris, 3 mmol/L K2HPO4, 1 mmol/L MgCl2, 1.4 mmol/L CaCl2, and 5 mmol/L glucose at pH 7.4) to a final cell density of 2.5 × 10^6 cells/ml. Cells were treated at room temperature for 1 h with either no compound (negative control), 100 nmol/L FTH-SAENTA, or 100 nmol/L FTH-SAENTA + 10 μmol/L NBMPR. At the end of 1 h, cells were collected by centrifugation and washed twice with sodium buffer. The cells were analyzed for fluorescence using a BD Flowcytometer.

**Analysis of cell surface hENT1/SLC29A1 sites by [3H]NBMPR binding.** Cell surface abundance of hENT1/SLC29A1 on CEM and CEM-AraC cells was assessed by equilibrium binding studies with 10 nmol/L [3H]NBMPR. Cells were washed free of medium, and cell suspensions containing 10^6 cells in 100 μL of sodium buffer were incubated with 10 nmol/L [3H]NBMPR in the absence or presence of 100 nmol/L FTH-SAENTA or 10 μmol/L unlabeled NBMPR at room temperature for 1 h. At the end of the incubation, cells in permeant mixture were centrifuged through oil (41 v/v% of silicon mineral oil) to remove free unbound [3H]NBMPR. The cell pellet was solubilized in 5% Triton X-100 and counted in a Beckman scintillation counter.

**Measurement of [3H]AraC uptake.** Assays of [3H]AraC uptake were conducted in PBS containing 10 μmol/L glucose and 2 mmol/L glutamine (PBS/GG) at 37°C. Briefly, 10^6 exponentially growing cells were collected, washed once with PBS, and resuspended in 500 μL of PBS/GG for 15 min, at...
after which [³H]AraC (final concentration, 200 nmol/L) was added and the reaction mixtures were incubated for 30 min. Each reaction mixture was then gently transferred to a prewarmed microcentrifuge tube containing 200 μL of oil cushion (see above), followed by centrifugation at 8,000 × g for 30 s. After removing supernatants, the cell pellets were washed twice with PBS, digested with 100 μL of 1 N NaOH, and neutralized by addition of 100 μL of 1 N HCl. The cell-associated radioactivity was measured by liquid scintillation counting. For assays under ATP depletion conditions, PBS was used instead of PBS/GG buffer, and cell suspensions were preincubated with rotenone (20 ng/mL) for 15 min and then 2-deoxyglucose (2 mmol/L) for another 15 min before the addition of [³H]AraC.

**DCK activity assay.** DCK activity was determined as described (18) with minor modifications. Briefly, 4 × 10⁶ cells (per reaction) were collected, washed twice with PBS, and lysed in 100 μL of CelLytic M (Sigma) following the manufacturer’s instructions. Cell lysates were clarified by centrifugation at 20,000 × g for 10 min and used either immediately for DCK assays or stored at −70°C. DCK activity was measured in reaction mixtures containing 10 μmol/L [³H]deoxycytidine, 5 mmol/L ATP, 5 mmol/L MgCl₂, 50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L NaF, 2 mmol/L DTT, and 1.8 mmol/L thymidine (buffer A). Reactions were started by addition of 3 μL of cell lysate to 500 μL of prewarmed buffer A (for 5 min) at 37°C. For time courses, 100-μL samples were pipetted onto Whatman DE-81 filter discs fitted to a vacuum device. Filters were then washed thrice with 5 mL of cold water each time and air-dried, and the radioactivity on the filters was measured by liquid scintillation counting. Each experiment was repeated at least thrice.

**Results**

**Development of AraC-resistant CCRF-CEM cell lines.** A stepwise selection protocol in drug-containing medium was used to derive AraC-resistant CEM cell lines. The IC₅₀ value of CEM cells for AraC was 0.025 μmol/L. CEM cells were initially passaged in small increments of AraC concentrations above this IC₅₀, leading after several months to the isolation of a subpopulation of CEM cells that retained excellent growth rates in 0.1 μmol/L AraC and were designated CEM/4×AraC. Further stepwise selection of this cell population in AraC concentrations up to 20× the IC₅₀ value resulted in the isolation of a second population of AraC-resistant cells, CEM/20×AraC. Both CEM/4×AraC and CEM/20×AraC showed no visible morphologic and size differences from their parental CEM cells. Cytotoxicity assays showed that both CEM/4×AraC-resistant and CEM/20×AraC-resistant cell lines were highly resistant to AraC compared with parental CEM cells, with

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**Figure 1.** Drug resistance profiles of CEM (●), CEM/4×AraC (□), and CEM/20×AraC (▲) cells. A, proliferating cell cultures were exposed to increasing concentrations of various drugs as indicated. Cell viability was determined over time by Trypan blue dye exclusion as described. Relative cell growth rate (in percentage) was expressed as the function of drug concentration. Points, mean of at least three independent experiments; bars, SE. B, summary of cytotoxicity of various drugs by comparing IC₅₀ values of each drug on individual cell lines. The IC₅₀ values were defined as described in Materials and Methods and deduced from A. N.D., not determined.

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<th>Drugs</th>
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increases in their IC₅₀ values of 125-fold and >200-fold, respectively (Fig. 1). Further drug resistance profiling analysis revealed that both cell lines were cross-resistant to several nucleoside drugs, including Gemcitabine, Cldarabine, Fludara, and Dideoxyctydine, with higher levels of resistance observed in the CEM/20×AraC cells. However, the two cell lines differed markedly in their degree of resistance to 5-fluorouridine and 5-fluoro-2’-deoxyuridine, with CEM/4×AraC being resistant to both compounds, whereas CEM/20×AraC remained as sensitive as the parental CEM cells. These differences in drug resistance profiles suggested a distinct mechanistic basis in the two cell populations. Both CEM/4×AraC and CEM/20×AraC remained sensitive to 5-FU (Fig. 1) and to a variety of natural products, including anthracyclines and etoposide (Fig. 1B). The lack of cross-resistance to natural products suggested that ABC transporters, such as ABCB1 (MDR1/P-glycoprotein) and multidrug resistance associated protein ABCC1 (MRP1), were not responsible for the resistance of these cells.

**Expression of candidate genes in AraC-resistant CEM cells.** To gain insight into possible mechanisms responsible for drug resistance in CEM/4×AraC and CEM/20×AraC cells, expression of mRNAs corresponding to several candidate genes known to be involved in transport and/or metabolism of nucleoside analogue drugs was investigated by RT-PCR. These genes encoded nucleoside transporters (SLC29A1 and A2, SLC28A1 and A2), ATP-dependent efflux pumps (ABCC4 and 5), and enzymes known to be involved in drug metabolism, such as DCK, cytidine deaminase (CDADC1), and ribonucleoside reductase large subunit (RRMI). These analyses failed to detect major differences in expression levels or sizes of cDNAs produced by the resistant isolates, with two notable exceptions. CEM/4×AraC cells expressed what seemed to be an altered SLC29A1 mRNA compared with both CEM (Fig. 2) and CEM/20×AraC (data not shown) cells. Using a primer pair (F1 and R3) designed to amplify the entire coding region of the SLC29A1 mRNA, CEM/4×AraC cells displayed a wild-type product similar in length to that detected in the parental cells (1380 bp) but also showed a shorter fragment of ~1260 bp (Fig. 2). Further RT-PCR studies with other primer pairs consisting of F1 as an anchor primer (exon 3) and other primers spanning different portions of the SLC29A1 cDNA showed that the altered product was only detected with exon 14-derived primers (F1 + R3) and not with further upstream primers, including one from exon 13 (F1 + R2). This suggested a possible deletion in the cDNA sequence derived from the exon 13 region in one or both copies of the SLC29A1 gene in CEM/4×AraC cells. No such alterations were noted in CEM/20×AraC cells.

**Genetic lesions in the SLC29A1 gene from CEM/4×AraC cells.** To delineate the nature of possible mutations found in SLC29A1 from CEM/4×AraC cells, the amplified RT-PCR products were cloned and sequenced. Two mutations were found in SLC29A1 cDNAs from CEM/4×AraC cells (Fig. 3A). A nonsense mutation was detected at the beginning of exon 4 at the Tyr¹¹ codon (TAC→TA4), resulting in a termination codon. The second mutation was the complete absence of exon 13 in some of the clones, consistent with the size differences in cDNA products from CEM/4×AraC detected in Fig. 2. Importantly, individual cDNA clones were found to contain either type of mutation, but not both, suggesting that the two alterations corresponded to different genetic lesions on each of the two alleles of the SLC29A1 gene in CEM/4×AraC cells. The mutations detected by cDNA sequencing were further validated by characterization of the corresponding genomic regions in the SLC29A1 gene, which were amplified by PCR and sequenced with [³²P]dATP (autoradiograms shown in Fig. 3B and C). These experiments validated the presence of a heterozygote nonsense mutation at the genetic codon for Tyr¹¹ of SLC29A1 in genomic DNA from CEM/4×AraC cells (Fig. 3B). Additionally, a complex pattern of sequence alterations was noted in a 15-nucleotide segment overlapping the exon 13/intron 13 junction of the SLC29A1 gene (Fig. 3C), including an alteration in the 5’ splice sequence (GUGAGU→GGGGGU) in intron 13 and a single-nucleotide deletion in the 3’ end of exon 13 (Fig. 3C). This complex mutation was verified by cloning the PCR products followed by DNA sequencing (Fig. 3C, right) and was, therefore, very likely to account for the altered splicing of exon 13 detected by cDNA sequencing from CEM/4×AraC RNA. That both types of

![Figure 2. Standard RT-PCR of the SLC29A1 gene from CEM (lane 1) and CEM/4×AraC (lane 2) cells, using two pairs of oligonucleotides. The top bar graph shows the schematic representation of the SLC29A1 transcript with the exons numbered and the coding region shaded. F1, R2, and R3 indicate forward and reverse oligonucleotides, respectively. Arrows, orientation and relative positions of the oligonucleotides in the transcript sequence. The left panel of the RT-PCR gel picture shows the SLC29A1 cDNA obtained using primer pair F1/R2, and the right panel shows the SLC29A1 cDNA products obtained using primer pair F1/R3. Lane S, negative control with primers only; lane M, a size marker. The drawing of the SLC29A1 transcript is not to scale.](image-url)
SAENTA staining followed by FACS analysis was conducted to quantify the hENT1/SLC29A1 protein expression using FTH-CEM/C2 further validated by the lack of fluorescent staining of isolated protein and detectable by confocal microscopy (Fig. 4). A probe known to specifically bind to cell surface hENT1/SLC29A1 was used to probe the absence of hENT1/SLC29A1 protein in CEM/C2. This species was undetectable in CEM/C2 but detected in extracts from CEM and HeLa/SLC29A1 transfectants, which shows the presence of immunoreactive species of predicted molecular mass length SLC29A1. In these experiments, HeLa cells transiently expressing a full-length cDNA were used as positive controls. Although an immunoreactive species of predicted molecular mass ~60 kDa was detected in extracts from CEM and HeLa/SLC29A1 transfectants, this species was undetectable in CEM/4×AraC cells (Fig. 4A). The absence of hENT1/SLC29A1 protein in CEM/4×AraC cells was further validated by the lack of fluorescent staining of isolated CEM/4×AraC clones incubated with FTH-SAENTA, a fluorescent probe known to specifically bind to cell surface hENT1/SLC29A1 protein, and detectable by confocal microscopy (Fig. 4B). Further quantification of hENT1/SLC29A1 protein expression using FTH-SAENTA staining followed by FACS analysis was conducted (Fig. 4C). Specific binding of FTH-SAENTA to parental CEM cells was shown by the complete inhibition of binding by preincubation of cells with the hENT1/SLC29A1-specific inhibitor, NBMPR (Fig. 4C, top). Similar analysis of an isolated CEM/4×AraC clone revealed no such fluorescent labeling on the cell surface in the absence of NBMPR (Fig. 4C, middle). Three additional CEM/4×AraC clones produced similar results (data not shown). Finally, results from additional [3H]NBMPR binding studies were consistent with those of the fluorescence labeling studies and showed binding of the probe to parental CEM cells (competable by FTH-SAENTA or unlabeled NBMPR) but not to a CEM/4×AraC cell clone (Fig. 4D). Taken together, the defects in the SLC29A1 gene of CEM/4×AraC cells abolished cell surface expression of the hENT1/SLC29A1 protein in this drug-resistant cell population.

**Drug uptake in CEM/4×AraC and CEM/20×AraC cells.** CEM cells possess a single type of nucleoside transporter, hENT1/SLC29A1 (37). The mutations in SLC29A1 found in CEM/4×AraC cells may have impaired nucleoside transport, causing reduced cellular accumulation and increased resistance to cytotoxic nucleoside drugs, including AraC. Therefore, uptake of [3H]AraC was measured in CEM, CEM/4×AraC, and CEM/20AraC cells. Results in Fig. 5A showed that neither CEM/4×AraC nor CEM/20×AraC cells accumulated [3H]AraC. This was consistent with mutations in the SLC29A1 gene of CEM/4×AraC cells but
surprising for CEM/20×AraC cells, which did not harbor such mutations and seemingly exhibited hENT1/SLC29A1 on their surfaces (Fig. 4B and C, bottom). Reduced drug uptake in both resistant cell lines was not affected by ATP depletion (Fig. 5A), suggesting that the reduced intracellular accumulation of [3H]AraC in the CEM/20×AraC cells was not caused by defects in unrelated ATP-dependent drug uptake or drug efflux mechanisms. Further transport studies using the normal physiologic nucleoside substrate [3H]uridine showed that both CEM and CEM/20×AraC cells accumulated uridine at similar levels, whereas CEM/4×AraC cells showed no accumulation of this nucleoside (data not shown). These results confirmed that the CEM/4×AraC cells were "null" for nucleoside import by hENT1/SLC29A1. On the other hand, CEM/20×AraC seemed to possess normal nucleoside transport activity (cell surface expression, transport of [3H]uridine), although they were highly resistant to cytotoxic concentrations of AraC and did not accumulate [3H]AraC in uptake assays.

An alternative drug resistance mechanism in the CEM/20×AraC cells. Because CEM/20×AraC cells did not harbor mutations in the SLC29A1 gene, exhibited hENT1/SLC29A1 on their surface, and could import [3H]uridine, additional candidate genes were investigated. DCK is responsible for the first modification required for metabolism of AraC. The kinase activity of DCK was assayed in CEM, CEM/4×AraC, and CEM/20×AraC cells. Although robust DCK activity was detected in CEM and CEM/4×AraC cells, it was largely absent in CEM/20×AraC cells (Fig. 5B). Cloning and sequencing the DCK cDNA from RNA of the CEM/20×AraC cells revealed two mutations (Fig. 6A). The first corresponded to a deletion of the first six nucleotides in exon 2, whereas the second was an insertion of 22 nucleotides at the exon 2 and exon 3 junction (Fig. 6A). To validate the mutations detected in cDNA clones from DCK of CEM/20×AraC cells, genomic DNA fragments overlapping the exon-intron junctions of intron 1 and exon 2, intron 2 and exon 3 were PCR-amplified, followed by cloning and nucleotide sequencing. A single-nucleotide substitution (a→c) was identified at the splice junction of intron 1 and exon 2 (Fig. 6B). This mutation was heterozygous and was likely responsible for the inappropriate splicing of intron 1, including deletion of some exon 2 sequence in the resulting mRNA/cDNA. A second complex alteration was noted at the junction of intron 2 and exon 3 of DCK (Fig. 6C). This mutation was also heterozygous and likely responsible for the altered splicing of exon 3. This may

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Figure 4. Analysis of hENT1/SLC29A1 protein expression by immunoblotting (A), fluorescent staining (B), FACS (C), and [3H]NBMPR binding (D). A, immunodetection of hENT1/SLC29A1 protein in CEM and CEM/4×AraC cells using hENT1-specific monoclonal antibodies. Lanes 1 and 2, crude membrane from CEM and CEM/4×AraC, respectively; lanes 3, 4, 5, and 6, whole-cell extracts from CEM, CEM/4×AraC, HeLa/vec, and HeLa/ENT1, respectively (50 μg protein per lane). B, fluorescent staining with FTH-SAENTA and confocal microscopy of CEM (top), CEM/4×AraC, clone #2 (middle), and CEM/20×AraC (bottom) cells in the absence (left) and presence (right) of NBMPR. C, FACS analysis of CEM (top), CEM/4×AraC, clone #2 (middle), and CEM/20×AraC (bottom) cells. D, cell surface [3H]NBMPR binding in CEM, CEM/4×AraC (clone #2), and CEM/20×AraC cells. See Materials and Methods for details.
Figure 5. Functional characterization of the drug resistance mechanisms in CEM/4×AraC and CEM/20×AraC cells. A, hENT1/SLC29A1-mediated cellular uptake of [3H]AraC was measured by cell-associated radioactivity when cells were incubated with radiolabeled drug under normal (empty columns) or ATP depletion (hatched columns) conditions, as described in Materials and Methods. B, determination of DCK activity in CEM (●), CEM/4×AraC (▲), and CEM/20×AraC (■) cells, as described in Materials and Methods. The data represent the means of at least three experiments; bars, SDs.

have resulted in the 22-bp insertion detected in the cDNA. It is noteworthy that both types of mutations were present in heterozygous fashion at genomic as well as cDNA levels. These results suggested that impaired DCK protein function was responsible for AraC resistance in CEM/20×AraC cells.

Discussion

In the present study, an AraC resistant cell line, CEM/4×AraC, was developed by continuous exposure of the CEM cell line to low concentrations of AraC. Selection of this cell population required several months of continuous culturing in the drug. CEM/4×AraC cells were then subcultured in increasing concentration of AraC until a second population of highly drug resistant cells (CEM/20×AraC) was isolated and stably passaged in the presence of the drug. The mechanism underlying drug resistance in these two cell lines was investigated at the molecular level. The early recognition that the drug resistance profiles of each population exhibited significant overlap, yet were distinct with respect to 5-fluorouridine and 5-fluoro-2′-deoxyuridine (in which the 20× line retained susceptibility compared with the 4× line), indicated that the two cell populations may exhibit different mechanisms of resistance.

Extensive molecular analyses showed that AraC resistance in CEM/4×AraC cells was caused by a complete loss of function of hENT1/SLC29A1, the nucleoside transporter used by AraC as a fraudulent substrate to enter cells. These results are based on the identification of obvious loss-of-function mutations in the SLC29A1 gene both at the cDNA and genomic levels and were phenotypically translated as loss of protein expression detected by immunoblotting and flow cytometry, lack of binding of known hENT1/SLC29A1 ligands, drastically reduced uptake of radioactive AraC, and cellular cross-resistance to other cytotoxic nucleoside drugs. The observation that the two mutations were found as heterozygotes in the CEM/4×AraC cell population while no full-length wild-type cDNA was produced suggested that each SLC29A1 gene copy had been sequentially inactivated during drug selection. This mechanism would imply that a reduction of 50% in activity of hENT1/SLC29A1 was sufficient to confer a selective growth advantage in medium containing low levels of the drug (near IC50 values). Therefore, it is tempting to speculate that silencing of a single copy of the SLC29A1 gene in leukemic cells may provide significant cellular resistance and concomitant growth advantage during treatment with AraC. Such biologically relevant silencing may be difficult to detect in vivo. Previous studies reported that lack of protein expression of hENT1/SLC29A1 is highly correlated with cellular resistance to AraC and its close relative, gemcitabine, in both in vitro selected drug-resistant cell lines and patients suffering from AML or mantle cell lymphoma (14, 16, 38–41). Conversely, transfection of SLC29A1 cDNA into otherwise hENT1-deficient cells sensitizes cells to AraC cytotoxicity (38). These studies support the contention that inactivation of hENT1/SLC29A1 may cause cellular resistance to nucleoside analogue drugs, not only in the ALL cells tested here but also in unrelated tumor cell types. Indeed, different tumor cell types have been shown to rely on the same molecular mechanism to achieve resistance to cytotoxic drugs in vitro and in vivo. A typical example is the overexpression of multidrug transporters MDR1 (ABCB1) and MRPI (ABCC1; for reviews, see refs. 42, 43). Importantly, the present study is a first example of a loss-of-function mutation in the SLC29A1 gene that causes cellular resistance to AraC.

The second cell population CEM/20×AraC, loosely derived from the 4× cell line by growth in increasing drug concentration, displayed a mechanism of AraC resistance distinct from that found in the 4× cells. Indeed, hENT1/SLC29A1 function was normal in CEM/20×AraC cells, including the absence of mutations in the gene, presence at the cell surface of a protein that could bind known hENT1/SLC29A1 ligands and capacity to transport uridine. Instead, CEM/20×AraC cells were found to carry obvious loss-of-function mutations in the DCK gene which is absolutely required for phosphorylation of AraC, a process necessary for AraC-mediated inhibition of RNA and DNA synthesis. The two mutations identified were heterozygous on each of the DCK alleles in the CEM/20×AraC cells, again suggesting sequential inactivation of the DCK gene during selection for high-level resistance to AraC. This in turn supports the notion that partial (50%) loss of DCK function may be sufficient to cause clinically relevant AraC resistance. Indeed, decreased DCK expression has been associated with increased AraC resistance in the later phase of drug selection in an in vitro selected human B leukemia cell line (18).

Both AraC-resistant cell lines displayed reduced levels of AraC accumulation. In CEM/4×AraC, mutations in the SLC29A1 gene blocked cellular uptake of AraC and of other nucleoside analogue drugs, establishing a causal relationship with cellular resistance to these drugs. In the CEM/20×AraC cell line, loss-of-function
mutations in the DCK gene explained resistance to AraC and other nucleoside analogue drugs (e.g., gemcitabine) that are substrates for this enzyme and hence are not phosphorylated in the resistant line. Although these nucleoside analogue drugs can enter cells via a functional hENT1/SLC29A1 protein, they may exit cells by the same route in the absence of phosphorylation due to the equilibrative nature of the transporter. On the other hand, 5-fluorouridine and 5-fluoro-2'-deoxyuridine are not substrates for DCK, can be phosphorylated by other nucleoside kinases, such as uridine kinase, and thus remain cytotoxic to the CEM/20x/C2AraC cells.

Functional studies and sequence analysis of archival samples corresponding to mass populations of CEM stocks frozen at different stages of drug selection has suggested the following scenario for the emergence of AraC resistance in the two CEM cell lines reported herein. The first AraC-selected cell line, CEM/4x/C2AraC, was probably a mixed population, consisting of a large majority of cells bearing SLC29A1 mutations, and a much smaller number of cells bearing DCK mutations (but wild type for SLC29A1). Loss of SLC29A1 function initially imparted low-level resistance to AraC in 4x/C2AraC cells. We speculate that loss of DCK function conferred higher level of resistance to AraC than that caused by loss of SLC29A1 function. Subsequent selection of CEM/4x/C2AraC cells in higher concentrations of AraC (e.g., 20x/IC50) caused an expansion of the DCK-/- cells. Further passages and/or subcloning to generate CEM/20x/C2AraC yielded a population of resistant cells highly enriched in DCK-/- cells and devoid of SLC29A1-/- cells. To test this hypothesis, we used an archival early specimen of CEM/4x/C2AraC cells and passaged it for a period of 3 months in 4x/IC50 of AraC, and each month, a sample was analyzed by FACS for FTH-SAENTA binding (Supplementary Fig. S1) as an indication of hENT1/SLC29A1 surface expression. Over this period, we observed a progressive shift from a population that expressed little hENT1/SLC29A1 to a population that expressed robust amounts, consistent with the notion that the culture was being taken over by a subpopulation of highly resistant DCK-negative cells.

DCK mutations have been previously associated with high levels of AraC resistance in other drug selected cell lines (44, 45). Conversely, introduction of DCK cDNA into the DCK-/- cells increased cellular sensitivity to AraC and other nucleoside analogue drugs (46–48). In addition, alternatively spliced DCK mRNAs have been detected in leukemic blasts from patients with resistant AML but not in sensitive AML, suggesting that these inactive DCK mRNAs might play a role in AraC resistance in vivo.
other mutations in these two genes and in members of the same
1. in general, and in leukemic cells, in particular. Moreover, these
findings in vitro have obvious implications for the parallel search of
other mutations in these two genes and in members of the same
pathway that can be associated with AraC resistance in vivo.

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