The Role of Human Equilibrative Nucleoside Transporter 1 on the Cellular Transport of the DNA Methyltransferase Inhibitors 5-Azacytidine and CP-4200 in Human Leukemia Cells

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ABSTRACT

The nucleoside analog 5-azacytidine is an archetypical drug for epigenetic cancer therapy, and its clinical effectiveness has been demonstrated in the treatment of myelodysplastic syndromes (MDS) and acute myelogenous leukemia (AML). However, therapy resistance in patients with MDS/AML remains a challenging issue. Membrane proteins that are involved in drug uptake are potential mediators of drug resistance. The responsible proteins for the transport of 5-azacytidine into MDS/AML cell lines are unknown. We have now systematically analyzed the expression and activity of various nucleoside transporters. We identified the human equilibrative nucleoside transporter 1 (hENT1) as the most abundant nucleoside transporter in leukemia cell lines and in AML patient samples. Transport assays using [14C]-5-azacytidine demonstrated Na+-independent uptake of the drug into the cells, which was inhibited by S-(4-nitrobenzyl)-6-thioinosine (NBTI), a hENT1 inhibitor. The cellular toxicity of 5-azacytidine and its DNA demethylating activity were strongly reduced after hENT1 inhibition. In contrast, the cellular activity of the 5-azacytidine derivative 5-azacytidine-5′-elaidate (CP-4200), a nucleoside transporter-independent drug, persisted after hENT1 inhibition. A strong dependence of 5-azacytidine-induced DNA demethylation on hENT1 activity was also confirmed by array-based DNA methylation profiling, which uncovered hundreds of loci that became demethylated only when hENT1-mediated transport was active. Our data establish hENT1 as a key transporter for the cellular uptake of 5-azacytidine in leukemia cells and raise the possibility that hENT1 expression might be a useful biomarker to predict the efficiency of 5-azacytidine treatments. Furthermore, our data suggest that CP-4200 may represent a valuable compound for the modulation of transporter-related 5-azacytidine resistances.

Introduction

Transport proteins that are involved in the entry and the efflux of chemotherapeutic agents and their metabolites across plasma membranes play important roles for the successful delivery of drugs to their intracellular targets (Giacomini et al., 2010). Reduced drug uptake into the cells or increased drug efflux from the cells can result in the development of drug resistances and poor treatment outcome (Gillet and Gottesman, 2010). Thus, the identification of processes for specific drugs may provide substantial insights to predict drug response.

5-Azacytidine and 5-aza-2′-deoxycytidine are cytosine analogs that function as inhibitors of DNA methylation and are currently the most advanced drugs for epigenetic cancer therapy. The clinical effectiveness of both drugs has been demonstrated during the treatment of myelodysplastic syndromes (MDS) and acute myelogenous leukemias (AML) with significant clinical response rates (Silverman, 2009; Griffiths and Gore, 2013). To effectively inhibit their molecular targets, these prodrugs need to be metabolized before they become incorporated into DNA and/or RNA (Rius and Lyko, 2012). The methylation reaction with azacytosine-containing DNA causes covalent trapping and depletion of DNA methyltransferases and, consequently, DNA demethylation and reversion of hypermethylation-induced gene silencing (Egger et al., 2004). The ability to induce DNA demethylation and epigenetic reprogramming is a specific characteristic of azanucleosides and distinguishes them from other nucleoside drugs (Iasa, 2007). However, little is known about the molecular identity of the proteins that mediate the transport of azanucleosides and their metabolites across the plasma membrane.

ABBREVIATIONS: AML, acute myelogenous leukemia; AVB, average β; CP-4200, 5-azacytidine-5′-elaidate; DB, delta β; FAB, French American British; hCNT, human concentrative nucleoside transporter family; hENT, human equilibrative nucleoside transporter family; MDCK, Madin-Darby canine kidney cells strain II; MDS, myelodysplastic syndrome; NBTI, S-(4-nitrobenzyl)-6-thioinosine; SLC, solute carrier family.
Several members of the solute carrier transporter (SLC) family represent bona fide candidates for the uptake of azanucleosides into cells. Members of the human concentrative nucleoside transporter (hCNT; SLC28A) and of the equilibrative nucleoside transporter (hENT; SLC29A) families are the known transporters for naturally occurring nucleosides and nucleoside analogs (Zhang et al., 2007). hCNT-mediated uptake of nucleosides into cells occurs in a Na⁺-dependent and unidirectional manner against the concentration gradient and with higher substrate affinity compared with the hENTs. In contrast, hENTs facilitate transport of nucleosides in a bidirectional manner, with broad selectivity but relatively low affinity compared with hCNT proteins. These characteristics make nucleoside transporters central candidates for the transport of 5-azacytidine and 5-aza-2'-deoxycytidine and for modulating drug resistances to these drugs. We previously showed that hCNT1 and hCNT3 can mediate the uptake of 5-azacytidine and 5-aza-2'-deoxycytidine in canine kidney cell models (Rius et al., 2009, 2010). However, hCNTs are expressed mainly in epithelial cells, whereas hENTs are ubiquitously expressed. This suggested that additional transporters might be involved in the transport of 5-azacytidine into nonepithelial cell types, including cells of patients with MDS and AML.

Studies on the resistance mechanisms of cancer cell lines to 5-aza-2'-deoxycytidine (decitabine) identified a combination of the transport resistances including hENT transporters and metabolizing enzymes (Qin et al., 2009). Furthermore, expression and activity of hENT1 were identified as factors that conferred resistance to the cytosine analog cytarabine in AML patients and in cancer cell lines (Galmarini et al., 2002; Sarkar et al., 2005; Zimmerman et al., 2009). Improvement of drug transport was also a driving factor in the development of an eladic acid derivative of 5-azacytidine (CP-4200; 5-azacytidine-5'-elaidate) that contains a fatty acid moiety to facilitate the nucleoside transporter-independent uptake of the parent drug. The demethylating activity of CP-4200 was proven in human cancer cell lines (Brueckner et al., 2010), which indicated comparable epigenetic effects to 5-azacytidine. These results indicate that knowledge of expression and function of specific nucleoside transporters should be central when devising effective epigenetic cancer therapies that target DNA methylation.

Taken together, these findings prompted us to study the transport mechanisms of 5-azacytidine and CP-4200 in leukemic syndromes and their effects on DNA methylation. For this aim, we used primary AML cells and leukemia cell lines. Expression profiling and transport assays identified hENT1 as the key transporter for 5-azacytidine uptake in leukemia-derived cells. A strong hENT1 dependence of the 5-azacytidine-induced demethylation was demonstrated by DNA methylation profiling. In addition, the CP-4200-induced demethylation was detected in the absence of hENT1 activity, which confirmed the transporter-independent activity of CP-4200 and the potential ability of CP-4200 to overcome resistance mechanisms related to the cellular uptake of 5-azacytidine.

**Materials and Methods**

**Compounds.** [5-³H]Uridine (0.9 T bq/mm mol) was purchased from Moravek Biochemicals (Brea, CA). [6-¹³C]5-Azacytidine was described previously (Rius et al., 2009). Unlabeled uridine, 5-azacytidine, dipyridamole, diltiazem, and dimethylsulfoxide were purchased from Sigma-Aldrich (St. Louis, MO). S-(4-nitrobenzyl)-6-thioinosine (NBTI) was obtained from Enzo Life Sciences (Farmingham, NY), and hygromycin B was from Invitrogen (Carlsbad, CA). CP-4200 was supplied by Clavis Pharma ASA (Oslo, Norway). Because of the poor solubility of CP-4200, dimethylsulfoxide was used as solvent in all experiments. All other chemicals were of analytical grade and purchased from Sigma-Aldrich, Merck (Darmstadt, Germany), or AppliChem (Darmstadt, Germany).

**Patient Samples.** Blasts from patients with AML (n = 12) were obtained at the time of diagnosis. Ethics committee approval was obtained with informed consent from all patients. All specimens were obtained from patients with AML at the time of diagnosis from the French American British (FAB) subtypes FAB-M1, FAB-M2, FAB-M4, and FAB-M5. Blasts were enriched by density centrifugation with an average >80% blast cells in the frozen specimens. Sample cells were thawed in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 100 units/ml penicillin/streptomycin and kept for 3 hours before the start of the experiment.

**Cell Lines and Cell Culture.** U937, K562, HL60, Jurkat, KG-1, W138, and PC3 cells were obtained from American Type Culture Collection (Manassas, VA). MLI cells were obtained from Jean-Pierre Issa (Temple University, Philadelphia, PA) and HCT116 cells from Bert Vogelstein (Johns Hopkins University, Baltimore, MD). U937, K562, ML1, HL60 and Jurkat cells were cultured in RPMI 1640 medium, KG-1 in Iscove’s modified Dulbecco’s medium, HCT116 in McCoy’s 5A medium, and W138 in Dulbecco’s modified Eagle’s medium. All media were supplemented with 10% (v/v) fetal bovine serum and 100 units/ml penicillin/streptomycin except for Iscove’s modified Dulbecco’s medium, which was supplemented with 20% (v/v) fetal bovine serum. Cells were kept at 37°C and 5% CO₂. All cell lines were reauthenticated after completion of the relevant experiments and periodically controlled for cell contamination using Multiplexion (Schmitt and Pawlita, 2009; Castro et al., 2013).

**cDNAs Encoding Human hENT1 Protein and Stable Transfection of hENT1 in Mammalian Cells.** The cDNA encoding hENT1 was cloned from human kidney using hENT1-specific primers (forward: 5'-ACCATGACACCACTGCACCC–3'; reverse: 5'-TCA-CAGAAATTGCGCAGAAG–3') based on the original sequence published by Griffiths et al. (1997) (NCBI accession number NM_001078174.1) and subcloned into the vector pcR2.1-TOPO (Invitrogen, Groningen, The Netherlands). The complete 2.1-kb cDNA insert was excised from the pcR2.1-TOPO vector and cloned into the XhoI and NotI restriction sites of the mammalian expression vector pcDNA3.1+ (Invitrogen). The hENT1 protein encoded by the cloned hENT1 cDNA was 100% identical to the reference sequence (NCBI accession number NP_001071642.1). Madin-Darby canine kidney cells strain II (MDCK) were cultured as described previously (Rius et al., 2009). MDCK cells were transfected with the pcDNA3.1+(−)hENT1 cDNA construct or vector only using Metafectene transfection agent (Biontex, München, Germany) according to the manufacturer’s instructions. Stable transfectants were selected using medium containing hygromycin B (0.5 mg/mL). Resistant clones were screened by immunoblot analysis and immunofluorescence microscopy for hENT1 expression.

**RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction.** RNA was isolated using the RNeasy RNA isolation kit (Qiagen, Hilden, Germany), and cDNA was prepared with the QuantiTect Reverse Transcription kit (Qiagen). Each cDNA sample was analyzed in triplicate using the QuantiFast SYBR Green polymerase chain reaction kit (Qiagen). QuantiTect Primer Assays (Qiagen) were used for hCNT1 (Hs_SLC28A1_1_SG, QT00070559), hCNT2 (Hs_SLC28A2_1_SG, QT01012333), hCNT3 (Hs_SLC28A3_1_SG, QT00024871), hENT1 (Hs_SLC29A1_1_SG, QT01000983), and hENT2 (Hs_SLC29A2_1_SG, QT00008316). Primer specific efficiencies were calculated from serial cDNA dilutions using the method of quantification of the LightCycler 480 instrument (Roche, Grenzach, Germany). For relative quantification we used the LightCycler.

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software and glyceraldehyde 3-phosphate dehydrogenase mRNA expression levels as a reference.

Confocal Immunofluorescence Microscopy of Human Blasts and Cultured Cells. Human blasts from AML patients and leukemia cells were seeded in 12-well plates at a density of 0.35×10^6 cells per well. Cells were cultured for 48 hours until confluent. Uptake studies examining cell lysis using 0.2% sodium dodecyl sulfate.

Results

hENT1 Mediates 5-Azacytidine Uptake in Primary AML Cells. As a first step toward the identification of the transport mechanisms for the therapeutic activity of 5-azacytidine, we analyzed the expression profile of various nucleoside transporters in primary bone marrow blasts from 12 AML patients. Quantitative reverse-transcription polymerase chain reaction analysis of hCNT1 and hENT2 clearly showed that hENT1 mRNA was the most abundant nucleoside transporter mRNA in bone marrow blasts (Fig. 1A). hCNT1, hCNT3, and hENT2 mRNA were expressed at substantially lower levels, whereas hCNT2 expression was not detectable (Fig. 1B). hENT1 expression was further analyzed by immunofluorescence microscopy. The specificity of the antibody against hENT1 was previously shown (Shan et al., 2012). In agreement with the mRNA expression profile, hENT1 protein was readily detectable in bone marrow blasts (Fig. 1B). Staining with an antibody against Na^+/K^+-ATPase, a plasma membrane protein, demonstrated colocalization of both proteins, suggesting that hENT1 is functional in bone marrow blasts (Fig. 1B). The expression of hENT1 protein in AML blasts was also demonstrated by immunoblot analysis using a polyclonal antibody against hENT1 (Komori et al., 2010; Tanaka et al., 2011) (Supplemental Fig. 1).

Confocal Immunofluorescence Microscopy of Human Blasts and Cultured Cells. The anti-ENT1 antibody (Abcam, Cambridge, MA) was diluted 1:100, the monoclonal anti-β-tubulin (Sigma-Aldrich) was used at a dilution of 1:5,000, and the horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antisera were diluted at 1:20,000. All antibodies were diluted in 1% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20.

Transport Experiments. For transport assays, primary AML blasts and leukemia cells were seeded at a density of 2×10^6 cells per vial before the start of the experiments. HCT116, PC3, and Wi38 cells were seeded in 12-well plates at a density of 0.35×10^6 cells per well and cultured for 48 hours until confluent. Uptake studies examining the Na^+-independent uptake of [^1^4^C^]5-azacytidine or [^3^H^]uridine were performed using Na^+-free buffer (100 mM choline chloride, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM Heps, pH 7.5). For uptake studies measuring the Na^+-dependent uptake, the choline chloride of the uptake buffer was replaced by equimolar concentrations of NaCl. Cells were first washed three times with Na^+-free buffer and then incubated with 1 mL of Na^+-free buffer containing the labeled substrate at the indicated concentration in the absence or presence of hENT inhibitors. After incubation at 37°C, cells were washed three times with ice-cold Na^+-free buffer. Intracellular radioactivity was determined by liquid scintillation counting after cell lysis using 0.2% sodium dodecyl sulfate.

Cytotoxicity Assays. Cytotoxicity of the test compounds was assessed by CellTiter-Blue assay (Promega, Mannheim, Germany). Cells were seeded (0.5×10^6 cells per well) in 96-well plates and incubated for 24 hours prior to exposure to graded concentrations of each drug every day for 72 hours as indicated. NBTI or dipirydamole was added at subtoxic concentrations (10 and 1 μM, respectively) 20 minutes before drug incubation. The IC_{50} concentration was defined as the drug concentration required to reduce cell survival to 50% as determined by the relative fluorescence of reduced resazurin.

DNA Methylation Analysis. Genomic DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen). Global DNA methylation levels were determined by capillary electrophoresis, as described previously (Stach et al., 2003). Genome-wide DNA methylation profiles were analyzed as described previously (Gronnier et al., 2010) using HumanMethylation27 DNA Analysis BeadChip arrays (Illumina, San Diego, CA) according to the manufacturer’s instructions. Results were analyzed using GenomeStudio (Illumina). Histograms, box plots, scatterplots, and heatmaps were drawn with R (http://www.R-project.org) or GenomeStudio (Illumina). The methylation status of specific cytosines is indicated by average β (AVB) values, where 1 corresponds to complete methylation and 0 to no methylation. Delta β (DB) values were calculated by subtracting AVB values of treated cells from AVB values of control cells. Microarray data were submitted to the ArrayExpress database (www.ebi.ac.uk/arrayexpress) with the accession number E-MTAB-1529.

Pharmacokinetic Evaluation of CP-4200. The pharmacokinetics of CP-4200 and its metabolite 5-azacytidine was investigated in male and female Sprague-Dawley rats and Beagle dogs. In rats, doses of 1.5, 4.4, and 9 mg/kg/day CP-4200 were administered, whereas in dogs the doses were 0.4, 0.9, and 1.5 mg/kg/day. In both species CP-4200 was administered as an intravenous bolus daily for two 7-day cycles with 7-day off-dose periods between the cycles. Blood was sampled after the first and last dose (days 1 and 21), and the plasma concentrations of CP-4200 and 5-azacytidine were determined by liquid chromatography-tandem mass spectrometry.

Statistical Analysis. For statistical analysis, the Student’s t test was used. P values of <0.01 were considered significant, P values of <0.001 were considered highly significant, and P values of >0.01 were considered not significant.
of hENT1 in blast cells and suggested an important role for hENT1 in the uptake of 5-azacytidine into AML blasts.

**5-Azacytidine Uptake in Leukemia Cell Lines Is hENT1-Dependent.** In subsequent experiments, we used human cancer cell lines to characterize in greater detail the role of hENT1 in 5-azacytidine uptake. To this end, a panel of six leukemia cell lines (U937, ML-1, K562, HL60, Jurkat, and KG-1) was selected. Additionally, two solid tumor-derived cancer cell lines (HCT116 and PC3) as well as a cell line from normal lung tissue (Wi38) were included as epithelial cell lines established in transport assays (Ward and Tse, 1999; Mangravite et al., 2003). First, quantitative reverse-transcription polymerase chain reaction was used to determine the mRNA expression of various nucleoside transporters. The results were similar to those observed in primary AML cells (Fig. 2, A–E). More specifically, hENT1 appeared to be the most abundantly expressed nucleoside transporter in all leukemia cell lines and also in the solid tumor cell lines (Fig. 2E). Interestingly, the normal fibroblast cell line (Wi38) showed the lowest levels of hENT1 mRNA expression, suggesting that cancer cells express high levels of hENT1. Indeed, Western analysis also showed high levels of hENT1 protein in all cancer cell lines, but only low levels in Wi38 cells (Fig. 2F; Supplemental Fig. 2). Further experiments focused on the U937, K562, and ML1 cell lines, because these cells represent established models for the study of AML and MDS (Lübbert et al., 2008). Immunolocalization of hENT1 in these three cell lines detected the protein in the plasma membrane (Fig. 2, G–I), which was consistent with its function as a nucleoside transporter.

The measurement of [14C]5-azacytidine uptake into leukemia cells confirmed that the nucleoside transport increased over time and that it was Na^+^-independent transport process (Fig. 3, A–C). These results indicated that the intracellular...
accumulation of 5-azacytidine in these cells was largely independent of hCNT transporters (Fig. 3, A–C). HCT116 cells, which were included as a representative solid tumor cell line, accumulated 5-azacytidine in a similar manner (Supplementary Fig. 3A). To confirm these findings, hENT activity was inhibited by the well-known inhibitors NBTI, dipyridamole, and dilazep. All inhibitors reduced 5-azacytidine uptake into three leukemia cell lines (Fig. 3, D–F) as well as into HCTT6 cells (Supplemental Fig. 3B). NBTI and dipyridamole were the most effective uptake inhibitors causing more than 50% hENT inhibition in all cell lines. However, intracellular accumulation of 5-azacytidine was still detectable after 5-minute hENT inhibition, which indicated basal (hENT-independent) nucleoside transport activities and/or the inhibition of nucleotide efflux transporters that have been shown to increase the intracellular accumulation of 5-azacytidine metabolites (Reid et al., 2003). Similar results were obtained using the natural occurring nucleoside uridine.

**Fig. 2.** Expression profile of nucleoside transporter proteins in leukemia cell lines, in cancer cell lines from solid tumors and in a normal fibroblast cell line. (A–E) Expression of nucleoside transporters mRNA transcript (hCNT1-3 and hENT1-2), as determined by quantitative real-time polymerase chain reaction analysis on cell lines. Expression values are means of triplicates reproduced independently at least twice and were calculated relative to β-actin (ACTB) expression. (F) Quantitative analysis of hENT1 signal normalized to the average Ponceau staining after immunoblot analysis of hENT1 in crude membranes. The blot was immunostained using a polyclonal antibody against hENT1. Representative immunoblot reproduced independently three times. (G–I) Immunolocalization of hENT1 in U937, K562, and ML1. hENT1 was stained using a polyclonal antibody against hENT1 (red), and nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) (blue). Scale bar, 10 μm.
in all cell lines (Supplemental Fig. 4). Consistent with the low level of hENT1 mRNA expression in Wi38 cells, these cells also showed the lowest level of uridine uptake (Supplemental Fig. 4). Taken together, these results demonstrated the predominant role of hENT1 as a nucleoside and 5-azacytidine transporter in cancer cells.

5-Azacytidine–Mediated Cytotoxicity Requires hENT1 Activity, but Not CP-4200. Cytotoxicity is a major effect of 5-azacytidine treatment, particularly at higher concentrations (Rius and Lyko, 2012). To characterize drug-induced cytotoxicity, cells were treated daily and dose-dependent cell viabilities were determined. The results established IC\textsubscript{50} values in the nanomolar range for all cell lines except for Wi38 (Table 1). Upon hENT1 inhibition by nontoxic concentrations of NBTI or dipyridamole (10 and 1 \(\mu\)M, respectively), cell viability increased 2- to 20-fold in all cell lines (Table 1), indicating that reduced hENT1 activities prevented the intracellular accumulation of 5-azacytidine at cytotoxic concentrations. The variable potency of the inhibitors in the different cell lines may be the result of altered affinity of mutated hENT1 proteins to NBTI and dipyridamole.

Fig. 3. hENT transport activity is required for cellular uptake of 5-azacytidine. (A–C) Cellular uptake of \([^{14}C]\)5-azacytidine (5 \(\mu\)M, \([^{14}C]\)Aza) in Na\textsuperscript{+} containing (open symbols) or Na\textsuperscript{+}-free (closed symbols) buffer. (D–F) Na\textsuperscript{+}-independent cellular uptake of \([^{14}C]\)5-azacytidine (5 \(\mu\)M) after preincubation of cells for 5 minutes with the hENT inhibitors dilazep, dipyridamole (Dipy), and NBTI (10 \(\mu\)M). Uptake as intracellular substrate accumulation was calculated as means \(\pm\) S.D. determined from a triplicate determination reproduced independently at least once. *\(P < 0.001\); **\(P < 0.01\) compared with control values in the absence of the inhibitor.
The role of hENT1 on cytotoxicity was further studied using the 5-azacytidine derivative CP-4200 (Fig. 4A), which had been designed to modulate 5-azacytidine uptake (Brueckner et al., 2010). Treatment of cell lines with CP-4200 resulted in slightly higher but comparable IC50 values to those obtained from cell lines treated with 5-azacytidine (Table 2). These results suggested that CP-4200 uptake into cells was efficient, causing comparable cytotoxicity. Interestingly, Wi38 cells, which expressed very low levels of hENT1 mRNA, showed a significantly more pronounced reduction of cell viability after treatment with 5-azacytidine or CP-4200 (Supplemental Tables 1 and 2).

To further characterize the influence of hENT transporters on the mode of action of 5-azacytidine and CP-4200, the IC50 values were measured for 5-azacytidine and CP-4200 in cell lines expressing different levels of hENT1. The IC50 values were determined by preincubation of cells for 20 minutes before drug treatment. Cell viability was determined with CellTiter-Blue assay, and the IC50 concentrations were calculated as the nucleoside analog concentration required to reduce cell viability by 50%. Every data point was measured in quadruplicate, and every experiment was reproduced independently at least once.

### Table 1

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>5-Azacytidine</th>
<th>5-Azacytidine Preincubated with 10 μM NBTI</th>
<th>5-Azacytidine Preincubated with 1 μM Dipyridamole</th>
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<tr>
<td>U937</td>
<td>45 ± 8</td>
<td>2101 ± 186*</td>
<td>306 ± 100**</td>
</tr>
<tr>
<td>K562</td>
<td>160 ± 55</td>
<td>243 ± 88***</td>
<td>571 ± 21*</td>
</tr>
<tr>
<td>ML1</td>
<td>276 ± 48</td>
<td>24,862 ± 4420*</td>
<td>12,603 ± 2846*</td>
</tr>
<tr>
<td>HL60</td>
<td>110 ± 25</td>
<td>640 ± 32†</td>
<td>1495 ± 299*</td>
</tr>
<tr>
<td>Jurkat</td>
<td>444 ± 48.1</td>
<td>1993 ± 164*</td>
<td>3849 ± 561*</td>
</tr>
<tr>
<td>KG-1</td>
<td>265 ± 86</td>
<td>524 ± 82**</td>
<td>859 ± 125*</td>
</tr>
<tr>
<td>HCT116</td>
<td>513 ± 41</td>
<td>5376 ± 909*</td>
<td>11,031 ± 3264*</td>
</tr>
<tr>
<td>PC3</td>
<td>731 ± 169</td>
<td>23,079 ± 5917*</td>
<td>21,366 ± 8256**</td>
</tr>
<tr>
<td>Wi38</td>
<td>2845 ± 762</td>
<td>19,094 ± 4752*</td>
<td>16,469 ± 3612*</td>
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*P < 0.001; **P < 0.01; and ***P = 0.1 compared with IC50 concentrations from the corresponding cell line in the absence of inhibitor.

To further characterize the influence of hENT transporters on the DNA demethylation activity of azanucleosides in U937 cells, we used Illumina HumanMethylation27 BeadChip arrays to interrogate the methylation status of 27,578 CpG sites representing 14,475-associated genes. Methylation of individual loci was determined by AVB values that ranged from 0 (unmethylated) to 1 (completely methylated), and methylation differences (DB values) were calculated for all probes by subtracting AVB values for treated cells from control AVB values. 5-Azacytidine- and CP-4200-treated cells showed similar DB values, which were negative, because of the drug-induced demethylation (Fig. 4C). After pretreatment with hENT inhibitors, the DB values for 5-azacytidine–treated cells were close to or even higher than 0, indicating that the methylation level of the treated cells was similar to the control cells (Fig. 4C). In contrast, pretreatment with CP-4200 and hENT inhibitors did not significantly influence DB values, which remained similar to the CP-4200–treated cells (Fig. 4C). These results are in agreement with the global DNA methylation levels that were obtained by capillary electrophoresis and provide further confirmation for the role of hENT1 in 5-azacytidine–induced DNA demethylation and the hENT1 independency of CP-4200–induced DNA demethylation.

High-resolution comparisons of individual DNA methylation profiles from U937 cells that were either treated with 5-azacytidine or remained untreated identified 1313 probes that became demethylated by 5-azacytidine (Fig. 4D).
After pretreatment with NBTI, the demethylation effect of 5-azacytidine was strongly reduced, resulting in only 63 probes that indicated DNA demethylation (Fig. 4E). These results strongly suggested that functional hENT1 was required for the DNA demethylation activity of 5-azacytidine. Because we observed a different hENT dependency for 5-azacytidine- and CP-4200-mediated cytotoxicity as well as global DNA methylation, methylation profiles for CP-4200-treated U937 cells were also analyzed. The results showed that 977 probes were demethylated by CP-4200 (Fig. 4F). However, after hENT1...
TABLE 2
IC_{50} concentrations for CP-4200 in cancer cell lines and normal fibroblast Wi38 cells after 72 hours of treatment

Cells were incubated for 72 hours with increasing concentrations of CP-4200. The effect of NBTI or dipyrnidamole was determined by preincubation of cells for 20 minutes before drug treatment. Cell viability was determined with CellTiter-Blue assay, and the IC_{50} concentrations were calculated as the nucleoside analog concentration required to reduce cell viability by 50%. Every data point was measured in quadruplicate, and every experiment was reproduced independently at least once.

<table>
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<th>Cell Line</th>
<th>IC_{50} CP-4200</th>
<th>IC_{50} CP-4200/5-Azacytidine</th>
<th>IC_{50} CP-4200 Preincubated with 10 μM NBTI</th>
<th>IC_{50} CP-4200 Preincubated with 1 μM Dipyrnidamole</th>
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<tr>
<td>U937</td>
<td>208 ± 38</td>
<td>4.6</td>
<td>17 ± 1.1*</td>
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<td>K562</td>
<td>209 ± 59</td>
<td>1.3</td>
<td>22 ± 3.2*</td>
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<td>270 ± 31</td>
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<td>37 ± 2.3*</td>
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<td>7.1</td>
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<td>Jurkat</td>
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<td>33 ± 1.5*</td>
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<td>2.4</td>
<td>19 ± 1.2*</td>
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<tr>
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<td>1644 ± 219</td>
<td>2.2</td>
<td>508 ± 25*</td>
<td>281 ± 20*</td>
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<tr>
<td>Wi38</td>
<td>356 ± 67</td>
<td>0.1</td>
<td>58 ± 15*</td>
<td>52 ± 9.4*</td>
</tr>
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</table>

*P < 0.001 compared with IC_{50} concentrations from the corresponding cell line in the absence of inhibitor.

inhibition with NBTI, 393 probes remained demethylated (Fig. 4G). The reduction in the number of demethylated probes could be explained as a consequence of the inhibited hENT1 that impaired the efflux and reuptake of intracellular nucleoside metabolites from CP-4200 such as 5-azacytidine. These differences were clearly reflected by analyzing the overlap of individual CGs that became demethylated after drug treatment (Supplemental Fig. 6). A substantial overlap of demethylated CGs could be detected after treatment of cells with both drugs (Supplemental Fig. 6A). In addition, 2% of the CGs demethylated by 5-azacytidine were still demethylated after hENT1 inhibition (Supplemental Fig. 6B). In contrast, 33% of CGs demethylated by CP-4200 was demethylated after hENT1 inhibition (Supplemental Fig. 6C). These results underscore that hENT transporters were not important for CP-4200-dependent DNA demethylation.

To obtain insights into the function of genes that became demethylated by 5-azacytidine and in a hENT-dependent fashion, ingenuity pathway analysis was performed on datasets obtained from U937 cells (Fig. 5). Demethylated genes were especially enriched in functional categories that could be associated with the terms cell proliferation and activation, regulation and maturation of the immune system. Selecting hENT1-dependent and 5-azacytidine–induced demethylated genes (Fig. 5A, region in blue) revealed a unique set of genes that could be associated with the term drug metabolism (Fig. 5B). These data supported the observed nucleoside transporter-dependent activity of 5-azacytidine. In contrast, analysis of excluded genes (Fig. 5C, region in gray) revealed conserved functional categories comparable with the total 5-azacytidine–induced hypomethylated genes (Fig. 5D). This supports the detection of a unique set of genes dependent on active hENT1 in drug metabolism, a key step in the mode of action of 5-azacytidine (Rius and Lyko, 2012).

Recombinant hENT1 Expression Confers Increased Drug Sensitivity to 5-Azacytidine and Facilitates DNA Demethylation in MDCK Cells. To validate the effects of hENT1 on cytotoxicity to 5-azacytidine and on 5-azacytidine–induced DNA demethylation, a stable clonal cell line (MDCK-hENT1) was generated. Expression of hENT1 was confirmed by immunoblotting (data not shown) and by immunofluorescence microscopy (Fig. 6A). Confocal laser scanning microscopy was performed using an antibody against hENT1 and an antibody against Na^+/K^+-ATPase, a plasma membrane protein. Intense green fluorescence for hENT1 was detected in the MDCK-hENT1 cells and hENT1 colocalized with Na^+/K^+-ATPase in these cells as demonstrated in the merged picture as a yellow staining (Fig. 6A). No green fluorescence for hENT1 was detected in vector-transfected MDCK-Co cells (Fig. 6A).

Uptake of [³H]uridine was measured in MDCK cells using Na^-free buffers to demonstrate the functionality of the transporter (Fig. 6B). The hENT1-mediated transport of uridine was unidirectional, time dependent (Fig. 6B), and saturable with a K_m value of 25 μM. NBTI inhibited the hENT1-mediated uptake of uridine (Fig. 6B). In contrast, the MDCK-Co cells did not mediate uridine uptake. Similar results were obtained with cytidine as substrate (data not shown).

The two major effects of this drug were assessed in the transfected MDCK cells (Fig. 6, C and D). Dose-dependent cell viability was determined after daily treatment of the cells. IC_{50} values of 4.7 ± 0.9 and 0.7 ± 0.1 μM were obtained for MDCK-Co and MDCK-hENT1 cells, respectively (Fig. 6C). The relative sensitivity of MDCK-hENT1 cells increased 6.5-fold compared with MDCK-Co cells (P < 0.001). Pretreatment of the cells with NBTI caused 16- and 32-fold increase in the cell viability of MDCK-Co and MDCK-hENT1, respectively (Fig. 6C). Furthermore, we analyzed the global methylation levels with capillary electrophoresis (Fig. 6D). The results showed that MDCK-Co and MDCK-hENT1 cells had a methylation level of 4.0 and 3.9%, respectively. After 72 hours of treatment with 0.5 μM 5-azacytidine, cytosine methylation levels of MDCK-hENT1 cells were significantly more reduced compared with controls (1.5 vs. 3%; Fig. 6D). The minor DNA demethylation in MDCK-Co cells (from 4 to 3%) after 5-azacytidine treatment can be explained by the presence of endogenous canine Ents (Hammond et al., 2004). After inhibition with NBTI, the demethylation activity of 5-azacytidine was completely blocked with methylation levels comparable with the levels without treatment (Fig. 6D). Thus, increased uptake of 5-azacytidine by hENT1 expression enhanced the relative sensitivity of the cells to the drug and
facilitated the 5-azacytidine-induced DNA demethylation. These results corroborate our findings with leukemia cell lines and further confirm a functional role of hENT1 in mediating the effects of 5-azacytidine.

Discussion

5-Azacytidine is a well-established nucleoside analog for the treatment of MDS and AML. These diseases are characterized by an overproliferation of poorly differentiated myeloid progenitor cells (Griffiths and Gore, 2013). Clinical resistance to 5-azacytidine is common, with more than half of all MDS/AML patients not responding to 5-azacytidine treatment, which ultimately leads to poor treatment outcome (Issa, 2007). An understanding of the resistance mechanisms to 5-azacytidine is hampered by the incomplete knowledge of the mode of action of this drug. Although the central cellular effects of 5-azacytidine are cytotoxicity as well as DNA and RNA demethylation (Rius and Lyko, 2012), the determinants that cause the chemoresistance to 5-azacytidine are unknown.

The expression of transporters has been closely linked to drug resistance, and therefore they represent important candidate biomarkers during chemotherapy (Gillet and Gottesman, 2010; Shaffer et al., 2012). Efflux transporters such as ABCB1, ABCC3, and ABCG2 have been suggested as the potential cause for treatment failure of AML (Benderra et al., 2004, 2005; Legrand et al., 2004). However, nucleoside analogs appear not to be substrates for these efflux transporters. Instead, the nucleoside transporter families hCNT and hENT are involved in transporting nucleoside analogs and their metabolites across cell membranes. hCNT1 and hCNT3 were previously identified to mediate the uptake of 5-azacytidine into cells (Rius et al., 2009, 2010). However, hCNT1 and hCNT3 are mostly expressed in epithelial cells, and their roles in leukemia cells are not known. Expression and activity of the nucleoside transporter hENT1 were previously reported to predict survival on gemcitabine treatment of pancreatic cancer (Marechal et al., 2009, 2012; Borbath et al., 2012), and hENT1 deficiency has been involved in the resistance to cytarabine in AML patients (Galmarini et al., 2002). Furthermore, low expression of hENT1, hENT2, and deoxycytidine kinase combined with high expression of cytosine deaminase have been correlated with the resistance to 5-aza-2'-deoxycytidine in cancer cell lines (Qin et al., 2009). Although hENT expression was analyzed, the transport activity of hENT1 in correlation to 5-azacytidine-induced DNA demethylation was not tested (Qin et al., 2009).

In the present study, we analyzed the expression profile of nucleoside transporters in primary AML samples and in leukemia cell lines. The results revealed that the expression of hENT1 specifically was high in primary AML cells. Comparable expression patterns were also obtained from a panel of leukemia cell lines. Interestingly, cancer cell lines from solid tumors also exhibited similar nucleoside transporter expression patterns, which indicated the predominant expression of the hENT1 transporter in cancer cell lines.

The 5-azacytidine transport activity in the presence of hENT1 inhibitors confirmed the hENT1-mediated uptake into primary AML-derived cells and various leukemia cell lines. When the cellular effects of 5-azacytidine on cell viability were analyzed, we observed decreased cytotoxicity upon hENT1 inhibition. Under these conditions, the DNA...
Fig. 6. Recombinant hENT1 expression in MDCK cells enhances 5-azacytidine sensitivity and DNA demethylation. (A) Immunolocalization of recombinant hENT1 and Na+/K+-ATPase in MDCK cells. MDCK cells were stably transfected with control vector (MDCK-Co) or hENT1 cDNA (MDCK-hENT1). Cells were grown on coverslips and stained using a polyclonal antibody against hENT1 (in green) and a monoclonal antibody against Na+/K+-ATPase (in red). Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI; in blue). Yellow staining shows colocalization of hENT1 and Na+/K+-ATPase. Scale bar, 10 μm. (B) Cellular uptake of [3H]uridine (1 μM) in Na+-free buffer in MDCK-Co and in MDCK-hENT1 without NBTI (control and hENT1, ■ and ●, respectively) and in MDCK-hENT1 preincubated for 20 minutes with NBTI (hENT1, ○). Uptake as intracellular substrate accumulation was calculated as means ± S.D. determined from a triplicate determination reproduced independently at least once. (C) MDCK-Co (■) and MDCK-hENT1 (●) cells were incubated for 72 hours with increasing concentrations of 5-azacytidine without (upper panel) or with 10 μM NBTI (lower panel). Cell viability was determined with CellTiter-Blue assay, and the IC50 concentrations were calculated as the 5-azacytidine concentration required to reduce cell viability by 50%. Every data point was measured in quadruplicate, and every experiment was reproduced independently at least once. (D) Determination of genomic cytosine methylation levels after treatment with 5-azacytidine for 72 hours in the presence or absence of NBTI (10 μM). *P < 0.01; **P < 0.001 compared with the corresponding untreated cells. Methylation levels were calculated as means ± S.D. determined from a quintuplicate determination reproduced independently at least once.
demethylation effects of 5-azacytidine were largely abolished. Pathway analysis of the different sets of hypomethylated genes revealed conserved functional categories. However, a unique set of hENT1-dependent and 5-azacytidine–induced demethylation genes was strongly enriched in the functional category for drug metabolism and transport. These findings are in line with the knowledge about cellular transport and metabolic activation of DNA methyltransferase inhibitors (Rius and Lyko, 2012).

Notably, the consequences of low hENT activity, i.e., the reduced cytotoxicity and low demethylation activity of 5-azacytidine, could be completely overcome by the use of CP-4200, a chemically modified 5-azacytidine derivative. CP-4200 is an elaidic acid ester of 5-azacytidine that was developed with the intention of reducing the dependency of the parent drug on membrane transporters (Brueckner et al., 2010). Interestingly, Wi38 cells as a cell line from noncancerous fibroblasts showed the lowest hENT1 expression combined with the lowest transport activity for nucleoside and the lowest cytotoxicity to 5-azacytidine compared with cancer cell lines from solid tumors. In contrast to cancer cell lines, the cellular toxicity of this noncancerous cell line to CP-4200 was higher than to 5-azacytidine, suggesting the relevant role of hENT1 in the uptake of 5-azacytidine. Thus, this study confirms this hypothesis and suggests that low hENT expression and/or activity might represent valuable patient stratification biomarkers for the clinical use of CP-4200. Because we previously showed that various formulations of CP-4200 showed increased therapeutic efficacy compared with equitoxic doses of 5-azacytidine (Brueckner et al., 2010), our new data explain this effectiveness by the ability of CP-4200 to overcome hENT-related resistance to 5-azacytidine.

Our results underscore the essential roles for specific transport proteins in epigenetic cancer therapy of leukemic syndromes. The present study identifies hENT1 as the most relevant nucleoside transporter for the uptake of 5-azacytidine, for 5-azacytidine–mediated cytotoxicity, and the resulting DNA demethylation in leukemia cells. Moreover, these findings correlate with the studies in stably transfected cells expressing recombinant hENT1. Although hENT1 seems to be very important, the residual accumulation of 5-azacytidine in hENT1–inhibited cells suggest additional cellular pathways that are involved in mediating drug resistance against 5-azacytidine in AML patients. These pathways need to be studied and validated in primary AML cells. Lastly, the hENT1–independent activity of the 5-azacytidine derivative CP-4200 suggests the potential use of this compound to overcome nucleoside transporter–related drug resistance to 5-azacytidine.

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