Human concentrative nucleoside transporter 1-mediated uptake of 5-azacytidine enhances DNA demethylation

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Abstract

The DNA methyltransferase inhibitors 5-azacytidine (5-azaCyd) and 5-aza-2′-deoxycytidine have found increasing use for the treatment of myeloid leukemias and solid tumors. Both nucleoside analogues must be transported into cells and phosphorylated before they can be incorporated into DNA and inactivate DNA methyltransferases. The members of the human equilibrative and concentrative nucleoside transporter families mediate transport of natural nucleosides and some nucleoside analogues into cells. However, the molecular identity of the transport proteins responsible for mediating the uptake of 5-azanucleosides has remained unknown. To this end, we have generated a stably transfected Madin-Darby canine kidney strain II cell line expressing recombinant hCNT1. An antiserum directed against hCNT1 specifically detected the protein in the apical membrane of hCNT1-expressing Madin-Darby canine kidney cells. Using [^{14}C]5-azaCyd, we show here that hCNT1 mediated the Na^+-dependent uptake of this drug with a K_m value of 63 μmol/L. Na^+-dependent transport of radiolabeled cytidine, uridine, and 5-fluoro-5′-deoxyuridine further showed the functionality of the transporter. hCNT1-expressing cells were significantly more sensitive to 5-azaCyd, and drug-dependent covalent trapping of DNA methyltransferase 1 was substantially more pronounced. Importantly, these results correlated with a significant sensitization of hCNT1-expressing cells toward the demethylating effects of 5-azaCyd and 5-aza-2′-deoxycytidine. In conclusion, our study identifies 5-azaCyd as a novel substrate for hCNT1 and provides direct evidence that hCNT1 is involved in the DNA-demethylating effects of this drug. [Mol Cancer Ther 2009;8(1):225–31]

Introduction

The 5-azanucleosides 5-azacytidine (5-azaCyd) and 5-aza-2′-deoxycytidine (5-aza-2′-dCyd; decitabine) exhibit significant activity in the treatment of leukemias and solid tumors (1, 2). It has been shown that both 5-azaCyd and 5-aza-2′-dCyd are able to inhibit DNA methyltransferases in patients. This effect has been shown to cause pronounced DNA demethylation and, in some cases, epigenetic reactivation of tumor suppressor genes. As such, 5-azaCyd and 5-aza-2′-dCyd represent prototypical drugs for epigenetic cancer therapies (3).

Clinical response rates to 5-azanucleosides are typically in the range of 30% to 40%. Also at the epigenetic level, a wide range of responses has been described. For example, a previous analysis of bone marrow samples from patients undergoing 5-aza-2′-dCyd therapy showed no detectable demethylation in 2 of 7 patients, whereas the remaining 5 patients showed demethylation levels ranging from 10% to 70% (4). Similar results were also obtained in solid tumors (2) and with 5-azaCyd (5, 6). These findings strongly indicate that responses to 5-azaCyd and 5-aza-2′-dCyd can be modified by patient-specific factors. However, these factors have remained unknown.

Cellular uptake mechanisms are critical modulators of drug responses and can thus play a role in the therapy outcome with 5-azaCyd and 5-aza-2′-dCyd. The members of the human equilibrative nucleoside transporter (hENT; SLC29A) and human concentrative nucleoside transporter (hCNT; SLC28A) families have been previously implicated in the cellular uptake of several chemotherapeutic nucleoside analogues and are considered the principal nucleoside transporters under physiologic and pathophysiologic conditions (7). Studies based on gene expression profiles suggested that nucleoside transporters may mediate transport of 5-azaCyd and 5-aza-2′-dCyd (8); however, these studies provided no direct demonstration of the molecular mechanism for the transport of 5-azaCyd and 5-aza-2′-dCyd across the plasma membrane. The hENT proteins facilitate transport of nucleosides in a bidirectional manner with broad selectivities but relatively low affinities (compared with hCNT proteins) and the hCNT proteins mediate uptake of nucleosides against the concentration gradient, in a Na^+-dependent manner, and with higher affinity compared with the hENTs (9, 10). In addition, both types of transporters are widely expressed (11). Lastly,

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hCNT proteins are involved in the regulation of nucleoside salvage, whereas the hENTs contribute to the nucleoside salvage in a more constitutive manner (12). Thus, we focused our study on hCNT proteins and, more specifically, on hCNT1.

Madin-Darby canine kidney (MDCK) cells stably expressing specific human transport proteins are a well-established cell system for the identification and functional characterization of drug transport mechanisms independent of the tissue-specific expression of the transport proteins (13–16). In this study, we show that expression of hCNT1 in MDCK cells is required for the uptake of 5-azaCyd. Importantly, expression of hCNT1 also sensitized MDCK cells to the cytotoxic effects of the drug, indicating a functional role of the protein in mediating drug responses. Consistent with this notion, hCNT1 also facilitated the degradation of cellular DNA methyltransferase 1 (DNMT1) protein and strongly sensitized the cells for the demethylating effects of 5-azaCyd. hCNT1-mediated sensitization of DNA demethylation was also observed for 5-aza-2′-dCyd, which showed that hCNT1 is functionally important for mediating the epigenetic effects of both 5-azaCyd and 5-aza-2′-dCyd.

Materials and Methods

Chemicals

[2-^{14}C]Cytidine (1.9 MBq/mmol), [5-^{3}H]uridine (0.6 TBq/mmol), and [6-^{3}H]5-fluoro-5′-deoxyuridine (5-F-5′-dUrd; 0.1 TBq/mmol) were purchased from Moravek Biochemicals. The synthesis of [6-^{14}C]5-azaCyd was done as published previously (17), with the following modification: the synthesis started from commercially available [^{14}C]dimethylformamide (50-62 mCi/mmol; GE Healthcare), thus obviating the conversion of ^{14}C-labeled sodium formate to [^{14}C]dimethylformamide. The specific activity of the final product was determined by dividing the radioactivity amount determined in a calibrated β-well-counter by the weight of the final crystalline compound. The purity of [^{14}C]5-azaCyd was 98% as determined by radio-TLC and radio high-performance liquid chromatography. Unlabeled cytidine, 5-azaCyd, 5-aza-2′-dCyd, and uridine were obtained from Sigma and 5-F-5′-dUrd was from Sequoia Research Products. All other chemicals were of analytic grade and purchased from Sigma, Merck, or AppliChem.

Antibodies

The peptide corresponding to the 23-amino-proximal amino acids of the hCNT1 sequence (*AEI*RSSWWSEAAPKPFSRWRNLQP; National Center for Biotechnology Information accession no. NP_004204) was synthesized and coupled to keyhole limpet hemocyanin (Peptide Specialty Laboratories). Rabbits were immunized with this conjugate to raise the AEI antiserum. The AEI antiserum was purified on an affinity column with the immobilized AEI peptide (Peptide Specialty Laboratories). The horseradish peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad and the Alexa Fluor 488-conjugated goat anti-rabbit IgG was from Molecular Probes.

Cell Culture and Stable Expression in Mammalian Cells

MDCK cells strain II were cultured as described previously (16). MDCK cells were transfected with the pcDNA3.1(+)-*hCNT1* cDNA construct, which was kindly provided by Marçal Pastor-Anglada (University of Barcelona; ref. 18) or vector only using Metafectene transfection agent (Biontex). Stable transfectants were selected using medium containing G418 (0.5 mg/mL). Resistant clones were induced with 10 mmol/L sodium butyrate for 24 h to enhance the expression of the recombinant protein (19) and screened by immunoblot analysis and immunofluorescence microscopy for hCNT1 expression.

Preparation of Crude Membrane Fractions, Immunoblot Analysis, and Deglycosylation

Crude membranes were prepared from transfected MDCK cells in the presence of proteinase inhibitors and analyzed by immunoblotting as described (19). The AEI antiserum was diluted 1:80,000 in PBS containing 0.05% Tween 20 and the horseradish peroxidase-conjugated goat anti-rabbit antiserum was used at a dilution of 1:20,000. Deglycosylation was done as described (19).

Immunofluorescence Microscopy of Cultured Cells

MDCK cells were grown on ThinCert membrane inserts as described previously (16). MDCK cells were fixed for 30 min with 2% paraformaldehyde in PBS and permeabilized for 30 min in 1% Triton X-100 in PBS. Cells were incubated with the affinity-purified AEI antiserum for 1 h at room temperature, washed in PBS, incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG for 1 h at room temperature, and then washed again in PBS. The membrane inserts were mounted onto glass slides using 50% glycerol in PBS. Confocal laser scanning micrographs of the cells were taken under the same conditions on a LSM510 apparatus (Carl Zeiss).

Transport Studies

MDCK cells were seeded in 6-well plates at a density of 2×10^6 per well and cultured for 24 h at confluence. Cells were induced with 10 mmol/L sodium butyrate for additional 24 h. Uptake studies examining the Na^+-dependent uptake of nucleosides were done at 37°C and using Na^+-containing buffer [100 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 10 mmol/L HEPES (pH 7.5)] and Na^+-free buffer in which NaCl was replaced by equimolar concentrations of choline chloride. Cells were first washed three times with Na^+-free buffer and then incubated with 1 mL Na^+-containing buffer containing the labeled substrate at the indicated concentration. 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 with 0.2% SDS. For determination of kinetic constants, transport rates of the nucleosides were measured at substrate concentrations ranging from 75 μmol/L to 1 mmol/L and after 20 s incubation of the substrate with the cells.

Cytotoxicity Assays

The sensitivity of MDCK cells to 5-azaCyd and 5-aza-2′-dCyd was assessed with AlamarBlue assays (Biosource).

MDCK cells were seeded (1×10^4 per well) in 96-well plates and incubated for 24 h before exposure to graded concentration of each drug for 72 h (13). The IC_{50} value was defined as the drug concentration required to reduce cell survival, as determined by the relative absorbance of reduced AlamarBlue, to 50%.

DNMT1 Trapping Assay

Cells were treated with 50 or 100 nmol/L 5-azaCyd for 6 or 24 h as indicated. Soluble protein extracts were prepared by freeze-thaw lysis as described previously (20) and further analyzed by immunoblotting using antibodies against DNMT1 and β-actin (Santa Cruz Biotechnology).

DNA Methylation Analysis

Genomic DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen). Global methylation levels were determined by capillary electrophoresis as described previously (21).

Statistical Analysis

For statistical analysis, the Student's t test was used. $P < 0.01$ was considered significant, and $P < 0.001$ was considered highly significant.

Results

Expression and Localization of Recombinant hCNT1 in MDCK Cells

A stable clonal cell line (MDCK-CNT1) was generated to investigate the localization of hCNT1 in polarized cells and to functionally characterize hCNT1. Expression of hCNT1 was analyzed by immunoblotting (Fig. 1A). The polyclonal AEI antiserum specifically detected a strong, broadband, characteristic for glycosylated proteins at 66 to 80 kDa in crude membranes from MDCK-CNT1 cells (Fig. 1A). The band at 66 to 80 kDa disappeared specifically in MDCK-CNT1 crude membranes when the AEI antiserum was preincubated with the synthetic AEI peptide that had been used to generate the AEI antiserum (data not shown). Deglycosylation of the hCNT1 protein using peptide *N*-glycosidase F shifted the 66 to 80 kDa band to a band with a molecular mass of ~60 kDa (Fig. 1B).

Confocal laser scanning microscopy using the affinity-purified AEI antiserum was done to study the localization of hCNT1 in stably transfected MDCK cells (Fig. 1C-F). Optical vertical sections showed intense green fluorescence for hCNT1 in the apical membrane of MDCK-CNT1 cells (Fig. 1D). No plasma membrane staining was observed in vector-transfected MDCK-Co cells (Fig. 1E and F). Thus, the affinity-purified AEI antiserum localized hCNT1 to the apical plasma membrane of polarized MDCK cells.

Transport of [^{14}C]5-azaCyd and Other Nucleosides Is Mediated by hCNT1

To experimentally address the possibility that hCNT1 might mediate the transport of 5-azaCyd across the plasma membrane, we synthesized a ^{14}C-labeled tracer molecule (see Materials and Methods). Uptake of [^{14}C]5-azaCyd was measured in MDCK-Co and MDCK-CNT1 cells using Na^+-containing and Na^+-free buffers to determine the Na^+-dependent uptake (Fig. 2A). The hCNT1-mediated

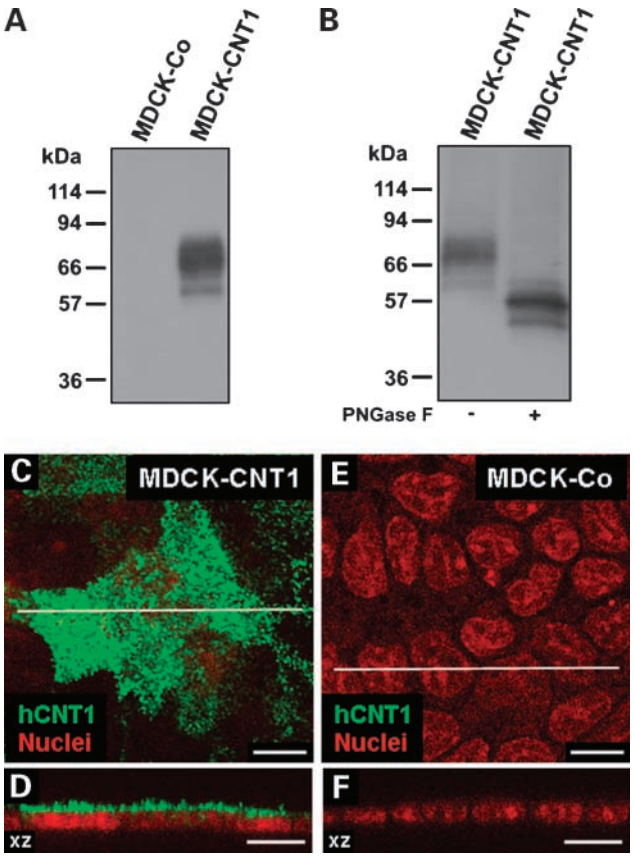

Figure 1. Expression and immunolocalization of recombinant hCNT1 in MDCK cells. **A,** immunoblot analysis of hCNT1 in crude membrane fractions prepared from MDCK cells stably transfected with control vector (MDCK-Co) or *hCNT1* cDNA (MDCK-CNT1; 10 μg protein/lane). The blot was immunostained using the polyclonal AEI antiserum against hCNT1. **B,** deglycosylation of hCNT1 in MDCK-CNT1 reduced the molecular mass of the recombinant protein to ~60 kDa. *PNGase F,* peptide *N*-glycosidase F. **C** to **F,** cells were grown in a polarized fashion on membrane inserts and analyzed by confocal laser scanning microscopy for the localization of hCNT1 (*green*) under the same conditions. *Red,* nuclei. **C** and **E,** 0.8 μm optical sections in the *x-y* plane. **D** and **F,** vertical sections in the *x-z* plane through the cell monolayers (*white lines* in **C** and **E**). Bar, 10 μm.

transport of 5-azaCyd was unidirectional, Na^+- and time-dependent, and saturable with a K_m value of 63 μmol/L (Fig. 2A; Table 1). The intracellular accumulation of [^{14}C]5-azaCyd at a concentration of 1 μmol/L amounted to 175.2 ± 9.8 and 4.1 ± 0.6 pmol/mg protein after 1 min in Na^+ and choline medium, respectively, in MDCK-CNT1 cells. In contrast, the MDCK-Co cells did not mediate Na^+-dependent uptake of 5-azaCyd (Fig. 2A; 4.8 ± 0.8 and 4.5 ± 0.5 pmol/mg protein after 1 min in Na^+ and choline medium, respectively).

Several established hCNT1 substrates were used to further characterize the MDCK transfectants. The MDCK-CNT1 cells were functionally active in nucleoside transport as evidenced by the time- and Na^+-dependent transport of cytidine, uridine, and 5-F-5′-dUrd (Fig. 2B-D), which amounted to 213, 257, and 141 pmol/mg protein/min in MDCK-CNT1 cells, respectively (Fig. 2B-D). hCNT1

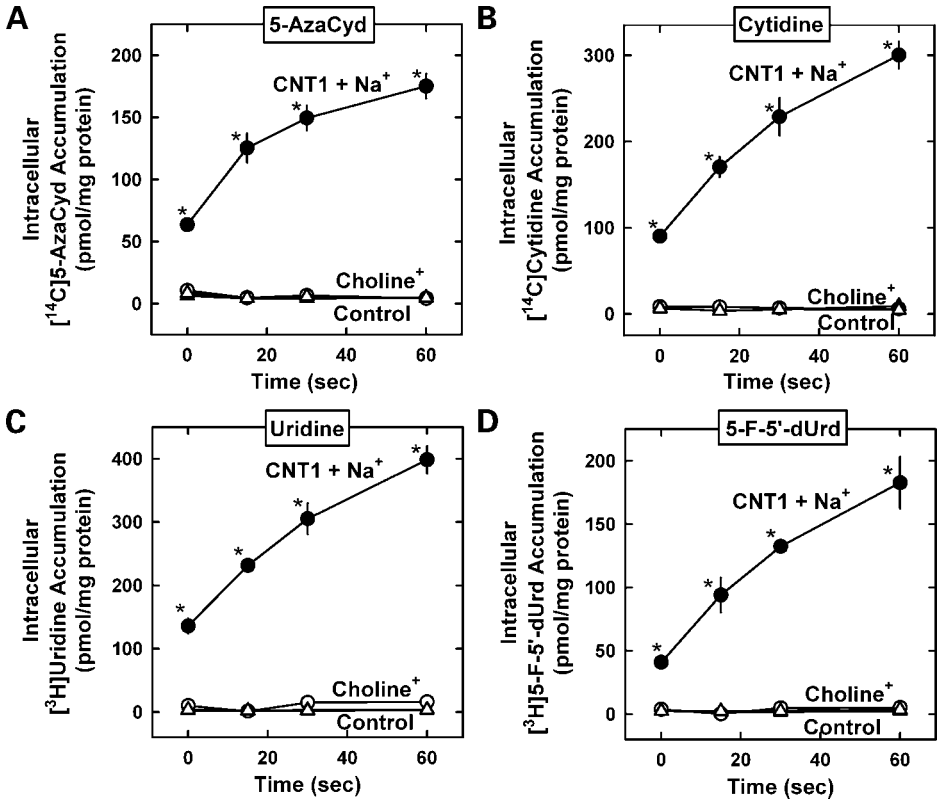

Figure 2. Transport of 5-azaCyd, [^{14}C]cytidine, [^{3}H]uridine, and 5-F-5′-dUrd. MDCK control cells (control; *triangles*) and hCNT1-expressing cells (CNT1; *circles*) were grown as described in Materials and Methods. Cells were incubated with 1 μmol/L [^{14}C]5-azaCyd (**A**), 1 μmol/L [^{14}C]cytidine (**B**), 1 μmol/L [^{3}H]uridine (**C**), or 1 μmol/L [^{3}H]5-F-5′-dUrd (**D**) in Na^+-containing (*Na^+*; *closed symbols*) or Na^+-free (*Choline$^+$*; *open symbols*) buffer. Because the data points for MDCK-CNT1 cells in Na^+-free buffer (*open circles*) and for MDCK control cells in Na^+-containing and in Na^+-free buffer (*closed* and *open triangles*, respectively) are in a similar range, the three different symbols overlap. Intracellular substrate accumulation was calculated as mean ± SD determined from triplicate measurements that were reproduced independently at least once. *, $P < 0.001$, compared with data points from MDCK-CNT1 cells in Na^+-containing buffer and from MDCK-Co cells in Na^+-containing and Na^+-free buffer.

showed K_m values for cytidine, uridine, and 5-F-5′-dUrd of 123, 467, and 403 μmol/L, respectively (Table 1).

hCNT1 Expression Confers Increased Drug Sensitivity to 5-azaCyd

The effect of 5-azaCyd into cells via hCNT1 was studied in cell viability assays (Fig. 3A). 5-azaCyd was added for 72 h to the MDCK-Co and MDCK-CNT1 cells growing on 96-well plates, cell viability was determined by the AlamarBlue assay, and IC_{50} values of 54 ± 5 and 1 ± 0.1 μmol/L were obtained for MDCK-Co and MDCK-CNT1 cells, respectively. The relative sensitivity of MDCK-CNT1 cells increased 54-fold compared with MDCK-Co cells ($P < 0.001$). Increased uptake of 5-azaCyd by hCNT1 expression therefore enhanced the relative sensitivity of the cells to the drug. Similarly, MDCK-CNT1 cells also showed increased sensitivity to 5-aza-2′-dCyd, with IC_{50} values 72 ± 10 μmol/L for MDCK-CNT1 cells and 331 ± 48 μmol/L for MDCK-Co cells.

hCNT1 Expression Facilitates 5-azaCyd-Dependent Trapping of DNMT1 and DNA Demethylation in MDCK Cells

Incorporation of 5-azanucleosides into DNA results in covalent trapping of DNA methyltransferase protein, which can be visualized by the depletion of DNMT1 from protein extracts (22). DNMT1 is highly conserved among vertebrates and antibodies against human DNMT1 can be used to specifically detect the canine orthologue in MDCK cells (Fig. 3B). To test if the hCNT1-mediated import of 5-azaCyd would facilitate the depletion of DNMT1, we determined DNMT1 protein levels by immunoblotting.

Table 1. Kinetic analysis for hCNT1-mediated uptake of labeled nucleosides

Substrate	K_m (μmol/L)	V_{max} (nmol/mg protein/min)	V_{max}/K_m (μL/mg protein/min)
5-azaCyd	63 ± 6*	15 ± 1*	238
Cytidine	123 ± 6*	62 ± 2*	504
Uridine	467 ± 50*	83 ± 31*	178
5-F-5′-dUrd	403 ± 35*	175 ± 43*	434

NOTE: Rates of Na^+-dependent intracellular accumulation of [^{14}C]5-azaCyd, [^{14}C]cytidine, [^{3}H]uridine, and [^{3}H]5-F-5′-dUrd were determined in *hCNT1*-transfected MDCK cells at six different substrate concentrations (0.075, 0.1, 0.15, 0.25, 0.5, and 1 mmol/L) and after an incubation time of 20 s. The K_m values were calculated from double-reciprocal plots of rate versus concentration. The statistical significance was calculated by fitting the linear regression of the double-reciprocal plots, with P values < 0.001 (indicated by asterisks). Mean ± SD from triplicate experiments.

Although DNMT1 was still detectable in control cells after 6 h treatment with 50 nmol/L 5-azaCyd, the protein was undetectable in MDCK-CNT1 cells (Fig. 3B). Similar results were obtained in parallel experiments with a higher concentration of 5-azaCyd (100 nmol/L; Fig. 3B). These results showed that overexpression of hCNT1 facilitates 5-azaCyd-dependent depletion of DNMT1 and further support the notion that hCNT1 plays a functional role in mediating the effects of this drug.

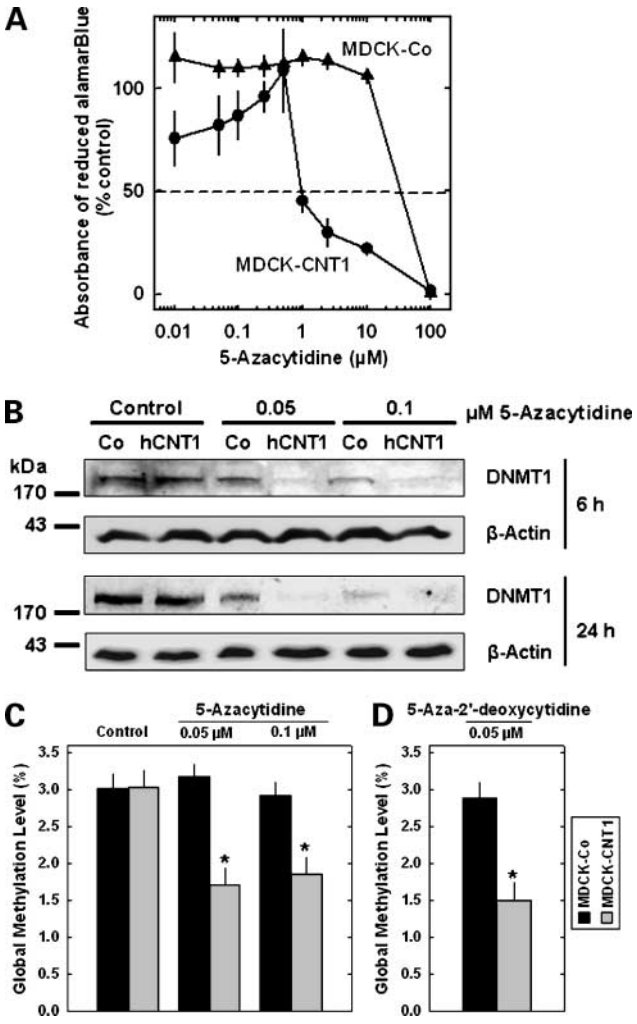

Figure 3. Expression of CNT1 enhances 5-azaCyd sensitivity, covalent trapping of DNMT1, and DNA demethylation. **A,** MDCK-Co and MDCK-CNT1 cells were incubated with different concentrations of 5-azaCyd and 5-aza-2′-dCyd for 72 h, and the cell viability was determined by AlamarBlue assays. Data are percentages of viable cells relative to untreated cells calculated as mean ± SD from 8 samples. The IC_{50} is the nucleoside analogue concentration required to reduce cell viability by 50%. **B,** immunoblot analysis of DNMT1 after treatment of MDCK-CNT1 or MDCK-Co with 5-azaCyd cells. Cells were treated with 50 or 100 nmol/L 5-azaCyd for 6 or 24 h as indicated. **C,** determination of genomic cytosine methylation levels after treatment with 5-azaCyd for 24 h. **D,** determination of genomic cytosine methylation levels after treatment with 5-aza-2′-dCyd. *, $P < 0.001$, compared with MDCK-Co cells and with untreated MDCK-CNT1 cells. All measurements were repeated at least three times. *Bars,* SD.

To determine whether the increased depletion of DNMT1 caused a more pronounced demethylation by 5-azaCyd, we analyzed the global methylation levels with capillary electrophoresis. The results showed that control MDCK cells had a methylation level of ~3.0%, which is very similar to the global methylation level of human cell lines and tissues (23, 24). After 24 h treatment with 50 nmol/L 5-azaCyd, cytosine methylation levels of MDCK-CNT1 cells were significantly reduced when compared with controls (1.7% versus 3.2%; $P < 0.001$; Fig. 3C). Similar results were obtained in parallel experiments with a higher concentration of 5-azaCyd (100 nmol/L; Fig. 3C). These results corroborate our findings with the DNMT1 trapping assay (Fig. 3B) and further confirm a functional role of hCNT1 in mediating the effects of 5-azaCyd.

We also sought to determine whether hCNT1 plays a similar role in the cellular uptake of the closely related DNA methyltransferase inhibitor 5-aza-2′-dCyd. To this end, we incubated MDCK-CNT1 and MDCK-Co cells with 50 nmol/L 5-aza-2′-dCyd and isolated genomic DNA after 24 h. Capillary electrophoretic analysis revealed 2.9% cytosine methylation in control cells but only 1.5% methylation in hCNT1-expressing cells ($P < 0.001$; Fig. 3D). These data strongly indicate that hCNT1 also mediates the cellular uptake of 5-aza-2′-dCyd.

Discussion

The effective cellular uptake of physiologic nucleosides and therapeutic nucleoside analogues across the plasma membrane needs to be mediated by transport proteins. Membrane transport of nucleosides and nucleoside analogues therefore represents an essential and often rate-determining step in many cell types that lack the ability to synthesize these molecules *de novo* and for the treatment of tumor cells with analogues (7, 25). The two nucleoside analogues, 5-azaCyd and 5-aza-2′-dCyd, are two emerging drugs that have found increasing use for the treatment of myeloid leukemias and other human cancers. However, the extended mode of action of these drugs is still largely unknown and the cellular uptake mechanisms remained to be identified (26). The members of the hCNT/SLC28 and hENT/SLC29 families have been described to mediate nucleoside and nucleoside analogue transport (7) and therefore were considered candidates for mediating cellular uptake of 5-azanucleosides. The ability of concentrative nucleoside transporters to mediate nucleoside uptake in a unidirectional manner, combined with previous reports (27, 28), established hCNT1 as a particularly attractive candidate for mediating the cellular uptake of 5-azaCyd. Consequently, the principal aim of our study was to establish a cellular model expressing hCNT1 for the study of the uptake of nucleosides and nucleoside analogues and thus to test the functional role of hCNT1 in mediating the drug effects of 5-azaCyd.

hCNT1 was expressed only in the MDCK-CNT1 transfectants and at the expected apparent molecular mass. The affinity-purified AEI antiserum localized this nucleoside

transporter to the apical plasma membrane of polarized MDCK cells. Until now, the localization of hCNT1 in polarized cells has only been studied in cell lines transfected with the hCNT1-XFP fusion constructs (14) or through the characterization of membrane vesicles from tissue epithelial cells (29). To our knowledge, the present stable MDCK-CNT1 transfectants therefore represent the first polarized cell line that express the recombinant untagged hCNT1 and sorted hCNT1 to the apical plasma membrane. In addition, the AEI antiserum represents a valuable tool for the detection of hCNT1 protein.

Using MDCK-CNT1 transfectants, we identified hCNT1 as a novel and thus far the first known nucleoside transporter for 5-azacytidine. The hCNT1-mediated transport of [^{14}C]5-azaCyd was unidirectional, Na^+- and time-dependent, and saturable. hCNT1 showed high affinity for 5-azaCyd with a K_m value of 63 μmol/L. The MDCK-CNT1 cells were also functionally active in nucleoside transport as evidenced by the time-dependent transport of cytidine, uridine, and 5-F-5′-dUrd and their saturable kinetics with K_m values. The K_m values obtained for these pyrimidine nucleosides are in the micromolar range as those reported previously (18, 30–32). Comparably minor differences may, in part, be explained by the expression of recombinant hCNT1 in different cell systems. Moreover, our results are in line with previous reports that suggested a common nucleoside transport system for 5-azaCyd as well as cytidine and uridine with similar efficiency (33). Further studies are required to determine whether additional hCNT proteins, such as hCNT3 (34), can also mediate 5-azaCyd transport.

For the characterization of DNA methyltransferase inhibitors, such as 5-azaCyd and 5-aza-2′-dCyd, human cancer cell lines have been used as experimental systems (6, 35). However, the cellular uptake systems for these drugs are not well defined. The present data show that the specific expression of hCNT1 in MDCK cells strongly enhances DNA demethylation by 5-azaCyd and by 5-aza-2′-dCyd. Of note, we also observed a detectable decrease in DNMT1 protein levels in control transfected MDCK cells treated with 5-azaCyd, thus suggesting the presence of a basal 5-azaCyd transport system in these cells. However, limited depletion of DNMT1 did not result in detectable DNA demethylation, which is in agreement with recent reports showing that low levels of DNMT1 protein are sufficient for the maintenance of comparably normal genomic DNA methylation in human cancer cell lines (36, 37). Complete depletion of DNMT1 could only be detected in hCNT1-transfected cells, which further underscores the significance of this transporter for the demethylation response to 5-azaCyd.

Other nucleoside transporters might also be involved in mediating cellular uptake of 5-azaCyd. Further studies are required to assess the effect of efflux transporters on intracellular nucleoside concentrations in addition to uptake transporters. It will be important to correlate the expression and subcellular localization of transport proteins such as hCNT1 to clinical and molecular response variables from patients undergoing demethylation therapy with 5-azanucleosides and to evaluate hCNT1 expression as a potential biomarker for 5-azaCyd and 5-aza-2′-dCyd responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Silverman LR, Mufti GJ. Methylation inhibitor therapy in the treatment of myelodysplastic syndrome. Nat Clin Pract Oncol 2005;2 Suppl 1:S12–23.

2. Appleton K, Mackay HJ, Judson I, et al. Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. J Clin Oncol 2007;25:4603–9.

3. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004;429:457–63.

4. Mund C, Hackanson B, Stresemann C, Lubbert M, Lyko F. Characterization of DNA demethylation effects induced by 5-aza-2′-deoxycytidine in patients with myelodysplastic syndrome. Cancer Res 2005;65:7086–90.

5. Gore SD, Baylin S, Sugar E, et al. Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 2006;66:6361–9.

6. Stresemann C, Bokelmann I, Mahlknecht U, Lyko F. Azacytidine causes complex DNA methylation responses in myeloid leukemia. Mol Cancer Ther 2008;7:2998–3005.

7. Zhang J, Visser F, King KM, Baldwin SA, Young JD, Cass CE. The role of nucleoside transporters in cancer chemotherapy with nucleoside drugs. Cancer Metastasis Rev 2007;26:85–110.

8. Huang Y, Anderle P, Bussey KJ, et al. Membrane transporters and channels: role of the transportome in cancer chemosensitivity and chemoresistance. Cancer Res 2004;64:4294–301.

9. Gray JH, Owen RP, Giacomini KM. The concentrative nucleoside transporter family, SLC28. Pflugers Arch 2004;447:728–34.

10. Baldwin SA, Beal PR, Yao SY, King AE, Cass CE, Young JD. The equilibrative nucleoside transporter family, SLC29. Pflugers Arch 2004; 447:735–43.

11. Pastor-Anglada M, Casado FJ, Valdes R, Mata J, Garcia-Manteiga J, Molina M. Complex regulation of nucleoside transporter expression in epithelial and immune system cells. Mol Membr Biol 2001;18:81–5.

12. Pastor-Anglada M, Cano-Soldado P, Molina-Arcas M, et al. Cell entry and export of nucleoside analogues. Virus Res 2005;107:151–64.

13. Cui Y, König J, Buchholz JK, Spring H, Leier I, Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol 1999;55:929–37.

14. Mangravite LM, Lipschutz JH, Mostov KE, Giacomini KM. Localization of GFP-tagged concentrative nucleoside transporters in a renal polarized epithelial cell line. Am J Physiol Renal Physiol 2001;280:F879–885.

15. Sasaki M, Suzuki H, Ito K, Abe T, Sugiyama Y. Transcellular transport of organic anions across a double-transfected Madin-Darby canine kidney II cell monolayer expressing both human organic anion-transporting polypeptide (OATP2/SLC21A6) and Multidrug resistance-associated protein 2 (MRP2/ABCC2). J Biol Chem 2002;277:6497–503.

16. Bartholome K, Rius M, Letschert K, Keller D, Timmer J, Keppler D. Data-based mathematical modeling of vectorial transport across double-transfected polarized cells. Drug Metab Dispos 2007;35:1476–81.

17. Chan KK, Staroscik JA, Sadee W. Synthesis of 5-azacytidine-6-^{13}C and -6-^{14}C. J Med Chem 1977;20:598–600.

18. Mata JF, Garcia-Manteiga JM, Lostao MP, et al. Role of the human concentrative nucleoside transporter (hCNT1) in the cytotoxic action of 5′-deoxy-5-fluorouridine, an active intermediate metabolite of capecitabine, a novel oral anticancer drug. Mol Pharmacol 2001;59:1542–8.

19. Rius M, Nies AT, Hummel-Eisenbeiss J, Jedlitschky G, Keppler D. Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. Hepatology 2003;38: 374 – 84.

20. Brueckner B, Boy RG, Siedlecki P, et al. Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. Cancer Res 2005;65:6305 – 11.

21. Stach D, Schmitz OJ, Stilgenbauer S, et al. Capillary electrophoretic analysis of genomic DNA methylation levels. Nucleic Acids Res 2003; 31:E2.

22. Weisenberger DJ, Velicescu M, Cheng JC, Gonzales FA, Liang G, Jones PA. Role of the DNA methyltransferase variant DNMT3b3 in DNA methylation. Mol Cancer Res 2004;2:62 – 72.

23. Gama-Sosa MA, Midgett RM, Slagel VA, et al. Tissue-specific differences in DNA methylation in various mammals. Biochim Biophys Acta 1983;740:212 – 9.

24. Paz MF, Fraga MF, Avila S, et al. A systematic profile of DNA methylation in human cancer cell lines. Cancer Res 2003;63:1114 – 21.

25. Plagemann PG, Shea MA. Transport as the rate-limiting step in the incorporation of uridine into mengovirus ribonucleic acid in Novikoff rat hepatoma cells. J Virol 1971;7:137 – 43.

26. Stresemann C, Lyko F. Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. Int J Cancer 2008;123:8 – 13.

27. Garcia-Manteiga J, Molina-Arcas M, Casado FJ, Mazo A, Pastor-Anglada M. Nucleoside transporter profiles in human pancreatic cancer cells: role of hCNT1 in 2′,2′-difluorodeoxycytidine-induced cytotoxicity. Clin Cancer Res 2003;9:5000 – 8.

28. Gloeckner-Hofmann K, Guillen-Gomez E, Schmidtgen C, et al. Expression of the high-affinity fluoropyrimidine-preferring nucleoside transporter hCNT1 correlates with decreased disease-free survival in breast cancer. Oncology 2006;70:238 – 44.

29. Patil SD, Unadkat JD. Sodium-dependent nucleoside transport in the human intestinal brush-border membrane. Am J Physiol 1997;272: G1314 – 20.

30. Ritzel MW, Yao SY, Huang MY, Elliott JF, Cass CE, Young JD. Molecular cloning and functional expression of cDNAs encoding a human Na^+-nucleoside cotransporter (hCNT1). Am J Physiol 1997;272: C707 – 14.

31. Graham KA, Leithoff J, Coe IR, et al. Differential transport of cytosine-containing nucleosides by recombinant human concentrative nucleoside transporter protein hCNT1. Nucleosides Nucleotides Nucleic Acids 2000;19:415 – 34.

32. Lostao MP, Mata JF, Larrayoz IM, Inzillo SM, Casado FJ, Pastor-Anglada M. Electrogenic uptake of nucleosides and nucleoside-derived drugs by the human nucleoside transporter 1 (hCNT1) expressed in *Xenopus laevis* oocytes. FEBS Lett 2000;481:137 – 40.

33. Plagemann PG, Behrens M, Abraham D. Metabolism and cytotoxicity of 5-azacytidine in cultured Novikoff rat hepatoma and P388 mouse leukemia cells and their enhancement by preincubation with pyrazofurin. Cancer Res 1978;38:2458 – 66.

34. Ritzel MW, Ng AM, Yao SY, et al. Recent molecular advances in studies of the concentrative Na^+-dependent nucleoside transporter (CNT) family: identification and characterization of novel human and mouse proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). Mol Membr Biol 2001;18:65 – 72.

35. Stresemann C, Brueckner B, Musch T, Stopper H, Lyko F. Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. Cancer Res 2006;66:2794 – 800.

36. Egger G, Jeong S, Escobar SG, et al. Identification of DNMT1 (DNA methyltransferase 1) hypomorphs in somatic knockouts suggests an essential role for DNMT1 in cell survival. Proc Natl Acad Sci U S A 2006; 103:14080 – 5.

37. Spada F, Haemmer A, Kuch D, et al. DNMT1 but not its interaction with the replication machinery is required for maintenance of DNA methylation in human cells. J Cell Biol 2007;176:565 – 71.

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