Vectorial Transport of Nucleoside Analogs from the Apical to the Basolateral Membrane in Double-Transfected Cells Expressing the Human Concentrative Nucleoside Transporter hCNT3 and the Export Pump ABCC4

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ABSTRACT:
The identification of the transport proteins responsible for the uptake and the efflux of nucleosides and their metabolites enables the characterization of their vectorial transport and a better understanding of their absorption, distribution, and elimination. Human concentrative nucleoside transporters (hCNTs/SLC28A) are known to mediate the transport of natural nucleosides and some nucleoside analogs into cells in a sodium-dependent and unidirectional manner. On the other hand, several human multidrug resistance proteins [human ATP-binding cassette transporter, subfamily C (ABCC)] cause resistance against nucleoside analogs and mediate transport of phosphorylated nucleoside derivatives out of the cells in an ATP-dependent manner. For the integrated analysis of uptake and efflux of these compounds, we established a double-transfected Madin-Darby canine kidney (MDCK) II cell line stably expressing the human uptake transporter hCNT3 in the apical membrane and the human efflux pump ABCC4 in the basolateral membrane. The direction of transport was from the apical to the basolateral compartment, which is in line with the unidirectional transport and the localization of both recombinant proteins in the MDCKII cells. Recombinant hCNT3 mediated the transport of several known nucleoside substrates, and we identified 5-azacytidine as a new substrate for hCNT3. It is of interest that coexpression of both transporters was confirmed in pancreatic adenocarcinomas, which represent an important clinical indication for the therapeutic use of nucleoside analogs. Thus, our results establish a novel cell system for studies on the vectorial transport of nucleosides and their analogs from the apical to the basolateral compartment. The results contribute to a better understanding of the cellular transport characteristics of nucleoside drugs.

Purine and pyrimidine nucleoside analogs are important drugs for the treatment of many tumors and viral infections (King et al., 2006). The identification and characterization of the transport proteins involved in the entry and the efflux of these substances and their metabolites across the plasma membrane represent an important contribution to a better understanding of their targeting to certain cells, their efficacy, and toxicity. In addition, down-regulation of the uptake transporter or up-regulation of the efflux pump, or a combination of both, can be a cause for the cellular resistance to nucleoside analogs and for therapeutic failure (Gottesman, 2002). Several proteins have been described as transporters for nucleosides, nucleoside analogs, and their metabolites (Deeley et al., 2006; for reviews, see King et al., 2006). Uptake of nucleosides and nucleoside analogs across the plasma membrane is mediated by, among others, the members of the human concentrative (CNTs/SLC28A) and equilibrative (ENTs/SLC29A) nucleoside transporter families, including hCNT1, hCNT2, and hCNT3, as well as hENT1 and hENT2 (Zhang et al., 2007). After intracellular phosphorylation, several members of the ATP-binding cassette (ABC) transporter superfamily, including ABCC4, ABCC5, and ABCC11, have been identified to mediate the efflux of these compounds from cells (Deeley et al., 2006). Thus far, the nucleoside transport proteins were mostly studied in transfected mammalian cells or by the use of Xenopus oocytes expressing only one recombinant transport protein (Schuetz et al., 1999; Wijnholds et al., 2000; Chen et al., 2001; Guo et al., 2003; Pratt et al., 2005). These cell systems lacked the potential to characterize the uptake transporters together

ABBREVIATIONS: hCNT, human concentrative nucleoside transporter; hENT, human equilibrative nucleoside transporter; ABCC, human ATP-binding cassette transporter, subfamily C; MDCK, Madin-Darby canine kidney; 5-F-5’-dUrd, 5-fluoro-5’-deoxyuridine; 5-azaCyd, 5-azacytidine; 5-aza-2’-dCyd, 5-aza-2’-deoxycytidine; NBTI, S-(4-nitrobenzyl)-6-thiinosine; NCBI, National Center for Biotechnology Information; PBS, phosphate-buffered saline.
with the export pumps in a single cell system and, in particular, in a polarized cell line useful for studies on vectorial transport.

Vectorial transport of endogenous and xenobiotic substances across epithelial cells results from the uptake into the cells and the subsequent unidirectional eflux of these substances, which may be unaltered or metabolized (Keppler, 2005). The intracellular and extracellular concentrations of substances and their half-life in the organism are controlled by vectorial transport. In the past years, double-transfected polarized cells expressing combinations of a basolateral uptake transporter together with an apical ATP-dependent eflux pump were introduced and have been widely used as in vitro cell systems to characterize transport proteins and to examine their substrate specificity (Cui et al., 2001; Sasaki et al., 2002; Kopplow et al., 2005; Mita et al., 2006). These cell systems expressing recombinant transport proteins represent valuable tools in drug discovery, and they reflect hepatic and renal epithelial vectorial transport, whereby the majority of compounds are taken up across the basolateral membrane and effluxed across the apical membrane. However, the reverse situation, i.e., the vectorial transport from the apical to the basolateral membrane in polarized cells expressing an apical uptake transporter and a basolateral ATP-dependent export pump, has not yet been studied, even though it resembles, for example, the uptake from the luminal side of the intestine, followed by basolateral efflux into blood. There are uptake transporters that are only expressed in the apical membrane of epithelial cells and export pumps that are only expressed in the basolateral membrane, which further underscores the significance of apical to basolateral vectorial transport. Thus, these cell systems allow studying the substrate specificity and the transport properties of uptake transporters and efflux pumps in a cell line that resembles the in vivo state of epithelial cells but also of nonepithelial cells that express several transport proteins.

The aim of the present work was to establish a polarized cell system with defined human uptake and export transport proteins for the nucleoside and nucleoside analog transport. As a nucleoside uptake transporter for this cell model, we selected hCNT3 because it transports purine and pyrimidine nucleosides and various therapeutic nucleoside analogs in a concentrative manner and with a broad substrate specificity (Ritzel et al., 2001; Sarkar et al., 2005; Errasti-Murugarren et al., 2007). In addition, hCNT3 seems to concentrate nucleosides intracellularly more efficiently than hCNT1 or hCNT2; thus, it is expected to play an important role in the pharmacokinetics of nucleoside analogs (Mangravite et al., 2003). As an export pump, we chose ABC4, which is known to confer resistance to nucleosides and nucleotide analogs and functions as an ATP-dependent organic anion transporter with broad substrate specificity (Russel et al., 2008). At the present time, the contribution of ABC4 to resistance against nucleoside analogs has been recognized (Schuetz et al., 1999), but further studies are required to prove its contribution under clinical conditions and in patient samples. Here we report our studies on vectorial transport in a double-transfected cell line stably expressing hCNT3 in the apical membrane and ABC4 in the basolateral membrane of polarized Madin-Darby canine kidney (MDCK) II cells.

Materials and Methods

Chemicals. [2-3H]Cytidine (1.9 MBq/mmol), [5-3H]Juridine (0.6 TBq/ mmol), and [6-3H]5-fluoro-5'-deoxyuridine (5-F-3H-dUrd; 0.1 TBq/mmol) were purchased from Moravek Biochemicals (Brea, CA). [6-3H]5'-Azacytidine (5-azaCyd) has been synthesized as described previously (Rius et al., 2003). The cDNA encoding hCNT3 was cloned from human ileum using hCNT3-specific primers (forward, 5'-AGCATGAGCTGAGGATACGC-3'; reverse, 5'-TCAAAAT- GTTATAGATCCCC-3') based on the original sequence published by Ritzel et al. (2001) (NCBI accession number NM_022127) and subcloned into the vector pCR2.1-TOPO (Invitrogen, Groningen, The Netherlands). The complete 2.1-kilobase cDNA insert was excised from the pCR2.1-TOPO vector and cloned into the HindIII and XhoI restriction sites of the mammalian expression vector pcDNA3.1-Neo (Invitrogen). The ABC4 protein encoding the cloned hCNT3 cDNA was 100% identical to the reference sequence (NCBI accession number NP_071410).

Cell Culture and Stable Expression in Mammalian Cells. MDCK II cells were cultured as described previously (Cui et al., 1999). MDCK cells were transfected with the pcDNA3.1(-)–hCNT3 cDNA construct or vector only using Metafectene transfection agent (Biontex, Munich, Germany) according to the manufacturer's instructions. Stable transfecants were selected using medium containing G418 (0.5 mg/ml). Resistant clones were induced with 10 mM sodium butyrate for 24 h to enhance the expression of the recombinant protein (Cui et al., 1999) and screened by immunoblot analysis and immunofluorescence microscopy. The hCNT3 expression. For generation of the CNT3/ABC4 double-transfected MDCK cells, the CNT3 single-transfected cells were transfected with the pcDNA3.1(-)–ABC4 cDNA construct using Metafectene transfection agent. Selection was carried out with hygromycin B (500 µg/ml). After induction with 10 mM sodium butyrate, resistant clones were screened by immunoblot analysis and immunofluorescence microscopy for ABC4 expression.

Preparation of Crude Membrane Fractions, Immunoblot Analysis, and Deglycosylation. Crude membranes were prepared from transfected MDCK cells in the presence of protease inhibitors and analyzed by immunoblotting as described previously (Rius et al., 2003). The NTS antisera was diluted 1:1000 in phosphate-buffered saline (PBS) containing 0.05% Tween 20, and the polyclonal SNG antisera was diluted as described previously (Rius et al., 2003). The horseradish peroxidase-conjugated goat anti-rabbit and anti-guinea pig antibodies were used at a dilution of 1:20,000. Deglycosylation was performed as described previously (Rius et al., 2003).

Preparation of Tissue Homogenates. Tissue (0.1–0.5 mg) was homogenized during thawing by 10-fold dilution with incubation buffer (250 mM sucrose and 10 mM Tris/HCl, pH 7.4) supplemented with protease inhibitors (0.3 μM aprotinin, 1 μM leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride). The resulting suspension was further homogenized with a Potter-Elvehjem homogenizer at 1000 rpm at 2 strokes/min for 30 strokes at 4°C.
homogenate was centrifuged at 1200 g for 10 min at 4°C, and the resulting supernatant was centrifuged at 100,000 g for 30 min at 4°C. The resulting pellet was homogenized in 10 mM Tris/HCl buffer, pH 7.4, supplemented with protease inhibitors. Aliquots were stored at −80°C.

**Immunofluorescence Microscopy of Cultured Cells and Tissue Samples.** MDCK cells were grown on ThinCert membrane inserts (diameter, 6 mm; pore size, 0.4 µm; pore density, 1 × 10^8/cm²; Greiner Bio-One, Frickenhausen, Germany) for 3 days at confluence and induced with 10 mM sodium butyrate for 24 h to enhance the expression of recombinant proteins. MDCK cells were fixed with 0.2% SDS by liquid scintillation counting. Kinetic analysis was not performed under these conditions by use of double-reciprocal plots and direct curve-fitting to the Michaelis-Menten equation. The IC₅₀ value was defined as the substrate concentration at half-maximal velocity of transport and was determined after lysing the cells with 0.2% SDS. Measurements at the time points indicated, radioactivity was measured in the opposite compartment. After incubation at 37°C, cells were washed three times with ice-cold Na⁺/K⁺ ATPase. To test the specificity of the NTS and SNG antibodies, the antibodies were preincubated for 2 h at room temperature with 90 µM (final concentration) of the synthetic antigenic NTS and SNG peptides, respectively, before their application to the tissue sections. Nuclei were stained with propidium iodide or with 4,6-diamidino-2-phenylindole. The membrane inserts were mounted onto glass slides using 50% glycerol in PBS. Images were taken with a confocal laser scanning microscope (LSM510 Meta; Carl Zeiss, Oberkochen, Germany, or SP5; Leica, Wetzlar, Germany).

**Cytotoxicity Assay.** The sensitivity of MDCK cells to 5-aza-Cyd and 5-aza-2'-dCyd was assessed with AlamarBlue assays (BioSource International, Camarillo, CA). MDCK cells were seeded (1 × 10⁴ cells/well) in 96-well plates and incubated for 24 h before exposure to graded concentrations of each drug between 10 nM and 100 µM for 72 h. The IC₅₀ value was defined as the drug concentration required to reduce cell survival, as determined by the relative absorbance of reduced AlamarBlue, to 50%.

**Uptake Studies.** MDCK cells were seeded in six-well plates at a density of 2 × 10⁵ cells/well and cultured for 24 h at confluence. Cells were induced with 10 mM sodium butyrate for an additional 24 h. Uptake studies examining the Na⁺-dependent uptake of nucleosides were performed using Na⁺-containing buffer (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.5) and Na⁺-free buffer in which NaCl was replaced by equimolar choline chloride. Cells were first washed three times with Na⁺-free buffer and then incubated with 1 µM of 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. For determination of kinetic constants in MDCK-CNT3 cells, transport rates were measured at five different substrate concentrations (0.075, 0.1, 0.15, 0.25, 0.5, and 1 mM) over a 20-s incubation time. Intracellular radioactivity was determined after lysing the cells with 0.2% SDS by liquid scintillation counting. Kinetic parameters were determined by the nonlinear least-squares fitting of the Michaelis-Menten equation. Kinetic analysis was not performed in MDCK-Co cells because they lacked significant levels of Na⁺-dependent uptake.

**Transcellular Transport.** MDCK cells were grown on ThinCert membrane inserts (diameter, 24 mm; pore size, 0.4 µm; pore density, 1 × 10^8/cm²; Greiner Bio-One) for 3 days at confluence and induced with 10 mM sodium butyrate for 24 h. The cells were washed in prewarmed (37°C) Na⁺-containing or Na⁺-free buffer with 5 mM glucose. The radiolabeled substrate was dissolved in Na⁺/glucose-containing or Na⁺-/glucose-buffer and added to the either the apical or basolateral compartment. After incubation at 37°C and at the time points indicated, radioactivity was measured in the opposite compartment. When NBTI was used, it was added to the basolateral compartment at a concentration of 10 µM. Cells were washed with ice-cold Na⁺- and glucose-containing or Na⁺-free but glucose-containing buffer. Intracellular radioactivity was determined after lysing the cells with 0.2% SDS. Measurements at several time points in cells expressing recombinant hCNT3 indicated that the transcellular transport from the apical to the basolateral compartment was linear at least within the first 15 min in the presence of sodium. The paracellular leakage was determined by the use of [3H]inulin (Biotrend, Cologne, Germany), and it amounted to less than 2% of the radioactivity added for all the MDCK cell lines. For statistical analysis, the Student’s t test was used.

**Results**

**Expression and Localization of Recombinant hCNT3 and ABCC4 in MDCK Cells.** The protein expression of hCNT3 and ABCC4 in the stably transfected MDCK cells was verified by immunoblot analyses (Fig. 1, A and B). The hCNT3 protein was specifically detected by the polyclonal NTS antibody as two dominant broad bands, characteristic for glycosylated proteins, with apparent molecular masses of 90 and 65 kDa in crude membranes from MDCK-CNT3 and MDCK-CNT3/ABCC4 cells (Fig. 1A). Deglycosylation of the NTS protein by PNGaseF shifted the two bands to a single band with a molecular mass of approximately 60 kDa (data not shown). ABCC4 was strongly expressed at 170 kDa in the MDCK-CNT3/ABCC4 cells (Fig. 1B). In the vector-transfected control cells, none of the two transport proteins was detectable. Control experiments showed that the NTS antiserum did not cross-react with the recombinant hCNT1 expressed in MDCK cells (data not shown). The cellular localization of the recombinant transport proteins in the MDCK transfecants was studied by confocal laser scanning microscopy (Fig. 1, C–N). The NTS antiserum localized hCNT3 in stably transfected MDCK cells (Fig. 1, C–H), and optical vertical sections showed intense red fluorescence for hCNT3 in the apical membrane domain of the cells expressing CNT3 and CNT3/ABCC4 (Fig. 1, D and F). No plasma membrane staining was observed in vector-transfected MDCK-Co cells (Fig. 1, G and H). ABCC4 was localized exclusively to the basolateral membrane domain of the CNT3/ABCC4 double-transfected MDCK cells (Fig. 1, K and L). Our localization studies are in line with previous work that showed apical localization of hCNT3 in MDCK cells (Errasti-Murugarren et al., 2007) and basolateral localization of ABCC4 in MDCK cells expressing recombinant ABCC4 (Lai and Tan, 2002).

**Transport of [¹⁴C]5-azaCyd and Other Nucleosides Mediated by hCNT3.** To test the functionality of hCNT3 in transfected MDCK cells, intracellular accumulation of several nucleosides was measured in MDCK-Co and MDCK-CNT3 cells. The results showed that the MDCK-CNT3 transfectants mediated the Na⁺-dependent uptake of the known substrates cytidine, uridine, and 5-F-5'-dUrd (Fig. 2, B–D) with Kₑ₅₀ values of 151, 51, and 615 µM, respectively (Table 1). Low transport rates of uridine were observed in the presence of choline in MDCK-CNT3 cells (Fig. 2C), which accounted only for 20% of the Na⁺-dependent uptake and were possibly the result of transport by endogenous transporters. Uptake of 5-azaCyd, a novel substrate identified recently for hCNT1 (Rius et al., 2009), was also analyzed in the cells expressing hCNT3 (Fig. 2A). The Na⁺-dependent intracellular accumulation of [¹⁴C]5-azaCyd at a concentration of 1 µM amounted to 221 pmol/mg protein after 1 min in MDCK-CNT3 cells (Fig. 2A). In contrast, the MDCK-Co cells did not mediate Na⁺-dependent uptake of 5-azaCyd (Fig. 2A). Kinetic analysis showed a Kₑ₅₀ value of 147 µM (Table 1). These results indicate that, in addition to established substrates, hCNT3 also mediates cellular uptake of the anticancer drug 5-azaCyd.

**hCNT3 Expression Enhances Inhibition of the Cell Growth by 5-azaCyd and 5-aza-2'-dCyd.** The uptake and action of 5-azaCyd by hCNT3 were further studied in viability assays with nonlabeled 5-azaCyd and with the structurally related derivative 5-aza-2'-dCyd. MDCK-Co and MDCK-CNT3 cells were incubated with various concentrations of 5-azaCyd or 5-aza-2'-dCyd for 72 h, and cell viability was determined by AlamarBlue assay (BioSource International). The viability of hCNT3-expressing MDCK cells decreased in a concentration-dependent manner in the presence of 5-azaCyd or 5-aza-2'-dCyd (Table 2). The IC₅₀ values obtained for MDCK-Co and MDCK-CNT3 cells after incubation with 5-azaCyd were 54 ± 5 µM.
and 0.8 ± 0.07 μM, respectively. Thus, hCNT3-expressing MDCK cells increased their relative sensitivity 68-fold compared with MDCK-Co cells. After incubation with 5-aza-2′-dCyd, MDCK-Co and MDCK-CNT3 showed IC_{50} values of 331 ± 48 μM and 25 ± 4 μM, respectively; thus, the relative sensitivity of MDCK-CNT3 cells increased 13-fold compared with MDCK-Co cells (Table 2).

Vectorial Transport of [^{14}C]Cytidine and [^{14}C]5-azaCyd in MDCK Cells Expressing hCNT3. MDCK cells were grown in a polarized fashion on cell culture inserts for studies on vectorial transport. Figure 3A shows schematically the role of the recombinant hCNT3 in the vectorial transport by the transfected MDCK cells. When MDCK cells were grown on filter membranes, the recombinant hCNT3 mediated the uptake of nucleosides from the apical compartment into the cells (Fig. 3A). To test whether vectorial transport of nucleosides takes place in the correct direction, transepithelial transport of nucleosides was measured across the apical and the basolateral membrane and in the opposite direction in MDCK-CNT3 and MDCK-Co cells (Fig. 3B). Incubation with [^{14}C]cytidine at a concentration of 1 μM in the apical compartment (apical→basolateral) was associated with an Na^+-dependent accumulation of radioactivity in the basolateral compartment of MDCK-CNT3 cells (Fig. 3B, left). In contrast, when radioactivity was added in the basolateral compartment (basolateral→apical), accumulation in the apical compartment was negligible (Fig. 3B, right). The Na^+-dependent apical-to-basolateral transport was 132-fold higher than that in the opposite direction in MDCK-CNT3 cells. Because we observed a substantial accumula-
tion in the basolateral compartment (Fig. 3B, left) without the expression of a recombinant efflux transporter in the basolateral membrane, we raised the question whether basolateral transporters for nucleosides were responsible for the release into the basolateral compartment and, in particular, whether the canine equilibrative nucleoside transporters (Ents) may play a role in the transport across the basolateral membrane of MDCK cells. Ents are expressed in many cell types and function as bidirectional nucleoside transporters that equilibrate intracellular and extracellular concentrations of nucleosides (Zhang et al., 2007). To identify a potential role of canine Ents in the release of nucleosides from the apical to the basolateral membrane was measured in the presence of 10 mM NBTI in the basolateral compartment, transcellular transport across cells was studied by measurement of the transcellular transport of the nucleoside analog [14C]5-azaCyd (Fig. 4, B–D), which is a substrate for hCNT3 (Fig. 2A). Thus, polarized MDCK cells grown on Thin-

![Fig. 2. Transport of [14C]5-azaCyd, [14C]cytidine, [1H]uridine, and [1H]5-fluoro-5′-deoxyuridine (5-F-5′-dUrd) by hCNT3. MDCK control cells (Control) and hCNT3-expressing cells (CNT3) were grown as described under Materials and Methods. Cells were incubated with 1 μM [14C]5-azaCyd (A), 1 μM [1H]uridine (B), 1 μM [1H]cytidine (C), or 1 μM [1H]5-F-5′-dUrd (D) in the presence of 100 mM NaCl (closed symbols) or in the presence of 100 mM choline chloride (open symbols). Intracellular substrate accumulation is presented as mean ± S.D., determined from a triplicate determination reproducibly twice.

### TABLE 1

Kinetic analysis for hCNT3-mediated uptake of labeled nucleosides

<table>
<thead>
<tr>
<th>Substrate</th>
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<tr>
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<tr>
<td>5-azaCyd</td>
</tr>
<tr>
<td>Cytidine</td>
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<tr>
<td>Uridine</td>
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<td>5-F-5′-dUrd</td>
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Rates of Na\(^+\) dependent intracellular accumulation of [14C]5-azaCyd, [14C]cytidine, [1H]uridine, and [1H]5-F-5′-dUrd were determined in hCNT3-transfected MDCK cells under substrate concentrations described under Materials and Methods. The \(K_{in}\) values were calculated from double-reciprocal plots. Data represent mean values ± S.D. from a triplicate determination.

### TABLE 2

Effect of 5-azaCyd and 5-aza-2′-dCyd on the sensitivity of hCNT3-expressing MDCK cells

<table>
<thead>
<tr>
<th>Nucleoside Analog</th>
<th>MDCK-Co</th>
<th>MDCK-CNT3</th>
<th>Relative Sensitivity</th>
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<tr>
<td>5-azaCyd</td>
<td>54 ± 5</td>
<td>0.8 ± 0.07</td>
<td>67.5</td>
</tr>
<tr>
<td>5-aza-2′-dCyd</td>
<td>331 ± 48</td>
<td>25 ± 4</td>
<td>13.2</td>
</tr>
</tbody>
</table>

\(^{a}\)IC\(_{50}\) is the nucleoside analog concentration required to reduce cell survival by 50%. \(^{b}\)The relative sensitivity is the IC\(_{50}\) for nucleoside analog treatment in MDCK-Co cells divided by the IC\(_{50}\) in MDCK-CNT3 cells.

In agreement with our previous findings, similar results were also obtained for the vectorial transport of 1 μM [14C]5-azaCyd (data not shown).

### Transcellular Transport of [14C]Cytidine and [14C]5-azaCyd Mediated by CNT3/ABCC4 Double-Transfected MDCK Cells

Finally, we sought to show that when the double-transfected MDCK cells were grown on filter membranes, the recombinant hCNT3 mediates the uptake of nucleosides from the apical to the intracellular space and the recombinant ABCC4 mediates efflux of compounds from the intracellular space to the basolateral compartment (Fig. 4A). The function of hCNT3 and ABCC4 in the double-transfected MDCK cells were studied by measurement of the transcellular transport of the nucleoside analog [14C]5-azaCyd (Fig. 4, B–D), which is a substrate for hCNT3 (Fig. 2A). Thus, polarized MDCK cells grown on Thin-
VECTORIAL TRANSPORT MEDIATED BY hCNT3 AND ABCC4

Cytidine transport by NBTI. [14C]Cytidine (1 μM) was added to the basolateral compartment. Transcellular transport was significantly detected only in the presence of Na⁺ and only in the MDCK cells expressing hCNT3 and ABC4 (Fig. 4C, bottom panel). In addition, we also measured transcellular transport of 5-azaCyd at a higher concentration of 10 μM and without NBTI (Fig. 4D). Under these conditions and in the absence of Na⁺-containing buffer, we observed a 2-fold higher transcellular transport in the double-transfected cells expressing hCNT3 and ABC4 than in the single-transfected cells expressing only hCNT3 (Fig. 4D, bottom panel, closed bars; p < 0.05). Because small amounts of 5-azaCyd also entered the cells in the presence of Na⁺-free (choline) buffer, we also observed a low level of transcellular transport in the absence of Na⁺ (Fig. 4D, bottom panel, open bars). This transcellular transport was approximately 3-fold higher in the MDCK-CNT3/ABCC4 compared with the MDCK-CNT3 cells (Fig. 4D, bottom panel, open bars). ABC4-mediated transcellular transport of 5-azaCyd at higher concentrations can be explained by the higher saturation of canine Ents, thus allowing for a higher rate of transport across the basolateral membrane by ABC4. Similar results were obtained by measurements of the transcellular transport of [14C]cytidine (data not shown). Taken together, these results show that the highest rate of transcellular transport was observed when recombiant ABC4 was expressed in the basolateral membrane. In summary, we have described the double-transfected MDCK-CNT3/ABCC4 cells as a model for apical to basolateral vectorial transport of nucleosides and nucleoside analogs.

Detection of hCNT3 and ABC4 in Human Pancreas and Pancreatic Carcinoma. To explore the relevance of our vectorial transport model for cancer therapy, we studied the expression pattern of hCNT3 and ABC4 in pancreatic tissues. Pancreatic tissue (three different normal pancreatic samples and three samples from pancreatic carcinomas) was selected for this analysis because nucleoside analogs are widely used in the chemotherapy of pancreatic carcinoma. hCNT3 and ABC4 expressions were analyzed in homogenates and cryosections from surgical specimens by immunoblot analysis and immunofluorescence microscopy using the antisera directed against hCNT3 and ABC4 (Fig. 5). Samples from normal kidney and renal clear cell carcinoma served as positive controls for both proteins. hCNT3 and ABC4 were detected in normal pancreatic tissue and pancreatic carcinoma, and their relative abundance varied strongly among the different tissue samples (Fig. 5A). The antisera against hCNT3 identified two major bands at 90 to 100 kDa, suggesting a higher specific band at 170 kDa. The hCNT3- and ABCC4-specific bands were detected in all the samples when the antisera was detected as a broad band at 170 kDa. The hCNT3- and ABCC4-specific signals were abolished in all the samples when the antisera was preincubated with the corresponding synthetic peptides before immunoblot analysis (data not shown).

In addition to immunoblot analysis, immunolocalization of both transporters was performed by immunofluorescence microscopy (Fig. 5, B–G). hCNT3 was localized in the basolateral and the apical membrane of the epithelia of ducts in normal pancreas and ductal pancreatic carcinoma (Fig. 5, B–D). However, ABC4 was predominantly localized only in the basolateral membrane of the ductular epithelia (Fig. 5, E–G), which is in accordance with a recently published study by König et al. (2005). In addition, hCNT3 and ABC4 were also localized in the plasma membrane of acinar cells.
Na+/K+-ATPase, a marker for the basolateral plasma membrane domain of epithelial cells, clearly colocalized with the basolateral staining of hCNT3 and ABCC4 in the ductular epithelia (Fig. 5, B–G). Preincubation of the antibodies with the corresponding synthetic peptides abolished the staining in the tissue cryosections (data not shown). Thus, these results show the coexpression of hCNT3 and ABCC4. Studies on expression and localization of the recombinant proteins by immunoblot and immunofluorescence microscopy confirmed that hCNT3 and hABCC4 are expressed in the apical and basolateral plasma membrane of the double-transfected MDCK cells, respectively (Fig. 1, C–N). Thus, the distinct localization of each transporter in different cell membrane domains of our cell system allows measurement of vectorial transport. This scenario reflects the in vivo state in some epithelial cells, and the differential membrane domain localization is also essential for the technically accurate transport measurements. Nevertheless, several nonepithelial tumor cells treated with nucleoside analogs showed coexpression of both transporters hCNT3 and ABCC4, or at least one of them, including leukemia cells (Schuetz et al., 1999; Molina-Arcas et al., 2003; Fotoohi et al., 2006; Peng et al., 2008; Guo et al., 2009) and solid tumor (Norris et al., 2005; Marechal et al., 2009) (Fig. 5).

For this aim, we established a stable single-transfected MDCK cell line expressing recombinant hCNT3 and a stable double-transfected MDCK cell line expressing recombinant hCNT3 together with ABCC4. Studies on expression and localization of the recombinant proteins by immunoblot and immunofluorescence microscopy confirmed that hCNT3 and hABCC4 are expressed in the apical and basolateral plasma membrane of the double-transfected MDCK cells, respectively (Fig. 1, C–N). Thus, the distinct localization of each transporter in different cell membrane domains of our cell system allows measurement of vectorial transport. This scenario reflects the in vivo state in some epithelial cells, and the differential membrane domain localization is also essential for the technically accurate transport measurements. Nevertheless, several nonepithelial tumor cells treated with nucleoside analogs showed coexpression of both transporters hCNT3 and ABCC4, or at least one of them, including leukemia cells (Schuetz et al., 1999; Molina-Arcas et al., 2003; Fotoohi et al., 2006; Peng et al., 2008; Guo et al., 2009) and solid tumor (Norris et al., 2005; Marechal et al., 2009) (Fig. 5).

The functionality of hCNT3 was examined in our MDCK-CNT3 transfectants. The MDCK-CNT3 cells were able to transport the known substrates cytidine, uridine, and 5-F-5'-dUrd (Fig. 2, B and C;
FIG. 5. Expression and immunolocalization of hCNT3 and ABCC4 in human normal and malignant pancreatic tissues. A, immunoblot analysis of hCNT3 and ABCC4 in homogenates (50 μg of total protein/lane) from normal pancreatic tissue and in pancreatic carcinoma and from human kidney, as a positive control. hCNT3 was detected with the NTS antiserum (anti-CNT3) as three major bands at 90 to 100 kDa, and ABCC4 was detected with the SNG antiserum (anti-ABCC4) as a broad band at 170 kDa. B–G, confocal laser scanning micrographs of cryosections from normal human pancreatic tissue (B, C, E, and F) and from human pancreatic ductal adenocarcinoma (D and G) stained with the NTS antiserum (B–D, green) and with the affinity-purified SNG antibody (E–G, green). The Na+/K+-ATPase (B–G, red) was used as a marker for the plasma membrane and the basolateral membrane domain of epithelial cells. B–D, localization of hCNT3 in the apical (a) and basolateral (b) membrane of the ductular epithelial cells (arrow) and in the plasma membrane of acinar cells (arrowhead). E–G, localization of ABCC4 in the basolateral membrane of the ductular epithelial cells (arrow) and in acinar cells (arrowhead). Colocalization of hCNT3 or ABCC4 (green) with the Na+/K+-ATPase (red) confirms the basolateral localization of these proteins (merged yellow color). Nuclei were stained with 4,6-diamidino-2-phenylindole (B–G, blue). Scale bars, 20 μm.
Table 1) (Ritzel et al., 2007). The kinetic analysis of these substrates revealed $K_m$ values in the micromolar range (Table 1), similar as described previously (Ritzel et al., 2001). We recently identified hCNT1 as a novel nucleoside transporter for the anticancer drug 5-azaCyd (Rius et al., 2009), a nucleoside analog, the transport of which has only been studied in cell systems with endogenous transporters (Huang et al., 2004; Rius et al., 2009). Our present work showed that hCNT3 also mediates 5-azaCyd uptake into the cells with a $K_m$ value of 147 $\mu$M (Fig. 2A; Table 1) and suggests that hCNT3 also mediates uptake of the structurally related drug 5-aza-2′dCyd (Table 2). In agreement with this notion, the expression of hCNT3 also resulted in a higher sensitivity of the cells to 5-azaCyd and 5-aza-2′-dCyd (Table 2). This finding is in line with the relatively broad substrate specificity of hCNT3. Because 5-azaCyd is an emerging epigenetic drug that has found increasing use for the treatment of myeloid leukemias and solid tumors (Silverman and Muffti, 2005; Appleton et al., 2007; Stresemann and Lyko, 2008), it will be interesting to determine whether the expression level of hCNT3 is relevant in the clinical response to 5-azaCyd.

The double-transfected polarized MDCK-CNT3/ABCC4 cells show an opposite localization as the transport proteins required for vectorial transport described previously, e.g., in the OATP1B3/ABCC2 double-transfected cells (Cui et al., 2001). In the work presented here, the uptake transporter hCNT3 was localized to the apical membrane of MDCK cells, whereas the export pump ABCC4 is localized to the basolateral membrane of MDCK cells (Fig. 1). Therefore, vectorial transport across these cells could be significant only from the apical to the basolateral compartment because both transporters mediate unidirectional transport (Figs. 3A and 4A). Indeed, vectorial transport of cytidine and 5-azaCyd by the MDCK-CNT3 and MDCK-CNT3/ABCC4 was detected significantly only from the apical to the basolateral membrane and in an $Na^+$-dependent manner, whereas transport from the basolateral to the apical chamber was insignificant (Fig. 3, B and C, and Fig. 4, B–D). As such, we describe here for the first time an in vitro cell model for measurements of the vectorial transport of nucleosides and nucleoside analogs by unidirectional transport proteins from the apical to the basolateral membrane.

A recent study reported that a single-transfected MDCK cell line expressing recombinant hCNT3 in the apical membrane showed increased apical-to-basolateral transport of cytidine (Errasti-Muragarren et al., 2007). However, the efflux transporters in the basolateral membrane of the cells were of endogenous origin. In contrast, our double-transfected cells express recombinant ABCC4 as an active $Na^+$-dependent efflux pump, able to transport substrates with high efficiency. Further studies are necessary to elucidate whether reduced glutathione might play a role in the efflux of derivatives of nucleosides and nucleoside analogs by ABCC4, as previously reported for other substrates (Lai and Tan, 2002) and especially for bile acids (Rius et al., 2003, 2008).

As expected, 5-azaCyd was significantly accumulated only in a sodium-dependent manner in the MDCK-CNT3 and MDCK-CNT3/ABCC4 cells and was detected only in the basolateral compartment of the MDCK-CNT3 and MDCK-CNT3/ABCC4 cells (Fig. 4, B–D). The detection of compounds in the basolateral compartment of MDCK-CNT3 cells indicated the presence of endogenous equilibrative or efflux transporters in the cells. MDCK cells are known to express a variety of endogenous transporters, and some of them have been identified as equilibrative uptake transporters, such as ENT1 (Hammond et al., 2004) and the canine efflux pump Abcc4 (Bartholomé et al., 2007). NBTH, which is an inhibitor of hENT1 at nanomolar concentrations and a micromolar inhibitor of hENT2 (Zhang et al., 2007), strongly increased the sodium-dependent intra-cellular accumulation of 5-azaCyd and cytidine in the MDCK-CNT3 and MDCK-CNT3/ABCC4 compared with MDCK-Co in the presence or absence of NBTH (Fig. 4). In contrast, the basolateral amount of 5-azaCyd and cytidine in the presence of NBTH was reduced compared with the absence of NBTH (Fig. 3C). Together, these data establish our MDCK-CNT3/ABCC4 cells as a model for vectorial transport from the apical to the basolateral membrane. In addition, the endogenous expression of the canine Entn in the MDCK cells reflects the ubiquitous expression of ENTs under physiological conditions. Thus, the apical-to-basolateral transport system may provide new insights into cell types that are clinically relevant. More importantly, coexpression of hCNT3 and ABCC4 could be shown in human pancreas and pancreatic carcinoma. The localization of hCNT3 in both plasma membrane domains of the pancreatic epithelial cells suggests a key role of the transporter in the basolateral membrane by taking up nucleoside-derived drugs from the blood side, whereas its expression in the apical membrane served a physiological function of uptake and salvage of nucleosides present on the luminal side of the epithelial ducts. Nucleoside analogs play an important role in the clinical management of pancreatic cancer, and it will be of interest to investigate the roles of hCNT3 and ABCC4 in therapy responses. It is of interest in this context that recent studies in patients with resected pancreatic adenocarcinoma undergoing chemotherapy with gemcitabine described hCNT3 as a marker for longer survival (Maréchal et al., 2009).


