Functional Characterization of a Nucleoside-Derived Drug Transporter Variant (hCNT3<sub>C602R</sub>) Showing Altered Sodium-Binding Capacity

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ABSTRACT

A novel cloned polymorphism of the human concentrative nucleoside transporter hCNT3 was described and functionally characterized. This variant consists of a T/C transition leading to the substitution of cysteine 602 by an arginine residue in the core of transmembrane domain 13. The resulting hCNT3<sub>C602R</sub> protein has the same selectivity and affinity for natural nucleosides and nucleoside-derived drugs as hCNT3 but much lower concentrative capacity. The insertion of the transporter into a polarized membrane seems unaffected in the variant. In a preliminary survey of a typical Spanish population, this variant showed an allelic frequency of 1%. The functional impairment of the hCNT3<sub>C602R</sub> polymorphism is attributable to the presence of an arginine rather than the loss of a cysteine at position 602, because an engineered hCNT3 protein with a serine residue at this position (hCNT3<sub>C602S</sub>) and hCNT3 have similar kinetic parameters. The sodium activation kinetic analysis of both transporters revealed a variation in the affinity for sodium and a shift in the Hill coefficient that could be consistent with a stoichiometry of 2:1 and 1:1 sodium/nucleoside, for hCNT3 and hCNT3<sub>C602R</sub>, respectively. In conclusion, the presence of an arginine residue in the core of transmembrane domain 13 is responsible for the different sodium affinity showed by the polymorphic transporter compared with the reference transporter. Individuals with the hCNT3<sub>C602R</sub> variant might show a lower nucleoside and nucleoside analog concentrative capacity, which could be clinically relevant.

Nucleoside transporters mediate the uptake of both natural nucleosides and most nucleoside-derived antitumoral drugs (Pastor-Anglada et al., 1998, 2005). There are two families of nucleoside carriers in mammalian cells, the equilibrative ENT/SLC29A transporters, with low affinity and wide selectivity, and the concentrative CNT/SLC28A transporters, with higher affinity and more restricted selectivity (Griffith and Jarvis, 1996; Baldwin et al., 1999). Because absorptive cells in the renal and intestinal epithelia express all members of the concentrative CNT family (Gutierrez and Giacomini, 1993; Ngo et al., 2001; Rodríguez-Mulero et al., 2005; Errasti-Murugarren et al., 2007), any change in their activity might be highly relevant in the pharmacokinetics of nucleoside-derived drugs.

CNT3 is the least well characterized concentrative nucleoside transporter. It shows wide substrate selectivity, accepting both purine and pyrimidine nucleosides, and has a 2:1 Na<sup>+</sup>/nucleoside coupling ratio, allowing it to concentrate nucleosides intracellularly 10 times more efficiently than CNT1 or CNT2, which show a 1:1 Na<sup>+</sup>/nucleoside stoichiometry (Ritzel et al., 2001). Its role in drug transport is not well known, being involved in the uptake of cladribine and fludarabine (Mangravite et al., 2003) and of cytosine arabinoside (Sarkar et al., 2005), as well as other non-nucleosidic drugs, such as the anthracine pirarubicin (Nagai et al., 2005). CNT3 has also been shown to transport gemcitabine and the antiviral drugs azidothymidine and ribavirin (Hu et
al., 2006; Yamamoto et al., 2007). Its location at the apical membrane of a polarized cell model (Mangravite et al., 2003; Errasti-Murugarren et al., 2007) together with its presence along the rat and human renal tubule (Rodríguez-Mulero et al., 2005; Damaraju et al., 2007) and human intestine (Ritzel et al., 2001) suggest that CNT3 plays an important role in the absorption and disposition of nucleosides and synthetic nucleoside analogs.

Because CNT3 seems such an important element in the disposition of physiological nucleosides and nucleoside analogs, changes that affect its activity might have very relevant clinical repercussions. Heterogeneous patient response to treatment could be explained, at least in part, by interindividual variability in the expression and/or activity of nucleoside transporters (Badagnani et al., 2005). An ex vivo analysis of nucleoside transporter activity and expression in patients with chronic lymphocytic leukemia showed that variation in the transport rates of fludarabine correlated with its chemosensitivity, with CNT3 expression being the most variable among individuals (Molina-Arcas et al., 2003). Badagnani et al. (2005) identified up to 56 variable sites in the coding and flanking regions of the CNT3 gene, SLC28A3, in a survey of 270 DNA samples from different U.S. populations (Badagnani et al., 2005). Ten of these coding variants resulted in amino acid changes, and three of them had total allele frequencies higher than 1%. The functional characterization of these variants suggested that CNT3 tolerates very little nonsynonymous change, at least in certain well defined positions (Badagnani et al., 2005).

Here, we report a new polymorphism of the human SLC28A3 gene, a T/C transition that consists of an amino acid change in the hCNT3 protein sequence, the cysteine at position 602 being changed to an arginine. In consequence, the resulting hCNT3 protein shows altered sodium binding capacity, bearing similar affinities for its substrates but much lower concentrative capacity. This variant, which shows an allelic frequency of 1% in a typical Spanish population, sheds some new light on the molecular mechanisms underlying hCNT3 function.

Materials and Methods

Plasmid Construction and Site-Directed Mutagenesis. A normal portion of a kidney bearing a renal carcinoma and removed at surgery, according to the ethics review board of the IUNA (Institut d’Urologia, Nefrologia i Andrologia—Fundació Puigvert), was used, when applicable, to calculate m values for uridine for the reference and C602R variants, respectively. Both primers annealed to the coding sequence in the arginine 602 region, and the corresponding reverse primer. The position of the codon for arginine 602, converted to a cysteine or to a serine residue, respectively, was underlined. The S575R substitution was introduced into hCNT1 using as a forward primer 5′-CTTCAGGGAGGCAGCTTCGTTCTCATGACAGCTGACGGCAGCTTCGTTCTCATGACAGCACCCTG-3′ or 5′-CTGATTGGGACCGCATTCCGTTCTCATGACAGCTGACGGCAGCTTCGTTCTCATGACAGCACCCTG-3′, respectively. Both primers annealed to the coding sequence in the arginine 575 region, and a compatible reverse primer. The position of the codon for serine 575 converted to an arginine residue was underlined. All constructions were verified by DNA sequencing in both directions and used for transient transfection.

Genotyping. Genomic DNA obtained from oral mucosa of 200 persons with their informed consent, with the use of a commercial kit (QIAGEN, Hilden, Germany), was a generous gift from Dr. Dolors Colomer (Hospital Clinic, IDIBAPS, Barcelona, Spain). Genotyping was performed using allele-specific TaqMan probes labeled with VIC and 5-carboxyfluorescein. Primers and probes were designed by the manufacturer with Primer Express software (Taqman Assays-on-Demand; Applied Biosystems, Foster City, CA). Amplification reactions were performed in optical 384-well plates, using 20 ng of DNA. After amplification, fluorescence was read in an ABI7700 sequence detector (Applied Biosystems). The presence of this variant was verified by DNA sequencing.

Cell Culture and Transfection. Human cervix carcinoma HeLa cells and Madin-Darby canine kidney epithelial cells (MDCK) were maintained at 37°C/5% CO2 in Dulbecco’s modified Eagle’s medium (Lonza Verviers SPRL, Verviers, Belgium) supplemented with 10% fetal bovine serum (v/v), 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM l-glutamine. HeLa cells were transiently transfected with the plasmid constructions described above using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and following the manufacturer’s protocol. Nucleoside transport, confocal microscopy, and flow cytometry analysis were carried out 24 h after transfection. MDCK cells were plated on transwell plates (12-mm diameter, 0.3-μm pore; Costar; Corning Life Sciences, Acton, MA) and transfected as described previously (Harris et al., 2004).

Nucleoside and Nucleobase Transport Assay. Uptake rates were measured as described previously (del Santo et al., 1998) by exposing replicate cultures at room temperature to the appropriate 3H-labeled nucleoside or nucleobase (1 μM, 1 μCi/ml; Moravek Biochemicals [Brea, CA] or GE Healthcare [Chalfont St. Giles, Buckinghamshire, UK]) in either a sodium-containing or a sodium-free transport buffer. Initial rates of transport were determined by an incubation of 1 min, and transport was terminated by washing in an excess of chilled buffer. Saturation kinetics were evaluated by nonlinear regression analysis, and the kinetic parameters derived from this method were confirmed by linear regression analysis. Sodium:nucleoside stoichiometry was calculated by measuring uridine uptake with varying concentrations of NaCl (0–100 mM) and then by analyzing the data with the Hill equation. Sodium concentrations were varied by adding the concentration of NaCl and choline chloride without changing the osmolality of the transport medium.

For inhibition kinetic studies cells were incubated with 1 μM [3H]-uridine (1 μCi/ml; GE Healthcare) and varying concentrations of unlabeled fludarabine (0–5000 μM), 5-fluorouridine (0–5000 μM), or cytarabine (0–10,000 μM). The Cheng-Prusoff equation was then used, when applicable, to calculate K values (Cheng and Prusoff, 1973), using calculated K values for uridine for the reference and polymorphic hCNT3 proteins (shown in Table 1).

Analysis of Nucleoside Transport and Nucleoside Vectorial Flux on Transwells. MDCK cells were grown on transwell filters and transfected, and uptake rates monitored, as described previously.
Membrane Insertion of hCNT3 and hCNT1 Proteins.

To determine whether the different hCNT3 and hCNT1 transporter variants were correctly inserted into the plasma membrane, confocal microscopy of GFP-fused chimeras was performed on a semi-confluent monolayer of transfected HeLa cells cultured on glass coverslips. Glass coverslip-grown cells were incubated with 1 μg/ml WGA-TRITC for 30 min at 4°C, rinsed three times in phosphate-buffered saline-Ca\(^{2+}\)-Mg\(^{2+}\), fixed for 15 min in 3% paraformaldehyde and 0.06 M sucrose, rinsed three times in phosphate-buffered saline, and then mounted with aqua-polymount coverslipping medium (Polysciences, Inc. Warrington, PA). Images were obtained using a laser-scanning confocal microscope (Fluoview 500; Olympus, Tokyo, Japan) equipped with helium-neon and argon lasers as the light source. The colocalization of GFP-fused chimeras with plasma membrane WGA-TRITC was determined by quantifying the extent of pixel colocalization of GFP with TRITC fluorescence using Meta-morph imaging software (Molecular Devices, Sunnyvale, CA), as described previously (Volpicelli et al., 2001; Murph et al., 2003). The background was subtracted from unprocessed images and the percentage of GFP pixels overlapping with TRITC pixels was measured. Data are presented as the mean ± S.E.M. of measurements from 10 cells per sample. Transfection efficiency and fluorescence intensity per cell were determined by flow cytometry using a flow cytometry system (Cytomics FC 500 MPL, Beckman Coulter, Fullerton, CA).

To analyze the polarized membrane insertion of both hCNT3 proteins, 1.7 × 10\(^5\) MDCK cells were grown in 12-well Costar polycarbonate Transwell filter inserts (Corning Life Sciences) for 24 h and then transfected as explained previously. Cells were fixed as described above, and then filters were excised and loaded onto a glass slide and covered with a coverslip. Between the slide and the coverslip, a 1-mm gap was filled with aqua-polymount coverslipping medium (Polysciences, Inc. Warrington, PA). Actin was stained using phalloidin-TRITC. Images were obtained as described above.

**TABLE 1**

<table>
<thead>
<tr>
<th>Nucleosides</th>
<th>hCNT3</th>
<th>hCNT3C602R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K(_m)</td>
<td>V(_{max})</td>
</tr>
<tr>
<td>Uridine</td>
<td>5.3 ± 1.2</td>
<td>484.6 ± 15.6</td>
</tr>
<tr>
<td>Cytidine</td>
<td>3.5 ± 0.4</td>
<td>401.7 ± 30.8</td>
</tr>
<tr>
<td>Thymidine</td>
<td>10.6 ± 2.2</td>
<td>645.2 ± 29.6</td>
</tr>
<tr>
<td>Adenosine</td>
<td>2.4 ± 1.1</td>
<td>579.2 ± 34.5</td>
</tr>
<tr>
<td>Guanosine</td>
<td>8.5 ± 2.2</td>
<td>458.5 ± 33.4</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.E.M. of measurements from 10 cells per sample. The cells on the filters were solubilized by 0.1% SDS and 100 mM NaOH. Support, and counted on a scintillation counter. The cells on the filters were washed in chilled buffer. The whole filter was wiped out and then aspirated the buffer, and filters were washed in chilled buffer. The whole filter was wiped out with tissue to remove any excess buffer, removed from the plastic support, and counted on a scintillation counter. The cells on the filters were solubilized by 0.1% SDS and 100 mM NaOH.

**Results**

hCNT3 cDNA was amplified from a kidney sample. When sequenced, an unexpected change was identified [a T/C transition at position 1804 (position relative to the ATG start site and based on the cDNA sequence)] resulting in a nonconservative replacement of cysteine 602 by an arginine). This variation is located at TMD13 of the transporter protein. As shown in Fig. 1, this cysteine is conserved among human, rat, mouse, and hagfish orthologs. Thus, this variation affects a highly conserved cysteine residue in the core of the TMD13. Because this change might have functional implications, we decided, first, to study whether the T/C variation (C602R) of the cloned transporter corresponded to a variant present in a typical Spanish population. The genotype analysis showed this variant to be present in two chromosomes of the 400 analyzed, thus yielding an allelic frequency of 1%, a value similar to that previously reported for other hCNT3 variants (Badagnani et al., 2005).

Second, we addressed whether this variant could affect the hCNT3-related function. To do so, we expressed the hCNT3C602R variant as well as the reference counterpart (hCNT3), corresponding to the published sequence with GenBank accession number AF305210. Figure 2 shows the sodium-dependent uptake rates of a panel of natural nucleosides (uridine, cytidine, thymidine, guanosine, and adenosine) and a nucleobase (hypoxanthine) measured in hCNT3- and hCNT3C602R-transfected HeLa cells. Both isoforms had identical selectivity, similar to that described previously (Ritzel et al., 2001), although transport rates were significantly lower in the polymorphic than in the reference transporter. When cysteine 602 was mutated to a serine, the engineered protein showed the same selectivity and transport rates as those found for hCNT3 (data not shown), suggesting that the presence of arginine at position 602, rather than the lack of cysteine, was responsible for the lower transport rate shown by the hCNT3C602R variant. Mutation of the equivalent residue in the other pyrimidine nucleoside-transporting isofrom, hCNT1, completely blocked the transport of all the nucleosides assayed, even when used at much higher concentrations (Fig. 2B).

Concentration-dependence curves for uridine, cytidine, thymidine, guanosine, and adenosine uptake in hCNT3- and hCNT3C602R-transfected HeLa cells, measured as initial rates of transport (1 min), were determined. The concentration-dependent uptake by both isoforms was saturable and conformed to Michaelis-Menten kinetics. Kinetic parameters, apparent K\(_m\) and V\(_{max}\), were calculated by fitting the transport data to the Michaelis-Menten equation, and the resulting values were confirmed by linear regression. The mean ± S.E.M. of triplicate determinations. The data were analyzed by nonlinear regression using GraphPad Prism software. Data are expressed as the mean ± S.E.M. of measurements from 10 cells per sample.
S.E.M. values (n = 3) for $K_m$ and $V_{\max}$ for both transporters are shown in Table 1. Thus, in the case of the polymorphic variant, the apparent $K_m$ for all the assayed natural nucleosides was identical to that observed for hCNT3, but $V_{\max}$ was nearly 4-fold lower than that found for the reference transporter. Kinetic parameters of hCNT3$_{C602R}$ were not significantly different from those obtained for hCNT3 (not shown).

We next investigated whether the reduced activity of the hCNT3$_{C602R}$ and the null activity of the mutated hCNT1 were due to impaired insertion into the membrane. Transiently transfected HeLa cells expressing GFP-fused chimeras of hCNT3, hCNT3$_{C602R}$, hCNT1, or hCNT1$_{S575R}$ were incubated with the plasma membrane marker WGA-TRITC for 30 min at 4°C to avoid Golgi staining. All the constructions showed a similar subcellular pattern (Fig. 3), being located predominantly at the plasma membrane and, to a lesser extent, at the Golgi complex, which may represent a newly synthesized transporter en route to the plasma membrane. To quantify GFP-fused transporter membrane insertion, we measured colocalization with the plasma membrane WGA-TRITC, using Metamorph image analysis (Volpicelli et al., 2001; Murph et al., 2003). All the constructions showed similar colocalization percentages (Table 2), and flow cytometry showed the same transfection rate and fluorescence intensity for both pairs of transporters (hCNT3 versus hCNT3$_{C602R}$ and hCNT1 versus hCNT1$_{S575R}$). Taken together, these results indicate that insertion into the membrane was unaltered in both engineered transporters.

To determine whether this hCNT3 variant could show altered sodium binding capacity, the initial rate of sodium-dependent uridine uptake (1 mM) was determined as a function of extracellular sodium concentration (0–100 mM) in HeLa cells transfected with cDNA encoding either hCNT3 or hCNT3$_{C602R}$. Figure 4 shows that the relationship for hCNT3 between uridine influx and extracellular sodium concentration was sigmoidal, whereas the sodium-dependent uridine uptake mediated by the hCNT3$_{C602R}$ was hyperbolic. Hill coefficients derived from these data were 2.2 ± 0.17 and 0.74 ± 0.2 for hCNT3 and its variant, respectively, whereas $K_0.5$ values for Na$^+$ were 18.04 ± 1.38 and 3.38 ± 1.02 mM, respectively. These results would suggest a sodium/nucleoside stoichiometry for hCNT3 of 2:1, whereas the hCNT3 variant would display a 1:1 coupling ratio.

Because hCNT3 is an apically located transporter in epithelia and its expression is known to determine nucleoside vectorial flux (Errasti-Murugarren et al., 2007) we proceeded to determine 1) whether the variant could be inserted in the same way into the membrane in a polarized manner and 2) whether this would affect vectorial flux of nucleosides across the epithelial barrier. Transfection of cDNA encoding GFP-fused transporter constructs showed that both hCNT3 and hCNT3$_{C602R}$ were inserted at the apical membrane of polarized MDCK cells grown in a transwell dish (data not shown), thus indicating that polymorphism did not affect proper insertion into a polarized membrane. Because of the important role of the hCNT3 in the transepithelial flux of nucleosides (Errasti-Murugarren et al., 2007), the vectorial flux of uridine was studied by using MDCK cells transfected with cDNA encoding either hCNT3 or hCNT3$_{C602R}$ or empty pcDNA3.1 vector, to analyze the effect of the variation on the net uridine flux across the epithelial barrier. Figure 5 shows the sodium-dependent transepithelial flux of uridine (A) and the intracellular accumulation (B) in hCNT3-, hCNT3$_{C602R}$-, and mock-MDCK cells. As expected, both vectorial flux and intracellular accumulation were significantly lower in epithelia expressing the polymorphic variant than in cells expressing the reference hCNT3. Nevertheless, a net sodium-dependent transepithelial flux and accumulation of uridine were still established in the presence of this variant.

We also examined the functional consequences of the variant using the antileukemic analogs fludarabine and cytarabine and the antineoplastic drug 5-fluorouridine. Figure 6 shows the uptake of 1 mM $[^3]$H]uridine in the presence of increasing concentrations of fludarabine (top), 5-fluorouridine (middle), or cytarabine (bottom) measured in hCNT3 and hCNT3$_{C602R}$ expressing HeLa cell line. The $K_i$ values revealed no differences in the interaction affinity of the assayed nucleoside analogs for both hCNT3 and the polymorphic variant.
Discussion

CNT3 plays an important role in the uptake and bioavailability of both natural and modified nucleosides as a result of its broad substrate selectivity and sodium/nucleoside stoichiometry (2:1 for CNT3 compared with 1:1 for CNT1 and CNT2), which allows concentrating its substrates 10-fold higher than the rest of the concentrative nucleoside transporters (Ritzel et al., 2001). Because of the capital role of CNT3 in maintaining the homeostasis of endogenous nucleosides, sequence variations affecting transporter activity could be relevant, not only because they might provide new insights into the structure-function relationship of the transporter protein, but also because these variants might have pathophysiological implications.

Human CNT3 cDNA is 2210 base pairs long (GenBank accession number AF305210) and encodes a protein of 691 amino acids. At the level of amino acid sequence, human CNT3 is 77% identical to rat CNT3, 78% identical to mouse CNT3, and 57% identical to hagfish CNT. Residues within TMDs 4 to 13 are particularly highly conserved among hCNT3 and other orthologs, suggesting that these regions contain the functional core of the proteins. The C602R variant identified herein results in a radical chemical change, with a Grantham value of 180 (Grantham, 1974), and is found at the core of transmembrane domain 13. This transmembrane domain may play a crucial role in transporter activity, as suggested by our results. When this variation was introduced into the hCNT1 transporter (hCNT1_{S575R}), an hCNT3 paralog, the resulting mutated transporter failed to transport any of the assayed substrates, which further supports the view that this TM domain is also important for hCNT1 function and is consistent with previous structure-function data, suggesting that these TMDs are relevant for substrate recognition and translocation (Zhang et al., 2006).

Because this variant was identified by serendipity, we were interested in assessing its occurrence in humans. A preliminary genomic analysis in a typical Spanish population revealed a T/C transition at position 1804 (leading to a non-conservative C602R substitution), a hitherto unreported polymorphic variant. It was detected in two chromosome of the 400 analyzed, thus yielding an allelic frequency for the C602R variant of 1%, similar to that described previously by Bagdanani et al. (2005) for some nonsynonymous hCNT3 variants. This variant was not identified in any of the previous studies on hCNT3 sequence analysis (Badagnani et al., 2005; Damaraju et al., 2005) suggesting a population specific incidence, as observed for other variants. The C602R change affected an evolutionarily conserved residue, which is also conserved in its orthologs in equivalent positions. The variant C602R has a reduced functional transport capability for

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Table 2

<table>
<thead>
<tr>
<th>Construction</th>
<th>Transfection Efficiency S.E.M.</th>
<th>Fluorescence Intensity S.E.M.</th>
<th>Colocalization S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCNT3</td>
<td>77.87</td>
<td>236.6</td>
<td>82.59</td>
</tr>
<tr>
<td>hCNT3_{C602R}</td>
<td>78</td>
<td>241.47</td>
<td>80.12</td>
</tr>
<tr>
<td>hCNT1</td>
<td>80.65</td>
<td>208.5</td>
<td>77.84</td>
</tr>
<tr>
<td>hCNT1_{S575R}</td>
<td>78.65</td>
<td>202.63</td>
<td>77.84</td>
</tr>
</tbody>
</table>

AU, arbitrary units.
both purine and pyrimidine nucleosides, although the affinity for both natural and derived nucleosides was identical in both transporters. It also resulted in a reduced transepithelial flux when transfected into a polarized model (MDCK) compared with reference transporter, as well as in a reduced intracellular accumulation. These observations are consistent with findings that hCNT3 is under strong negative selection, as deduced from the few nonsynonymous variants it presents, most of which are singletons (Badagnani et al., 2005) and because changes at evolutionarily conserved residues are most likely to be deleterious to protein function (Miller and Kumar, 2001; Leebman et al., 2003). Because of its broad selectivity and high concentrative capacity and its presence in absorptive epithelia-like intestine (Ritzel et al., 2001) and renal tubules (Rodríguez-Mülero et al., 2005; Damaraju et al., 2007), CNT3 could play a pivotal role in nucleoside homeostasis and in the pharmacokinetics of nucleoside-derived drugs.

The expression of this transporter variant may result in reduced intestinal absorption as well as defective renal reabsorption of nucleosides, both natural and synthetic, that are effectively transported by CNT3. However, the possibility that the other two CNT proteins would compensate for a decreased efficiency of CNT3 function cannot be ruled out but could not be tested because we genotyped a blind genomic DNA biobank.

Biochemical evidence was provided showing that the decreased $V_{\text{max}}$ of the hCNT3 variant was the result of altered sodium binding capacity, because expression at the plasma membrane was unaffected. The polymorphic variant was also inserted into the proper membrane compartment when studied in a polarized epithelial cell line such as MDCK, showing an apical localization as described previously (Mangravite et al., 2003). The sodium dependence analysis of uridine uptake revealed a sigmoidal kinetics for hCNT3, whereas this relationship was hyperbolic for hCNT3$_{C602R}$. The Hill coefficient for hCNT3 was similar to those described previously (close to 2) (Ritzel et al., 2001; Toan et al., 2003), thus consistent with a 2:1 sodium/nucleoside stoichiometry. However, this coefficient for hCNT3$_{C602R}$ and the $K_m$ for sodium pointed toward a shift from a 2:1 to a 1:1 sodium/nucleoside concentrative nucleoside transporter, similar to the behavior of hCNT1, for which a 1:1 stoichiometry has been reported (Gutierrez and Giacomini, 1993). Thus, the lower concentrative ability of the polymorphic C602R variant compared with the hCNT3 could

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**Fig. 4.** Sodium dependence of influx of uridine mediated by recombinant hCNT3 and hCNT3$_{C602R}$. Transporter-mediated uptake of $[^3H]$uridine (1 μM, 1 min) by hCNT3 (A) and hCNT3$_{C602R}$ (B) was measured in transport medium containing 0–100 mM NaCl using choline chloride to maintain isosmolality. Mediated transport was calculated as uptake in transporter transfected cells minus mock transfected cells in which uridine uptake is not sodium-dependent. Inset, Hill plots of hCNT3 and hCNT3$_{C602R}$ data, respectively. $K_m$ values and Hill coefficients ($n$) were 18.04 ± 1.38 mM and 2.2 ± 0.17, respectively, for hCNT3 and 3.38 ± 0.12 mM and 0.74 ± 0.2 for the polymorphic transporter. Data are expressed as the mean ± S.E.M. of three experiments carried out on different days on different batches of cells.

**Fig. 5.** Transepithelial flux of uridine in MDCK cells transfected with cDNA encoding either hCNT3 or hCNT3$_{C602R}$ or empty pcDNA3.1. (A) Sodium-dependent transepithelial flux of $[^3H]$uridine (1 μM) in MDCK cells transfected with cDNA encoding either hCNT3 (■), polymorphic transporter (▲), or empty pcDNA3.1 (▼) from the apical to the basolateral compartment. (B) Sodium-dependent intracellular accumulation of uridine measured in hCNT3- (open bar), hCNT3$_{C602R}$- (close bar), and mock- (striped bar) transfected MDCK-cells after 20 min. Data are expressed as the mean ± S.E.M. of uptake values obtained in three wells or filter inserts. Data are representative of three experiments carried out on different days on different batches of cells.
be due to its conversion into a 1:1 sodium/nucleoside transporter, which in principle should be 10-fold less concentrative than a 2:1 carrier. However, another possible explanation was that this variant could be affecting $V_{\text{max}}$ for reasons other than a change in stoichiometry. Maybe with an increased affinity for sodium, a transport cycle takes longer because it takes a longer time for the sodium to dissociate from the binding site. At steady state, uptake is determined by the balance between influx and leak back out of the cell. If influx is decreased and leak is unchanged, then net steady-state uptake will be decreased. Smith et al. (2005) showed that hCNT3 is functionally active in the presence of a proton gradient across the plasma membrane with a proton/nucleoside stoichiometry of 1:1, and it seems that both sodium and proton binding sites are located in the same region of hCNT3. Their results suggest that hCNT3 might translocate substrates in a 1:1 sodium/nucleoside coupling ratio; such a possibility is also suggested here by analysis of this novel variant of hCNT3. Thus, these results could be consistent with a sequential mechanism of transport in which sodium binds to the transporter first, increasing its affinity for the nucleoside, which then binds to the transporter (Jauch and Lauger, 1986; Klamo et al., 1996; Mackenzie et al., 1996). They also suggest that the second sodium ion that binds CNT3 is involved in increasing the concentrative ability of the transporter by enhancing its $V_{\text{max}}$.

Taking all our results together, it seems that the presence of arginine residue in the core of TMD13 would be responsible for the altered sodium binding capacity of the hCNT3<sub>C602R</sub> variant. When the cysteine at position 602 was mutated into a serine, kinetic parameters of this hCNT3<sub>C602S</sub>-engineered protein were similar to those obtained for hCNT3, thus suggesting that the feature responsible for low activity was the presence of the arginine residue rather than the lack of the cysteine residue at position 602. This observation suggested that it was the presence of a positive charge (arginine at physiological pH is protonated) inside the TMD13 helix rather than the lack of cysteine that caused the reduced activity, either because of an electrostatic effect or to structure changes, or both. The lack of a crystal structure for hCNT3 or any other CNT protein makes it difficult to advance further in this hypothesis. Yamashita et al. (2005), working with the bacterial homolog of the mammalian Na<sup>+</sup>/Cl<sup>−</sup> neurotransmitter transporter, described the presence of two separate sodium-binding sites, each formed by several amino acids from various TM domains that spatially composed a sodium-binding pocket. Considering a similar model for hCNT3, a positive charge near one of the two sodium-binding sites could result in electrostatic repulsion between the sodium ion and the arginine residue. Alternatively, the presence of arginine at position 602 could compromise the TMD13 structure, affecting one of the sodium binding sites and thus preventing sodium from binding to it. These data were consistent with a recent finding (Zhang et al., 2006) that suggests an important structural role for this TM segment as well as a possible role in the recognition of substrates. The possibility that TMD13 is involved, at least in part, in sodium recognition is also consistent with recent data suggesting that structural determinants of sodium/nucleoside stoichiometry reside in the C-terminal half of the protein (Smith et al., 2005).

In summary, the present study reports the existence of a polymorphic variant of the human concentrative nucleoside transporter CNT3 (the C602R substitution) that severely affects its functionality and that could be of physiological and pharmacological relevance. A single substitution in TMD13 results in reduced $V_{\text{max}}$ and impaired vectorial flux and accumulation of nucleosides across epithelia. This variant also highlights the important role that TMD13 might play in sodium binding and translocation. Further genotyping of hu-
man populations is required to figure out to what extent this variant is abundant in different ethnic groups or in patients with particular diseases.

Acknowledgments

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5: 22–23.

1504–1511.

1149: 202–208.

1356: 32678–32683.

1150: 1549–1553.

3099–3108.

326: 398–403.

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