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The Human Concentrative Nucleoside Transporter-3 C602R Variant Shows Impaired Sorting to Lipid Rafts and Altered Specificity for Nucleoside-Derived Drugs[S]

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ABSTRACT

The human concentrative nucleoside transporter-3 C602R (hCNT3C602R), a recently identified human concentrative nucleoside transporter-3 (hCNT3) variant, has been shown to interact with natural nucleosides with apparent K_m values similar to those of the wild-type transporter, although binding of one of the two sodium ions required for nucleoside translocation is impaired, resulting in decreased V_{max} values (*Mol Pharmacol* **73:**379–386, 2008). We have further analyzed the properties of this hCNT3 variant by determining its localization in plasma membrane lipid domains and its interaction with nucleoside-derived drugs used in anticancer and antiviral therapies. When expressed heterologously in HeLa cells, wild-type hCNT3 localized to both lipid raft and nonlipid raft domains. Treatment of cells with the cholesterol-depleting agent methyl-β-cyclodextrin resulted in a marked decrease in hCNT3-related transport activity that was associated with the loss of wild-type hCNT3 from lipid rafts. It is noteworthy that although exogenously expressed hCNT3C602R was present in nonlipid raft domains at a level similar to that of the wild-type transporter, the mutant transporter was present at much lower amounts in lipid rafts. A substrate profile analysis showed that interactions with a variety of nucleoside-derived drugs were altered in the hCNT3C602R variant and revealed that sugar hydroxyl residues are key structural determinants for substrate recognition by the hCNT3C602R variant.

Cellular uptake of nucleoside analogs, including those used for anticancer and antiviral therapies, depends on the activity of sodium-coupled nucleoside transporters of the *SLC28* family. One such transporter, human concentrative nucleoside transporter-3 (hCNT3/*SLC28A3*), plays an important role in the absorption and disposition of nucleosides and synthetic nucleoside analogs. hCNT3 accepts both natural and chemically modified purines and pyrimidines, shows a 2:1 sodium/nucleoside coupling ratio, and is localized to the apical membrane of renal tubule epithelial cells (Ritzel et al., 2001; Damaraju et al., 2007; Errasti-Murugarren et al., 2007).

Response to therapy with nucleoside-derived drugs shows interindividual variability that can be accounted for by genetic variants of the transporters and enzymes implicated in drug uptake and activation. Concentrative nucleoside transporters in general tend to be highly conserved, and this is particularly true for hCNT3, suggesting that this transporter could be biologically more important than the two other *SLC28* family members (Badagnani et al., 2005). For this reason, and because hCNT3 may be an important determinant of whole-body nucleoside homeostasis and nucleoside-derivative disposition, genetic polymorphisms that affect the biological activity of hCNT3 can have clinical repercussions.

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ABBREVIATIONS: hCNT3, human concentrative nucleoside transporter 3; HA, hemagglutinin; MDCK, Madin-Darby canine kidney cells; MES, 2-(*N*-morpholino)ethanesulfonic acid; 5′-DFUR, 5′-deoxy-5-fluorouridine; MβCD, methyl-β-cyclodextrin; AZT, azidothymidine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; hENT, human equilibrative nucleoside transporter; PAGE, polyacrylamide gel electrophoresis; hCNT3C602R, human concentrative nucleoside transporter-3 C602R.

In this regard, we have characterized recently a novel, nonsynonymous polymorphism, C602R, that is located in the middle of the hCNT3 transmembrane domain 13 and shows an allelic frequency of 1% in a typical Spanish population (Errasti-Murugarren et al., 2008). As a consequence of this substitution, the resulting hCNT3 protein shows changes in kinetic behavior consistent with a shift from a 2:1 to a 1:1 substrate translocation stoichiometry and unaltered affinity for natural substrates (Errasti-Murugarren et al., 2008). The profound effects of this substitution predict a central role for this transmembrane domain in transporter function (Zhang et al., 2006; Errasti-Murugarren et al., 2008).

Transmembrane domains play an important role in substrate binding and recognition as well as in the stabilization of protein structure and subcellular localization of mature proteins (Lai et al., 2005; Zhang et al., 2006; Yao et al., 2007). The role of transmembrane domains in targeting proteins to particular plasma membrane lipid microdomains has been studied widely (Coffin et al., 2003; Zhang et al., 2003a; Barman et al., 2004). Localization of nucleoside transporters to lipid rafts in apical membranes of human syncytiotrophoblasts has been reported recently for hCNT1 and hENT1 (E. Errasti-Murugarren, P. Díaz, V. Godoy, G. Riquelme, and M. Pastor-Anglada, unpublished results), although raft-targeting determinants remain unknown. These observations indicate that nucleoside transporter proteins are not only heterogeneously distributed between apical and basal domains in polarized epithelia (Mangravite et al., 2001, 2003) but are also heterogeneously distributed within the apical domain. Although the physiological significance of this nonuniform distribution is not yet clear, the differential subcellular distribution of nucleoside transporters might affect their biological function.

Here, we report a novel dual effect of the C602R polymorphism on hCNT3 biological function, showing that it affects both selectivity for nucleoside analogs and plasma membrane localization. In particular, the hCNT3C602R variant shows reduced affinity for several nucleoside derivatives and impaired targeting to plasma membrane lipid rafts. These observations highlight the concept that particular residues in a membrane transporter can differentially alter substrate specificity and impair appropriate localization to membrane domains that foster optimal transporter activity.

Materials and Methods

Reagents. [5,6-^{3}H]Uridine (35–50 Ci/mmol) was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Radiolabeled nucleoside-derived drugs were purchased from Moravek Biochemicals (Brea, CA). Uridine, cytidine, adenosine, thymidine, nucleoside derivatives, and methyl-β-cyclodextrin (MβCD) were obtained from Sigma-Aldrich (St. Louis, MO).

Plasmid Construction and Site-Directed Mutagenesis. A 2.2-kilobase fragment of human renal CNT3 (hCNT3) was amplified from a kidney sample as described previously (Errasti-Murugarren et al., 2007). The QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) was used to convert codons for Arg602 in cloned hCNT3C602R to lysine and serine residues, according to the manufacturer's protocol. Green fluorescent protein- and hemagglutinin (HA)-tagged constructs of hCNT3C602R in pcDNA3.1 (Errasti-Murugarren et al., 2009) were used as templates. The R602K and R602S substitutions were introduced into hCNT3C602R using the forward primers 5′-CTG ATT GCG GGG ACC GTG GCC AAA TTC ATG ACA GCC TGC ATC G-3′ and 5′-CTG ATT GCG GGG ACC GTG GCC AGC TTC ATG ACA GCC TGC ATC G-3′, respectively, and a compatible reverse primer. Both primers annealed to the coding sequence in the Arg602 region; the underlined positions show the location of the Arg602 codon in the C602R polymorphism and the introduced sequence that converts it to a lysine or serine residue, respectively. Constructs were verified by DNA sequencing in both directions before using them in transient transfections.

Cell Culture and Transfection. HeLa cells were maintained at 37°C in a humidified 5% CO_2 environment in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (v/v), 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. HeLa cells were transiently transfected with the plasmid constructs described above using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described by the manufacturer. Nucleoside transport analyses were carried out 24 h after transfection. For cytotoxicity assays, HeLa cells were transfected using calcium phosphate.

MDCK cells, maintained as described for HeLa cells, were plated on 12-mm diameter, 0.3-μm pore Transwell plates (Corning Life Sciences, Lowell, MA) and transfected as described previously (Harris et al., 2004). In all cases, transfection efficiencies and expression of both constructions was similar, as reported previously (Errasti-Murugarren et al., 2008).

Nucleoside Uptake Measurements. Uptake rates were measured as described previously (del Santo et al., 1998). In brief, replicate cultures were exposed at room temperature to the appropriate ^{3}H-labeled uridine (1 μM, 1 μCi/ml) or nucleoside-derived antineoplastic or antiviral drugs [gemcitabine, fludarabine, 5′-deoxy-5-fluorouridine (5′-DFUR), azidothymidine (AZT), or ribavirin; 1 μM, 3 μCi/ml] in a sodium-replete (137 mM NaCl) or sodium-free (137 mM choline chloride) transport buffer containing 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM HEPES, pH 7.4. Initial rates of transport were determined using an incubation period of 1 min, and transport was terminated by washing with an excess volume 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 of the derived Eadie-Hofstee plots. To assess whether a selected nucleoside or nucleoside derivative interacted with the hCNT3 transporter protein and to determine IC_{50} values, we monitored sodium-dependent uridine uptake in the presence of increasing concentrations of the putative inhibitor. The Cheng-Prusoff equation was then used to calculate K_i values as described previously (Wallace et al., 2002).

Analysis of Nucleoside-Derived Drug Transport and Vectorial Flux in Transwells. MDCK cells were grown on Transwell filters, and uptake rates were monitored 48 h after transfection, as described previously (Harris et al., 2004; Errasti-Murugarren et al., 2007). In brief, filter inserts were washed three times with sodium-replete or sodium-free buffer, and then 1 μM (3 μCi/ml) ^{3}H-nucleoside-derived antineoplastic or antiviral drug (gemcitabine, fludarabine, 5′-DFUR, AZT, and ribavirin) was added to the apical side. Transport experiments were conducted using buffer containing either sodium or choline on both sides of the Transwell filters (0.5 ml in both apical and basal compartments). At various time points (up to 20 min), 50 μl of buffer was collected from the basal compartment. The transport experiments were terminated by aspirating the buffer and washing filters with chilled buffer. The entire filter was wiped with tissue to remove excess buffer, removed from the plastic support, and counted on a scintillation counter. The cells on the filters were solubilized with 0.1% SDS/100 mM NaOH.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide Cytotoxicity Assays. HeLa cells were seeded at a density of 10^4 cells/cm^2 in 96-well culture plates and transfected with hCNT3 or hCNT3C602R cDNAs using calcium phosphate. Twenty-four hours after transfection, cells were exposed to increasing concentrations of fludarabine (1–150 μM) or 5′-DFUR (10 μM to 10 mM) for 90 min. Cultures were then incubated for 48 h, and cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazo-

lium bromide (MTT) assay. After removing medium, 100 μl of MTT reagent (7.5 mg/ml) was added to each well, and plates were incubated for 45 min at 37°C. The MTT reagent was then discarded and the dark blue formazan crystals were solubilized in dimethyl sulfoxide. The absorbance of formazan was measured spectrophotometrically at 595 nm using a microplate reader. The number of surviving cells is directly proportional to the quantity of the formazan product generated. Data were fitted to a dose-response curve using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA) to obtain IC_{50} values of the fludarabine and 5′-DFUR.

Isolation of Lipid Rafts and Western Blot Analysis. HeLa cells transfected with HA-tagged hCNT3 or hCNT3C602R were trypsinized, washed with ice-cold phosphate-buffered saline, and centrifuged at 1200 rpm for 4 min. The cell pellet was resuspended and lysed with ice-cold MBS buffer (25 mM MES and 150 mM NaCl, pH 6.5) containing 0.5% Triton X-100 and a protease inhibitor cocktail (Complete MINI; Roche Diagnostics, Indianapolis, IN). Cells were then disrupted by passing through a 25-gauge needle 30 times. The resulting cell extract was adjusted to 40% sucrose in MBS buffer, placed in a 12-ml centrifuge tube, and then overlaid successively with 2 ml each of 35, 25, 15, and 5% sucrose in MBS buffer. After centrifugation at 39,000 rpm for 20 h at 4°C, 11-ml gradient fractions were collected. Aliquots (20 μl) of sucrose gradient fractions from hCNT3- and hCNT3C602R-transfected HeLa cells were separated by SDS-PAGE on 10 to 12% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA). After incubation with anti-HA high-affinity antibody (diluted 1:2000), proteins were detected using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit (GE Healthcare). Blots were analyzed densitometrically using Quantity One software (Bio-Rad Laboratories, Hercules, CA). For cholesterol depletion assays, transfected cells were first treated with increasing concentrations of MβCD for 30 min at 37°C before trypsinization and fractionation.

Data Analysis. 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 using different batches of cells.

Results

Effect of the C602R Substitution on hCNT3 Transport Activity and Interaction with Nucleoside Analogs. As noted above, the hCNT3C602R variant has the same selectivity and affinity for natural nucleosides as does hCNT3, but it has a much lower transport capacity (Errasti-Murugarren et al., 2008). To address whether this substitution affects hCNT3 interaction with some commonly used antiviral and antineoplastic nucleoside-derived drugs, we measured drug uptake in HeLa cells transiently transfected with expression constructs of either hCNT3 (GenBank accession number AF305210) or hCNT3C602R. Figure 1A shows the rates of sodium-dependent uptake of a selection of nucleoside-derived drugs (gemcitabine, 5′-DFUR, fludarabine, AZT, and ribavirin) and a natural nucleoside (uridine) 24 h after transfection. Expression of the wild-type transporter resulted in sodium-dependent nucleoside transport activity with broad selectivity, similar to that described previously and consistent with hCNT3-type transporters (Ritzel et al., 2001; Hu et al., 2006). The hCNT3C602R transporter variant showed lower transport rates, as described previously (Errasti-Murugarren et al., 2008). In contrast to results obtained with natural nucleosides, the magnitude of this decrease (23, 0.25, 22, 13, and 44% of hCNT3 activity for gemcitabine, 5′-DFUR, fludarabine, AZT, and ribavirin, respectively) was not similar among nucleoside-derived drugs (Fig. 1A). In fact, whereas 5′-DFUR uptake by hCNT3 was the highest among all substrates tested, 5′-

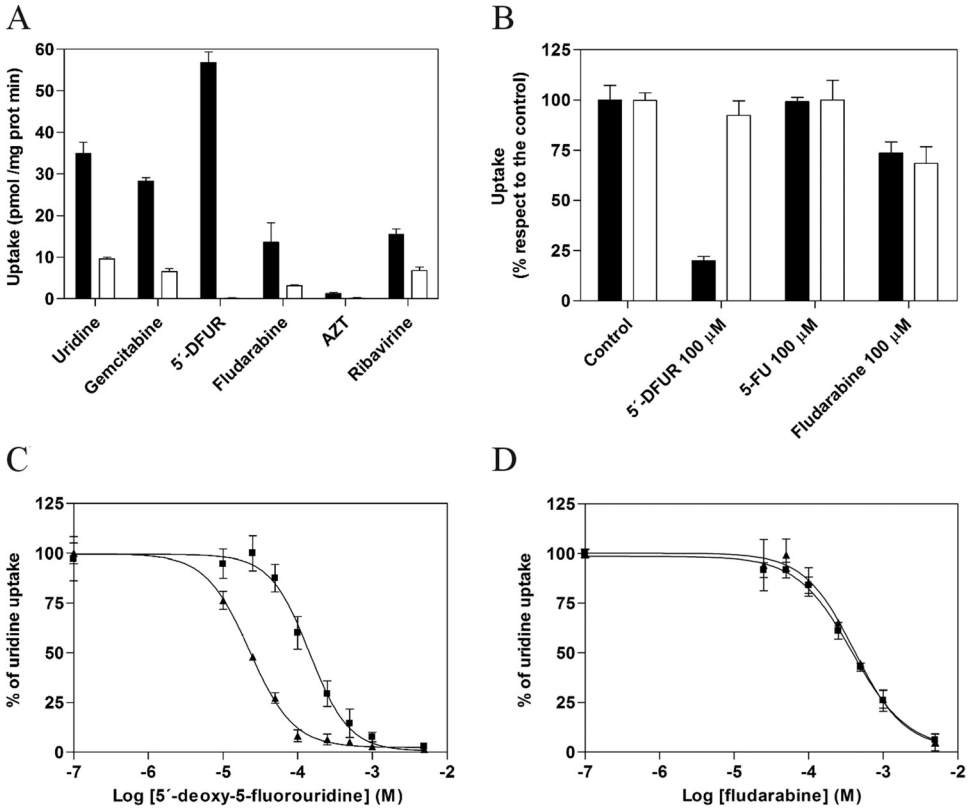

Fig. 1. Sodium-dependent uptake and interaction of nucleoside-derived drugs with recombinant hCNT3 and hCNT3C602R transporters expressed in HeLa cells. A, uptake of ^{3}H-labeled nucleoside-derived drugs and uridine (1 μM, 1 min) by hCNT3 (■) or hCNT3C602R (□) was measured in transport medium containing 137 mM NaCl or 137 mM choline chloride. Sodium-dependent transport was calculated as uptake in NaCl medium minus uptake in choline chloride medium. B, transporter-mediated uptake of [^{3}H]uridine (1 μM, 1 min) by hCNT3 (■) and hCNT3C602R (□) was inhibited with 100 μM 5′-DFUR, 5-fluorouracil, or fludarabine. C and D, transporter-mediated uptake of [^{3}H]uridine (1 μM, 1 min) by hCNT3 (▲) and hCNT3C602R (■) was inhibited with increasing concentrations of 5′-DFUR (C) or fludarabine (D). K_i values were determined by fitting data by nonlinear regression using GraphPad Prism 4.0 software. For 5′-DFUR, K_i values for hCNT3 and hCNT3C602R were 22.63 and 114.29 μM, respectively; corresponding values for fludarabine were 390.2 and 405.2 μM. Data were normalized to control uptake values and are expressed as the mean ± S.E.M. of three experiments carried out on different days using different batches of cells.

DFUR uptake by the variant was almost negligible. Inhibition of [^{3}H]uridine uptake by 100 μM 5′-DFUR, fludarabine, and 5-fluorouracil (a nucleobase that is not an hCNT3 substrate) suggested a reduced interaction of 5′-DFUR but not fludarabine with the hCNT3C602R variant (Fig. 1B). To confirm these results, we measured the uptake of 1 μM [^{3}H]uridine in HeLa cells expressing hCNT3 or hCNT3C602R in the presence of increasing concentrations of either 5′-DFUR or fludarabine. The K_i values obtained revealed a highly reduced affinity of 5′-DFUR for the hCNT3C602R variant but no differences between transporters in the interaction of fludarabine (Fig. 1, C and D). The presence of an arginine at position 602 is responsible for these effects because the mutation of cysteine 602 to a serine resulted in an engineered protein with the same selectivity (Table 1) and transport rates as those found for hCNT3 (data not shown).

Functional Analysis of the Interactions of Nucleoside-Derived Drugs with hCNT3 and hCNT3C602R. To determine the effects of the C602R polymorphism on substrate recognition and interaction, we assayed natural nucleosides and derivatives at increasing concentrations for their ability to inhibit [^{3}H]uridine uptake in hCNT3-, hCNT3C602R-, hCNT3C602S-, and hCNT3C602K-transfected HeLa cells. The K_i values obtained from concentration-effect curves are listed in Table 1. Consistent with previous results (Errasti-Murugarren et al., 2008), we found no significant differences in the affinity of natural nucleosides among wild-type or Cys602-substituted hCNT3s. However, nucleoside derivatives containing substitutions in the ribose moiety showed a tendency toward reduced interaction with the hCNT3C602R transporter variant compared with those with substitutions in the nucleobase. For example, 5′-DFUR and 5′-deoxyadenosine but not 5-fluorouridine exhibited a reduced affinity for the polymorphic variant, indicating that a change in the ribose moiety is responsible for the altered interaction of 5′-DFUR with the transporter variant.

It is noteworthy that some of the nucleoside analogs commonly used in antiviral (AZT and ribavirin) and antineoplastic (gemcitabine, 5′-DFUR) therapies showed reduced interactions with the hCNT3C602R transporter variant compared with those observed for the wild type. As noted above, the C602S mutant showed a kinetic behavior identical with that of the wild-type transporter (Table 1), indicating that this reduction in the affinity of the transporter for nucleoside analogs is due to the presence of an arginine at position 602 rather than to the lack of a cysteine. Moreover, mutation of Cys602 to lysine had an even greater impact on substrate specificity than that found for the C602R variant, one that even affected interaction with natural nucleosides. Together, these results indicate that the positive charge at position 602 is a major determinant of dysfunction in the C602R and C602K variants, although a potential steric effect of the bulky side chain cannot be excluded.

Effect of the C602R Polymorphism on 5′-DFUR and Fludarabine Cytotoxicities. Altered interaction of the hCNT3C602R variant with nucleoside-derived drugs predicts that drug-induced cytotoxicity would be altered. To demonstrate this, we characterized the effects of the C602R substitution on the hCNT3-mediated cytotoxicity of the anticancer analogs fludarabine and 5′-DFUR (Fig. 2). As expected, cells expressing hCNT3 were more sensitive to 5′-DFUR and fludarabine than mock-transfected cells, which show only endogenous equilibrative transport activity (ENT type). Consistent with the results described above, cells expressing hCNT3 were significantly more sensitive to 5′-DFUR than those expressing the C602R polymorphism ($p < 0.05$) (Fig. 2A), whereas fludarabine

TABLE 1

Affinity of natural nucleosides (purine and pyrimidine) and nucleoside-derived drugs for hCNT3, hCNT3C602R, hCNT3C602S, and hCNT3C602K measured in transiently transfected HeLa cells

Transporter-mediated uptake of [^{3}H]uridine (1 μM, 1 min) by hCNT3, hCNT3C602R, hCNT3C602S, and hCNT3C602K was inhibited with increasing concentrations of natural nucleosides or nucleoside-derived drugs. K_i values were determined by fitting data by nonlinear regression using GraphPad Prism 4.0 software. For hCNT3C602S and hCNT3C602K mutants, K_i values were only obtained for natural nucleosides and the most commonly used nucleoside analogs. Data are expressed as the mean ± S.E.M. of three experiments carried out on different days using different batches of cells. Statistical analysis was carried out comparing K_i values for hCNT3 versus those obtained for mutated transporters.

Substrate	K_i			
	hCNT3	hCNT3C602R	hCNT3C602S	hCNT3C602K
		μM		
Adenosine	2.4 ± 0.2	2.2 ± 0.3	2.3 ± 0.8	4.3 ± 0.2**
2-Fluoroadenosine	12.4 ± 0.9	16.6 ± 3.4		
5′-Deoxyadenosine	12.4 ± 1.1	49.3 ± 6.6**		
Fludarabine	390 ± 9.2	405 ± 25.4	333.7 ± 43.2	1200 ± 210**
Cytidine	3.5 ± 0.9	3.9 ± 0.8	3.7 ± 0.8	7.7 ± 0.4*
2′-Deoxycytidine	6.2 ± 1.1	5.8 ± 0.7		
2′,3′-Dideoxycytidine	3400 ± 800	3700 ± 1100	3500 ± 500	N.D.
Cytarabine	16,200 ± 2300	13,400 ± 2800	15,600 ± 2100	N.D.
Gemcitabine	14.7 ± 1.1	119 ± 13.2**	12.6 ± 1.3	410 ± 20.2***
Thymidine	10.6 ± 1.2	9.5 ± 1.2	11.6 ± 14	58.8 ± 6.6**
AZT	414 ± 20	5340 ± 965**	435 ± 40.2	N.D.
Uridine	3.5 ± 0.5	3.3 ± 0.4	3.9 ± 0.8	6.2 ± 0.9
5′-DFUR	22.6 ± 3.4	114 ± 13.2**	23.0 ± 2.2	422 ± 32.2***
5-Fluorouridine	5.7 ± 0.8	6.5 ± 0.9		
2′-Deoxyuridine	2.9 ± 0.7	21.0 ± 3.3**		
Guanosine	11.3 ± 2.1	9.3 ± 1.1	10.5 ± 1.5	22.4 ± 2.2*
Ribavirin	21.3 ± 3.4	55.1 ± 6.4**	21.8 ± 2.2	102.9 ± 11.2**

N.D., not determined because of insufficient interaction of analog with transporter protein.
* $p \leq 0.05$.
** $p \leq 0.01$.
*** $p \leq 0.001$.

cytotoxicity was similar regardless of which variant was expressed (Fig. 2B).

Effect of the C602R Polymorphism on Transepithelial Flux and Intracellular Accumulation of Nucleoside-Derived Drugs. hCNT3 is known to determine nucleoside vectorial flux because of its apical localization in polarized models (Errasti-Murugarren et al., 2007). To determine whether this novel hCNT3 variant would affect vectorial flux of nucleoside-derived drugs across epithelia, we grew hCNT3-, hCNT3C602R-, or mock-transfected MDCK cells on Transwell inserts and analyzed the transepithelial flux of nucleoside-derived drugs from the apical to the basolateral domain. Figure 3 shows the effect of the variant on net sodium-dependent flux across the epithelial barrier and intracellular accumulation of nucleoside derivatives with either antiviral (A and C) or antineoplastic (B and D) activities. As expected, both vectorial flux and intracellular accumulation were significantly lower in epithelia expressing the C602R polymorphism than in cells expressing wild-type hCNT3. Despite a clear reduction, this variant nevertheless mediated a net sodium-dependent transepithelial flux and accumulation of both antiviral and antineoplastic nucleoside-derived drugs.

Lipid Raft Association of hCNT3. Amino acid changes in transmembrane domains can alter the affinity of transporter proteins for lipid rafts, which in turn may affect their biological activity (Murtazina et al., 2006; Nguyen et al., 2007). To first establish the distribution of hCNT3 in membrane lipid domains, for which no information is currently available, we assessed hCNT3 protein levels in purified lipid microdomains by Western blot analysis. Detergent-resistant and nonresistant membrane fractions from HeLa cells transiently transfected with hCNT3 were isolated by sucrose density centrifugation, as described under *Materials and Methods* (Fig. 4A). hCNT3 immunoreactivity was detected in both detergent-resistant (lipid raft) and nonresistant fractions. Caveolin-1, a known lipid raft protein, was detected in the low-density fractions (fractions 4 and 5), whereas clathrin, which is a nonraft protein, was associated with high-density fractions (fractions 7–11). To determine whether microdomain localization is involved in hCNT3 function, we depleted hCNT3-transfected HeLa cells of cholesterol, which

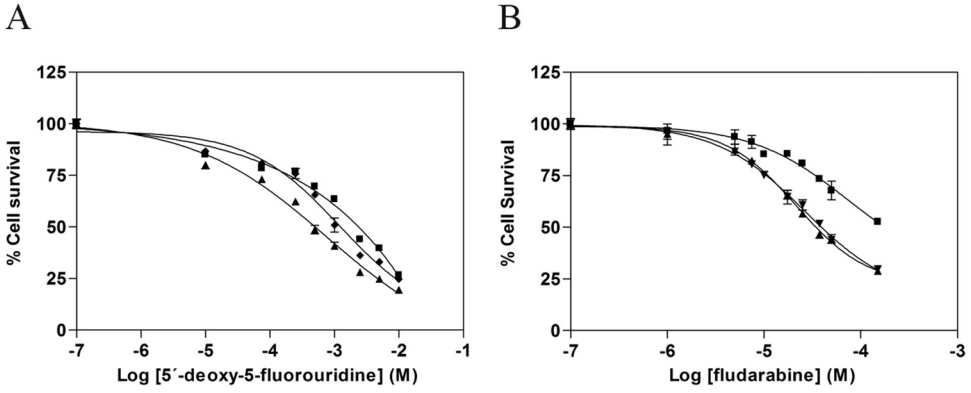

Fig. 2. Cytotoxicity of nucleoside derivatives in hCNT3- and hCNT3C602R-transfected HeLa cells. Cytotoxicity of 5′-DFUR (A) and fludarabine (B) in cells transfected with hCNT3 (▲), hCNT3C602R (▼), and in mock-transfected cells (■). Cell viability was assessed 72 h after a 90-min exposure to drug. IC_{50} values of 5′-DFUR in hCNT3-, hCNT3C602R-, and mock-transfected cells were 404 ± 4.4, 711 ± 105, and 1371 ± 156 μM, respectively; the corresponding IC_{50} values for fludarabine were 20.1 ± 2.2, 26.6 ± 4.3 and 46.9 ± 3.5 μM. Data are expressed as the mean ± S.E.M. of three experiments carried out on different days using different batches of cells.

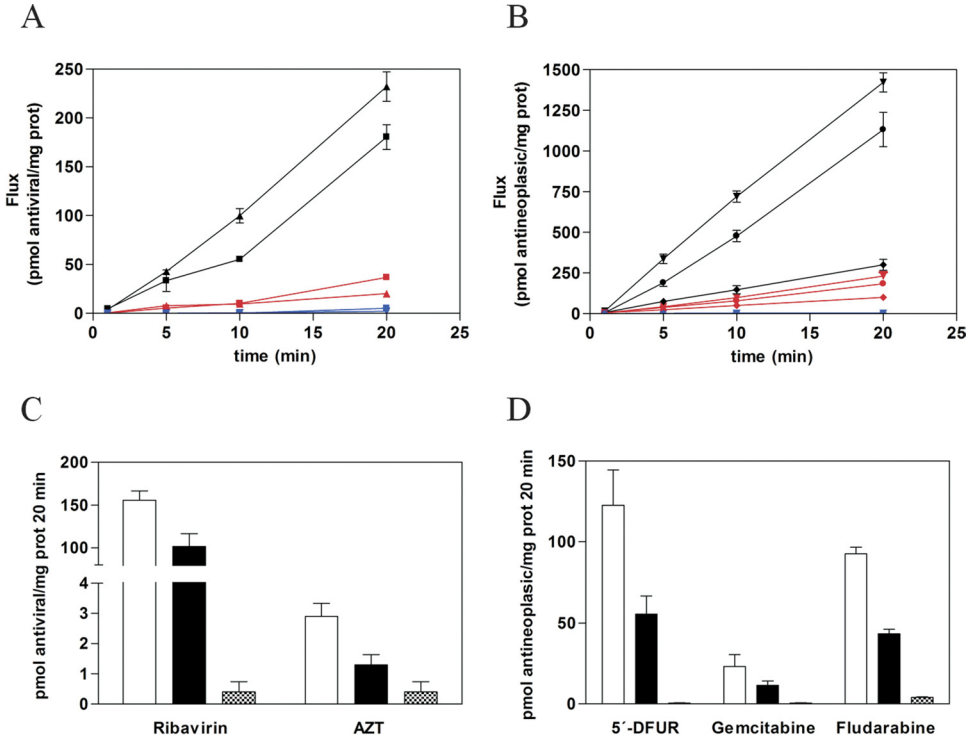

Fig. 3. Transepithelial flux of nucleoside-derived drugs in hCNT3-, hCNT3C602R-, and mock-transfected MDCK cells. Sodium-dependent transepithelial flux of ^{3}H-labeled antiviral (A) and anticancer (B) nucleoside-derived drugs (1 μM) from the apical to the basolateral compartment in MDCK cells transfected with wild-type hCNT3 (black symbols), hCNT3C602R (red symbols), or in mock-transfected cells (blue symbols). A, ribavirin (▲) and AZT (■); B, 5′-DFUR (▼), fludarabine (♦), and gemcitabine (●). Sodium-dependent intracellular accumulation of antiviral (C) or anticancer (D) drugs measured in MDCK cells transfected with hCNT3 (□), hCNT3C602R (■), and in mock-transfected cells (▨) after 20 min. Data show the intracellular accumulation of the antiviral or the antitumoral labeled drug added to the apical side of the epithelial barrier and their derived metabolic products. 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 using different batches of cells.

is an essential component of lipid rafts, by treating cells with increasing concentrations of MβCD for 30 min at 37°C and then measured ^{3}H-labeled uridine uptake. MβCD induced a dose-dependent reduction in hCNT3 activity (Fig. 4B), although transporter affinity was unaffected by the treatment (Supplementary Fig. 1). The activity of endogenous, non–raft-associated equilibrative nucleoside transporters was used as a control for MβCD effects (data not shown). A concentration of 10 mM MβCD was the highest concentration that affected raft-associated hCNT3 activity without affecting equilibrative nucleoside transporter activities. The reduction in hCNT3 activity by cholesterol depletion was associated with a reduction in the lipid-raft targeting of the transporter protein. In hCNT3-transfected HeLa cells pretreated with 10 mM MβCD for 30 min, hCNT3 was fully solubilized in Triton X-100 at 4°C (Fig. 4A).

Effect of the C602R Polymorphism on Association of hCNT3 with Lipid Rafts. Similar experiments were then performed using cells transiently transfected with the hCNT3C602R variant. Transmembrane domain 13, where the substitution is located, may be involved in maintaining the structural integrity of the transporter (Zhang et al., 2006; Errasti-Murugarren et al., 2008), although a potential role in lipid raft targeting had not been tested previously. Figure 5A shows representative profiles of hCNT3 and hCNT3C602R distribution in submembrane fractions. A densitometric analysis of sucrose-gradient fractions from hCNT3- or hCNT3C602R-transfected cells showed that the amount of hCNT3C602R variant protein associated with lipid rafts (fractions 4 and 5) was reduced approximately 4-fold compared with that of the hCNT3 protein, with no change in the amount of transporter protein in the nonraft fractions (Fig. 5B). Moreover, comparisons with the activity and lipid-raft targeting of the wild-type hCNT3 transporter showed that the reduced sodium-dependent [^{3}H]uridine uptake of the C602R polymorphism correlated precisely with its reduced lipid-raft distribution (Fig. 5C). Consistent with this, the activity of the hCNT3C602R variant was largely unaffected by MβCD treatment, whereas MβCD-mediated cholesterol depletion markedly reduced the function and lipid-raft localization of wild-type hCNT3 (Fig. 5D). Finally, to determine whether the presence of a cysteine at position 602 was responsible for enabling appropriate transporter associations with lipid rafts, we tested the serine-substituted hCNT3C602S mutant. This mutant showed a lipid raft distribution profile identical with that of the wild-type transporter (Fig. 5, A and B), indicating that a cysteine residue at position 602 is not required for transporter association with lipid rafts.

Discussion

Genetic variants that affect transporter activity could potentially explain differences among patients in the uptake and disposition of commonly used anticancer and antiviral drugs (Cropp et al., 2008; Hesselson et al., 2009; Kroetz et al., 2010). Although a number of variants of the *SLC28* and *SLC29* gene families, encoding hCNT and hENT proteins, respectively, that affect the interaction of nucleoside-derived drugs with transporter proteins have been described recently, their impact on drug action is still unclear (Gray et al., 2004; Badagnani et al., 2005; Owen et al., 2005; Li et al., 2007). As introduced above, among these transporters, hCNT3 seems to play a pivotal role in the pharmacokinetics of most nucleoside-derived drugs. We identified recently a novel, nonsynonymous polymorphism of the *SLC28A3* gene that affects sodium binding and hCNT3-related activity (Errasti-Murugarren et al., 2008). Although the hCNT3C602R variant had much lower transport capacity for natural nucleosides than its wild-type counterpart, it showed no difference in the apparent K_m value for natural substrates (Errasti-Murugarren et al., 2008). However, in the current study, we demonstrate that this variant has a reduced affinity for some, but not all, nucleoside derivatives. It is noteworthy that Cys602 is predicted to be located in transmembrane domain 13 near other residues that are not believed to be relevant for purine or pyrimidine specificity (Zhang et al., 2006). When this residue was mutated to serine, the uptake rates for all assayed nucleoside-derived drugs were similar to those observed for the wild-type transporter, indicating that the presence of an arginine rather than the lack of a cysteine is responsible for reduced uptake of nucleoside derivatives. Analysis of a selection of nucleoside analogs suggests that, in general, substitutions in the pentose ring strongly affect in-

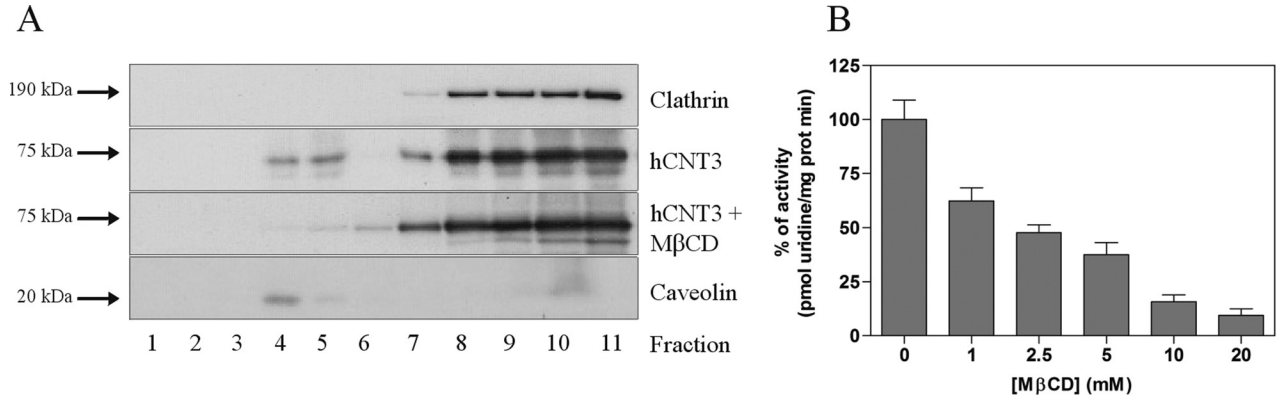

Fig. 4. Characterization of hCNT3 distribution in membrane lipid fractions isolated from transfected HeLa cell. Lipid rafts were isolated from hCNT3-transfected HeLa cells by solubilization in ice-cold 1% Triton X-100 followed by sucrose gradient fractionation. A, equal volumes of each sucrose gradient fraction were separated by SDS-PAGE on 10% gels and analyzed by Western blotting using an anti-HA antibody. Caveolin and clathrin were used as raft and nonraft markers, respectively. A representative Western blot showed a bimodal distribution of hCNT3 in both lipid raft and nonlipid raft fractions. Also shown is the effect of treatment with 10 mM MβCD for 30 min at 37°C. B, dose-dependent effect of MβCD (30 min at 37°C) on hCNT3 activity in transfected HeLa cells. Data are representative of three experiments carried out on different days using different batches of cells.

teractions of nucleoside analogs with the hCNT3C602R variant but not with the wild-type counterpart. As suggested previously (Zhang et al., 2003b), the substrate pocket in the hCNT3 protein primarily binds the 3′-OH group, which is essential for transportability. Our observations suggest that slight changes in structural determinants close to the 3′-OH preclude high-affinity substrate interaction. This conclusion is also in agreement with the view that substitution of an arginine—or even more strikingly, a lysine—at Cys602 somehow alters the substrate-binding pocket. In fact, variable interaction of nucleoside-derived drugs with particular polymorphic variants will also contribute to heterogeneity in drug-responsiveness, thus anticipating that pharmacokinetics could further depend on the drug used. For instance, reduced transport of all nucleoside-derived drugs is expected for hCNT3C602R, but the pharmacokinetics might be different depending on whether a patient bearing the mutation was treated with gemcitabine or the prodrug capecitabine, for which 5′-DFUR is a metabolite and the immediate precursor of 5-fluorouracil. Moreover, heterologous expression of hCNT3 in HeLa cells resulted in increased drug-induced sensitivity, in agreement with previous data showing a similar effected triggered by hCNT1 (Mata et al., 2001). This supports the view that high-affinity drug uptake is a key determinant of nucleoside analog-induced cytotoxicity in cancer cells. Accordingly, expression of the mutated transporter resulted in reduced 5′-DFUR-triggered cytotoxicity. It is noteworthy that this was not the case for fludarabine, thus suggesting an additional role for metabolizing enzymes as limiting factors in drug-induced cell death.

This study also shows that hCNT3 associates with both lipid-raft and non–lipid-raft fractions in HeLa cell membranes. Moreover, cholesterol depletion with MβCD induced a significant decrease in hCNT3 activity, suggesting that hCNT3 transporter activity is highly dependent on association with lipid rafts. Although previous studies of the distribution of nucleoside transporters in the apical plasma membrane of human syncytiotrophoblasts have shown that nucleoside transporter proteins (in particular hCNT1 and hENT1) are present in lipid rafts (E. Errasti-Murugarren, P. Díaz, V. Godoy, G. Riquelme, and M. Pastor-Anglada, unpublished observations), no information about the functional targeting of nucleoside transporter proteins to particular plasma membrane microdomains is available. Associations between lipid-raft localization and functional activity have been described for other transporter proteins. In particular, it has been shown that cholesterol depletion of non-polarized cells results in a decrease in NHE3 and hPept1 activities (Murtazina et al., 2006; Nguyen et al., 2007). Our analysis showed that lipid-raft localization of the hCNT3C602R variant was impaired. Because plasma membrane levels of hCNT3 and

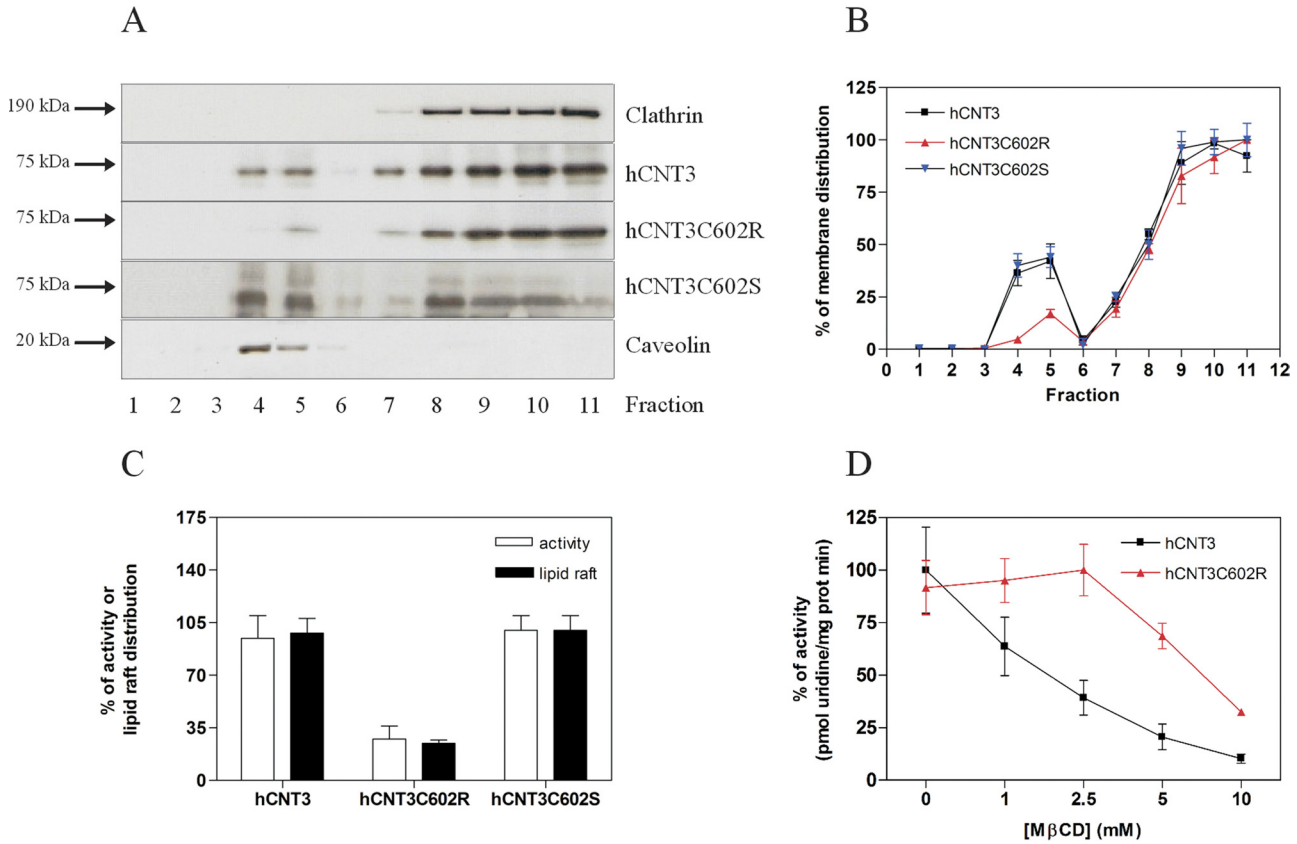

Fig. 5. Effect of the C602R polymorphism on hCNT3 lipid-raft distribution in transfected HeLa cells. Lipid rafts were isolated from hCNT3- and hCNT3C602R-transfected HeLa cells by solubilization in ice-cold 1% Triton X-100 followed by sucrose gradient fractionation. A, the distribution of hCNT3, hCNT3C602R, and hCNT3C602S proteins in the gradient fractions extracted from transiently transfected HeLa cells was analyzed by SDS-PAGE on 10% gels and Western blotting using an anti-HA antibody. B, densitometric analysis of sucrose gradient fractions containing hCNT3, hCNT3C602R, and hCNT3C602S. Data were normalized considering 100% the higher densitometric value for each construction. C, comparison of sodium-dependent [^{3}H]uridine uptake and lipid raft distribution of wild-type hCNT3, hCNT3C602R, and hCNT3C602S transporter proteins. Uptake values and lipid raft distribution are normalized to those of the wild-type transporter (100%). D, effect of MβCD treatment (30 min at 37°C) on hCNT3 and hCNT3C602R uridine uptake activity in transfected HeLa cells. Uptake values are normalized to those of untreated controls (100%). Data are representative of three experiments carried out on different days using different batches of cells.

hCNT3C602R are essentially identical (Errasti-Murugarren et al., 2008), it is reasonable to infer that the C602R protein has been redistributed from lipid-raft to non–lipid-raft fractions. This reduced association with lipid rafts is corroborated by the fact that cholesterol depletion, in this case by MβCD, had little effect on hCNT3C602R-related transporter activity compared with the marked effect of cholesterol depletion on the activity of the wild-type protein. It is tempting to speculate that the presence of putative hCNT3-interacting proteins in lipid rafts and/or the interaction of cholesterol molecules with the transporter might determine the overall transporter structure and thus favor transporter activity. However, additional studies will be required to determine the physiological mechanisms that regulate hCNT3 activity in lipid rafts.

Taken together, these results indicate a role for transmembrane domain 13 in targeting (and/or retention) of the hCNT3 transporter to plasma membrane lipid-raft microdomains. This interpretation is in agreement with previous reports showing that selected transmembrane domains are important determinants of plasma membrane lipid-raft localization (Coffin et al., 2003; Zhang et al., 2003a; Barman et al., 2004). For hCNT3, the highly hydrophobic nature of transmembrane 13, together with the absence of exposed residues for substrate binding, make this domain a suitable candidate for protein-lipid interactions. It has been suggested that transmembrane proteins associate with lipid rafts through direct interactions between transmembrane amino acids and cholesterol-sphingolipid complexes (Anderson and Jacobson, 2002). Some proteins, such as the HA of influenza virus, reside permanently in lipid-raft microdomains (Scheiffele et al., 1997). It is noteworthy that the distal region of hCNT3 transmembrane domain 13 strongly resembles the C terminus of the HA transmembrane domain responsible for lipid-raft interaction. This suggests that both domains adopt a three-dimensional structure with a higher affinity for lipid-raft components, thus reinforcing the putative role of transmembrane domain 13 in hCNT3-raft association. However, Cys602 is not likely to be involved in protein-lipid interactions because its substitution by a serine, which is a hydrophilic amino acid not expected to interact with cholesterol, had no effect on transporter activity or lipid raft association. These results indicate that the C602R substitution causes a conformational change that leads to a decrease in the affinity of the transporter for lipids surrounding transmembrane 13, which is in agreement with the explanation discussed above for the altered nucleoside-analog interaction of the hCNT3C602R variant. The finding that a transmembrane polymorphism in the Fcγ RIIB inhibitory receptor decreases the affinity of the protein for lipid rafts is also consistent with this explanation (Floto et al., 2005; Kono et al., 2005).

In summary, the present study reports the pharmacological characterization of hCNT3C602R, a recently defined hCNT3 polymorphism. This polymorphic variant exhibits severely, but differentially, altered interactions with nucleoside-derived drugs. The analysis of this variant also highlights the role that transmembrane domain 13 might play in hCNT3 transporter structure and subcellular localization as a potentially major determinant of lipid-raft targeting and/or retention. This novel finding is of crucial relevance because it suggests that hCNT3 retention in lipid rafts is a key determinant of its biological function.

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