FEBS 24095 FEBS Letters 481 (2000) 137–140

# Electrogenic uptake of nucleosides and nucleoside-derived drugs by the human nucleoside transporter 1 (hCNT1) expressed in *Xenopus laevis* oocytes

M. Pilar Lostao[a], Joao F. Mata[b], Ignacio M. Larrayoz[a], Simone M. Inzillo[a], F. Javier Casado[b], Marçal Pastor-Anglada[b,*]

[a] *Departamento de Fisiología y Nutrición, Universidad de Navarra, Pamplona, Spain*
[b] *Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, Barcelona 08028, Spain*

Received 14 July 2000; accepted 14 August 2000

Edited by Maurice Montal

**Abstract The concentrative pyrimidine-preferring nucleoside transporter 1 (hCNT1), cloned from human fetal liver, was expressed in *Xenopus laevis* oocytes. Using the two-electrode voltage-clamp technique, it is shown that translocation of nucleosides by this transporter generates sodium inward currents. Membrane hyperpolarization (from −50 to −150 mV) did not affect the $K_{0.5}$ for uridine, although it increased the transport current approximately 3-fold. Gemcitabine (a pyrimidine nucleoside-derived drug) but not fludarabine (a purine nucleoside-derived drug) induced currents in oocytes expressing the hCNT1 transporter. The $K_{0.5}$ value for gemcitabine at −50 mV membrane potential was lower than that for natural substrates, although this drug induced a lower current than uridine and cytidine, thus suggesting that the affinity binding of the drug transporter is high but that translocation occurs more slowly. The analysis of the currents generated by the hCNT1-mediated transport of nucleoside-derived drugs used in anticancer and antiviral therapies will be useful in the characterization of the pharmacological profile of this family of drug transporters and will allow rapid screening for uptake of newly developed nucleoside-derived drugs.** **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

*Key words:* hCNT1; Nucleoside; Anticancer drug; Electrophysiology

## 1. Introduction

Most nucleoside-derived drugs used in antiviral and anticancer therapies may be substrates of one or more of the cloned CNT and ENT transporters [1–4]. Two ENT transporter isoforms have been cloned, ENT1 and ENT2. The former is inhibited by nanomolar concentrations of the nucleoside analog NBTI whereas the latter is insensitive to this inhibitor. The two ENT isoforms identified so far show broad substrate specificity and appear to mediate equilibrative translocation of nucleosides across the plasma membrane with relatively low affinity [1–4]. Similarly, two CNT isoforms have been cloned, CNT1 which is pyrimidine-preferring and CNT2 which is purine-preferring [1–4]. Heterologous expression of these membrane proteins in mammalian cells and *Xenopus laevis* oocytes has recently been used to determine the pharmacological profile of the CNT and ENT transporters, by measuring either the influx of radiolabeled substrates or the interaction with the carrier by inhibition of the uptake of the natural substrate [2–9]. The first approach is direct but requires a labeled drug and is time-consuming, which may not be suitable for pharmacological screenings. The second approach, in contrast, does not define the substrate specificity of the carrier. Since CNT transporters are Na-dependent, the coupling of Na influx with nucleoside uptake should induce measurable currents which may facilitate an electrophysiological approach to the pharmacological properties of these relatively new family of drug transporters. Previous evidence suggests that nucleoside transport through these systems is dependent on the transmembrane electrical gradient and may be consistent with a stoichiometry of one nucleoside per sodium ion being cotransported [10,11].

In this report, it is shown that human nucleoside transporter 1 (hCNT1) expression in *X. laevis* oocytes results in the induction of currents due to the cotransport of sodium with some nucleosides. The substrate specificity is that expected for a pyrimidine-preferring transporter. The evidence that CNT-related activity can be measured electrophysiologically opens the possibility for massive screening for transporter specificity of nucleoside derivatives suitable for the treatment of a variety of human viral and neoplastic diseases.

## 2. Materials and methods

### 2.1. Cloning of the hCNT1 cDNA

The hCNT1 clone was obtained by amplification of an oligo(dT)-primed cDNA library from human fetal liver (Marathon Ready cDNA, Clontech), using a PCR approach with Advantage 2 Polymerase Mix (Clontech). Primers HC1E (5′-GCTGCACTGCATG-GTTGCTGCTGGATGTGTTG-3′) and HC1D (5′-GGGAAGGGG-CATGTGAGGGACCCCAGGAGA-3′), derived from the reported sequence for hCNT1 (GenBank™ accession number U62966), were used in a touchdown PCR that produced a unique DNA band with an approximate molecular weight of 2 kb. This PCR product was gel-purified, used in an additional reaction of dATP added to the 3′ end with TaqPolymerase (Promega), again purified by a DNA clean up column (Wizard DNA Cleanup System, Promega) and finally ligated to the plasmid pGEM-Teasy (pGEM-Teasy Vector System II, Promega) with T4 DNA ligase (New England Biolabs). The construct pGEMT-hCNT1 after both strands Taq DyeDeoxy terminator cycle sequencing (DNA sequencing kit, dRhodamine Terminator Cycle Sequencing, Perkin Elmer) and aligned with a published sequence previously described, confirmed the nature of the hCNT1 cDNA. The hCNT1 cDNA was then inserted into the pBSII(KS) vector, which

*Corresponding author. Fax: (34)-93-4021559.
E-mail: mpastor@porthos.bio.ub.es

0014-5793/00/$20.00 © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0014-5793(00)01983-9

contained a poly-A tail of 54 bp from the original plasmid pBSII(KS)-hSGLT1 [12] as follows. pBSII(KS)-hSGLT1 was digested with *Nsi*I, blunted with Klenow fragment, purified through a DNA clean up column, digested with *Sal*I and gel-purified. The construct pGEMT-hCNT1 was digested in parallel. The two fragments were ligated with T4 DNA ligase as previously described and the product, pBSII(KS)-hCNT1-poly-A tail, was used to transform *Escherichia coli* XL-1 Blue. Plasmid pBSII(KS)-hSGLT1 was kindly donated by Dr. E.M. Wright (UCLA).

*2.2. Expression of hCNT1 in Xenopus oocytes*

The plasmid containing the hCNT1 cDNA was linearized with *Xba*I and cRNA synthesized using the Ambion T3 MEGAscript kit (Austin, TX, USA) in the presence of m7G(5′)ppp(5′)G. 50 ng of cRNA was injected into *X. laevis* oocytes as previously described [13] and maintained at 18°C in Barth's medium for 3–5 days. Uridine uptake assays were performed by a radiotracer method [14]. Briefly, groups of 6–10 oocytes were incubated for 1 h at room temperature in 400 μl of $Na^+$ buffer containing 50 μM [$^3$H]uridine (3 μCi/ml, specific activity 36.0 Ci/mmol) (Amersham Pharmacia Biotech, Buckinghamshire, UK), either in the absence or in the presence of nucleosides at the indicated concentrations. After the incubation period had elapsed, the $^3$H content of each oocyte was determined by liquid scintillation counting. Uptakes are expressed as pmol/h per oocyte.

Electrophysiology experiments were performed to examine substrate-induced $Na^+$ inward currents using the two-electrode voltage-clamp method [15]. The oocyte membrane potential ($V_m$) was held at −50 mV and continuous current data were recorded using Axoscope V3. To obtain current/voltage ratios, 11 pulses of potential between +50 and −150 mV in 20 mV decrements were applied for 100 ms, using pClamp 6 software (both from Axon Instruments, Foster City, CA, USA).

The apparent affinity constant ($K_{0.5}$) and the maximal current for saturating uridine ($I_{max}$) were obtained by fitting the steady-state currents at each membrane potential to the equation:

$$I = I_{max} \cdot [S]/(K_{0.5} + [S]),$$

where [S] is the substrate concentration, using the non-linear fitting method in SigmaPlot 4 (SPSS, Chicago, IL, USA).

In the experiments performed in the absence of sodium, NaCl was replaced by choline Cl.

All the nucleosides used in these experiments were purchased from Sigma (St. Louis, MO, USA).

## 3. Results and discussion

Fig. 1 shows the inward currents evoked by saturating concentrations of natural nucleosides and nucleoside derivatives used in cancer treatment in an oocyte clamped at −50 mV expressing the hCNT1 transporter. Uridine, a common substrate for hCNT1 and hCNT2, elicited the highest currents, whereas cytidine, a pyrimidine which presumably is a hCNT1-specific substrate, induced Na inward currents which were 50% of those induced by uridine (Fig. 1; Table 1). Gemcitabine, a fluoropyrimidine currently used in the treatment of a variety of solid tumors, including pancreatic cancer, also generated currents but with significantly lower intensity than the natural substrates (Fig. 1; Table 1). This agrees with previous flux measurements of radiolabeled substrates performed on *Xenopus* oocytes independently expressing the four nucleoside transporters cloned so far. Formycin B and guanosine, hCNT2-specific substrates, did not evoke inward currents (Fig. 1). Similarly, fludarabine, a nucleoside-derived drug currently used in the treatment of lymphoproliferative malignancies, did not induce currents either. Fludarabine monophosphate was also checked as a putative substrate because this is the chemical form of the prodrug administered to the patients.

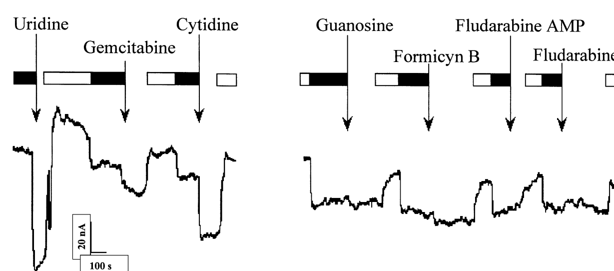


Fig. 1. $Na^+$ currents evoked by different nucleosides and nucleoside-derived drugs in a single oocyte expressing hCNT1. Oocytes were held at −50 mV and continuously perfused with $Na^+$ buffer in the absence of substrate (black box). The current registered under that condition corresponds to the base-line current (20 nA). The addition of nucleosides or derivatives (0.5 mM) is indicated by an arrow. After perfusion with each nucleoside, oocytes were washed out in $Na^+$-free medium (blank box). A representative result in a single oocyte expressing hCNT1 is shown.

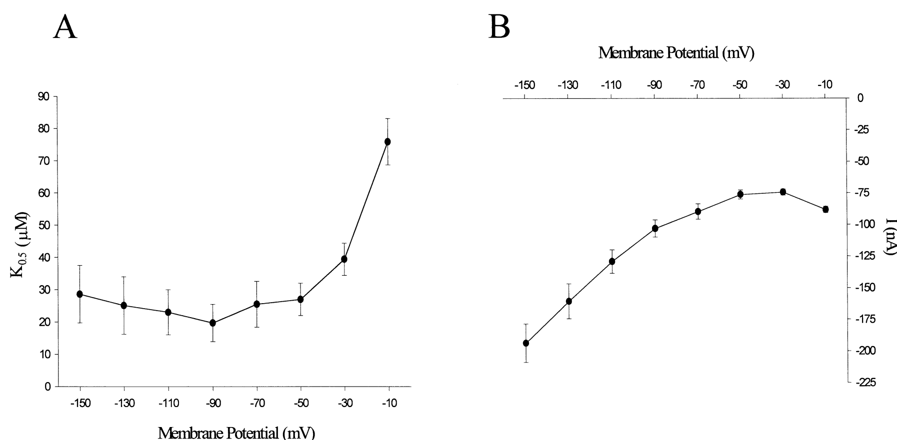


Fig. 2. $K_{0.5}$ and $I_{max}$ for uridine as a function of membrane potential. Experiments were routinely performed in hCNT1 expressing oocytes, 3 days after cRNA injection. $K_{0.5}$ and $I_{max}$ were obtained at every membrane potential by fitting the steady-state current obtained at six different concentrations of uridine (from 10 to 500 μM) to the equation in Section 2. A representative experiment using a single oocyte expressing hCNT1 is shown. The error bars correspond to the error of the fitting. (A) $K_{0.5}/V$ curve. $K_{0.5}$ values are very similar at each membrane potential between −50 mV and −150 mV. (B) $I_{max}/V$. Maximal current increases with more negative potentials from $-76 \pm 3.5$ nA at −50 mV to $-190 \pm 15$ nA at −150 mV. Similar results were obtained with oocytes from two different frog donors.

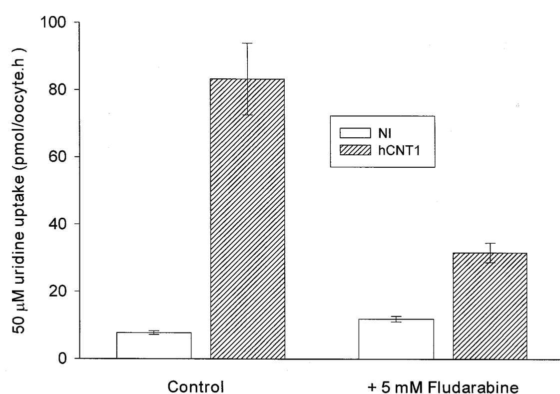


Fig. 3. Effect of fludarabine on radiolabeled uridine uptake into oocytes expressing the hCNT1 transporter. Transport measurements were performed 3 days after the injection of oocytes with hCNT1 cRNA. The substrate, uridine, was added at a concentration of 50 μM, and the putative inhibitor, fludarabine, was added at a concentration 100 times greater (5 mM). Data represent the mean of 8–9 measurements and the error bars indicate standard errors. Uridine uptake by non-injected oocytes (NI) is also shown. Similar results were obtained with oocytes from three different frog donors.

As expected for a nucleotide, this compound did not induce any currents in oocytes expressing hCNT1.

Data in Fig. 1 also show that the shift in inward current as a result of sodium addition in oocytes expressing hCNT1 is significant and much higher than the shift observed in control oocytes (not shown). This observation suggests that there is a slippage of the cation through the transporter, as previously reported for other Na-dependent cotransporters like the Na/glucose cotransporter SGLT1 [16].

Interestingly, addition of a hCNT1 substrate in the absence of sodium also resulted in the induction of a small current (not shown). For instance, 0.5 mM uridine in choline chloride medium produced 20% of the current in NaCl medium at −50 mV membrane potential, which suggests that the transporter is also coupled to an ion other than sodium. Studies are in progress to determine which other ion(s) are involved in nucleoside transport mediated by hCNT1.

Substrate-evoked currents at −50 mV membrane potential were measured at increasing substrate concentrations. The pattern followed Michaelis–Menten type saturation kinetics, thus allowing the calculation of $K_{0.5}$ values for uridine, gemcitabine and cytidine (Table 1). hCNT1 shows higher affinity for the drug than for natural substrates but the intensity of the current is much lower, suggesting that gemcitabine binds better to the transporter but that translocation is lower. These observations are in agreement with the apparent $K_m$ values derived from flux measurements recently performed by others [9]. Similarly, a glucose derivative, indican (indoxyl-B-D-glucopyranoside), shows a much higher affinity for SGLT1 than the natural substrates but only generates 14% of the maximal current [17], which suggests that increasing the size of the conjugate reduces the turnover rate of the transporter although its chemical structure favors the binding to the carrier.

Table 1
$K_{0.5}$ values of the transported substrates were obtained by fitting the currents obtained at seven concentrations (10 μM–1 mM) to the equation in Section 2

| | $K_m$ (μM) | Uridine current (%) (nA) |
|---|---|---|
| Uridine | 56 ± 9 | 100 |
| Gemcitabine | 17 ± 2 | 33 ± 5 |
| Cytidine | 34 ± 7 | 50 ± 4 |

Gemcitabine (0.5 mM) and cytidine (0.5 mM) currents are expressed as % of the current generated by 0.5 mM uridine. Values are the mean of 2–6 measurements performed in oocytes from 2–6 different batches, and the error bars indicate standard errors.

In another set of experiments, the $K_{0.5}$ for uridine was shown to be largely independent of $V_m$, increasing only at −10 mV by nearly 2.5-fold (Fig. 2). However, $I_{max}$ was voltage-dependent at hyperpolarized membrane potentials and did not approach saturation at the membrane potential range used. This suggests that agents that hyperpolarize the plasma membrane also increase the transport rate mediated by hCNT1. This evidence agrees with previous experiments using isolated rat hepatocytes, in which it was shown that glucagon and ionophores able to hyperpolarize the plasma membrane induce a transient activation of Na-dependent nucleoside transport into liver parenchymal cells [11]. Hepatocytes express at least CNT1 and CNT2, and their expression is known to be regulated by cell differentiation and transformation [18–20]. In other cell types, such as human B-lymphocytes, activation is also associated with up-regulation of CNT activities [21]. Since the pattern and activity of nucleoside transporters appear to be regulated in mammalian cells and this may be a putative mechanism by which cells develop resistance to anticancer and antiviral treatments, we believe it was important to set up a rapid method to establish the pharmacological profile of these transporters. Such a method is presented in this study. As discussed above, measurement of flux of radiolabeled tracers is time-consuming and may be limited by the availability of labeled drugs. Moreover, inhibition studies cannot be reliable. As shown in Fig. 3, fludarabine, a nucleoside-derived drug which, according to the present data, is not an hCNT1 substrate, can inhibit uridine uptake in oocytes expressing the hCNT1 transporter, which suggests that there might be interaction of certain nucleosides with transporters at the plasma membrane, without substrate translocation.

In summary, this study provides the first electrophysiological characterization of a nucleoside transporter, which is involved in the uptake of fluoropyrimidines, such as gemcitabine, currently used in the treatment of solid tumors.

*Acknowledgements:* This study has been funded by Grants SAF99-0155 and 2FD97-1268 to M.P.-A. and PIUNA (University of Navarra) to M.P.L. Joao F. Mata is a fellow from the Praxis XXI programme (Portuguese Government). I.M. Larrayoz is a fellow of the Asocaición de Amigos (University of Navarra). We thank Asunción Redín for her technical assistance.

## References

[1] Griffith, D.A. and Jarvis, S.M. (1996) Biochim. Biophys. Acta 1286, 153–181.
[2] Wang, J., Schaner, M.E., Thomassen, S., Su, S.F., Piquette-Miller, M. and Giacomini, K.M. (1997) Pharm. Res. 14, 1524–1532.
[3] Pastor-Anglada, M., Felipe, A. and Casado, F.J. (1998) Trends Pharmacol. Sci. 19, 424–430.
[4] Baldwin, S.A., Mackay, J.R., Cass, C.E. and Young, J.D. (1999) Mol. Med. Today 5, 216–224.
[5] Huang, Q., Yao, S.Y.M., Ritzel, M.W.L., Paterson, A.R.P., Cass, C.E. and Young, J.D. (1994) J. Biol. Chem. 269, 17757–17760.
[6] Fang, X., Parkinson, F.E., Mowles, D.A., Young, J.D. and Cass, C.E. (1996) Biochem. J. 317, 457–465.

[7] Yao, S.Y.M., Cass, C.E. and Young, J.D. (1996) Mol. Pharmacol. 50, 388–393.
[8] Schaner, M.E., Wang, J., Zevin, S., Gerstin, K.M. and Giacomini, K.M. (1997) Pharm. Res. 14, 1316–1321.
[9] Mackey, J.R., Yao, S.Y.M., Smith, K.M., Karpinski, E., Baldwin, S.A., Cass, C.E. and Young, J.D. (1999) J. Natl. Cancer Inst. 91, 1876–1881.
[10] Ruiz-Montasell, B., Casado, F.J., Felipe, A. and Pastor-Anglada, M. (1992) J. Membr. Biol. 128, 227–233.
[11] Gómez-Angelats, M., del Santo, B., Mercader, J., Ferrer-Martínez, A., Felipe, A., Casado, J. and Pastor-Anglada, M. (1996) Biochem. J. 313, 915–920.
[12] Hediger, M.A., Coady, M.J., Ikeda, T.S. and Wright, E.M. (1987) Nature 330, 379–381.
[13] Birnir, B., Loo, D.D.F. and Wright, E.M. (1991) Pflüg. Arch. 418, 79–85.
[14] Ikeda, T.S., Hwang, E.-S., Coady, M.J., Hirayama, B.A., Hediger, M.A. and Wright, E.M. (1989) J. Membr. Biol. 110, 87–95.
[15] Hirayama, B.A., Lostao, M.P., Panayotova-Heiermann, M., Loo, D.D.F., Turk, E. and Wright, E.M. (1996) Am. J. Physiol. 270, G919–G926.
[16] Lostao, M.P., Hirayama, B.A., Loo, D.D.F. and Wright, E.M. (1994) J. Membr. Biol., 161–170.
[17] Díez-Sampedro, A., Lostao, M.P., Wright, E.M. and Hirayama, B.A. (2000) J. Membr. Biol. (in press).
[18] Felipe, A., Valdés, R., del Santo, B., Lloberas, J., Casado, J. and Pastor-Anglada, M. (1998) Biochem. J. 330, 997–1001.
[19] del Santo, B., Valdés, R., Mata, J., Felipe, A., Casado, F.J. and Pastor-Anglada, M. (1998) Hepatology 28, 1504–1511.
[20] Dragan, Y., Valdés, R., Gómez-Angelats, M., Felipe, A., Casado, F.J., Pitot, H.C. and Pastor-Anglada, M. (2000) Hepatology (in press).
[21] Soler, C., Felipe, A., Mata, J.F., Casado, F.J., Celada, A. and Pastor-Anglada, M. (1998) J. Biol. Chem. 273, 26939–26945.