Identification of Cys¹⁴⁰ in helix 4 as an exofacial cysteine residue within the substrate-translocation channel of rat equilibrative nitrobenzylthioinosine (NBMPR)-insensitive nucleoside transporter rENT2

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The human and rat equilibrative nucleoside transporter proteins hENT1, rENT1, hENT2 and rENT2 belong to a family of integral membrane proteins with 11 potential transmembrane segments (TMs), and are distinguished functionally by differences in transport of nucleobases and sensitivity to inhibition by nitrobenzylthioinosine (NBMPR) and vasoactive drugs. In the present study, we have produced recombinant hENT1, rENT1, hENT2 and rENT2 in *Xenopus* oocytes and investigated uridine transport following exposure to the impermeant thiol-reactive reagent *p*-chloromercuriphenyl sulphonate (PCMBs). PCMBs caused reversible inhibition of uridine influx by rENT2, but had no effect on hENT1, hENT2 or rENT1. This difference correlated with the presence in rENT2 of a unique Cys residue (Cys¹⁴⁰) in

the outer half of TM4 that was absent from the other ENTs. Mutation of Cys¹⁴⁰ to Ser produced a functional protein (rENT2/C140S) that was insensitive to inhibition by PCMBs, identifying Cys¹⁴⁰ as the exofacial Cys residue in rENT2 responsible for PCMBs inhibition. Uridine protected wild-type rENT2 against PCMBs inhibition, suggesting that Cys¹⁴⁰ in TM4 lies within or is closely adjacent to the substrate-translocation channel of the transporter. TM4 has been shown previously to be within a structural domain (TMs 3–6) responsible for interactions with NBMPR, vasoactive drugs and nucleobases.

Key words: *p*-chloromercuriphenyl sulphonate, PCMBs, uridine transport, *Xenopus* oocyte.

INTRODUCTION

Most natural and synthetic nucleosides are relatively hydrophilic, and specialized plasma-membrane nucleoside transporter (NT) proteins are required for their uptake or release from cells [1,2]. NT-mediated cellular uptake is a critical determinant of nucleoside metabolism and the pharmacological actions of anti-neoplastic and anti-viral nucleoside drugs [3–5]. By regulating adenosine concentrations in the vicinity of cell-surface receptors, NTs also have profound effects on neurotransmission, vascular tone and other processes [6,7]. In human and other mammalian cells, seven plasma-membrane nucleoside-transport processes that differ in their cation dependence, permeant selectivities and inhibitor sensitivities have been observed. The major (*cit* and *cif*) and minor (*cib*, *csg*, *cs*) concentrative NTs [where *c* is concentrative, *s* and *i* are sensitive and insensitive to inhibition by nitrobenzylthioinosine {6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine, NBMPR}], respectively, *f* is formycin B (non-metabolized purine nucleoside), *t* is thymidine, *g* is guanosine and *b* is broad selectivity] are inwardly directed Na⁺-dependent processes and have been demonstrated functionally in specialized cells such as intestinal and renal epithelia, liver, choroid plexus, other regions of the brain, splenocytes, macrophages and leukaemic cells [1,2]. The equilibrative (bidirectional; *e*) transport processes (*es* and *ei*) have generally lower substrate affinities than the concentrative transporters and occur in most, possibly all, cell types [1,2].

Molecular cloning and *Xenopus* oocyte expression studies have identified cDNAs encoding the human and rat proteins responsible for each of the major concentrative and equilibrative NT processes (*cit*, *cif*, *es* and *ei*) operative in mammalian cells [8–17]. These proteins comprise two previously unrecognized families of integral membrane proteins (concentrative nucleoside transporters, CNTs, and equilibrative nucleoside transporters, ENTs) with quite different predicted architectural designs [1,2]. The relationships of these NT proteins with the processes defined by functional studies are as follows: CNT1 and *cit*, CNT2 and *cif*, ENT1 and *es*, and ENT2 and *ei*. The human and rat ENT1 proteins (hENT1, rENT1) and ENT2 proteins (hENT2, rENT2) transport both purine and pyrimidine nucleosides and are distinguished functionally by a difference in sensitivity to inhibition by NBMPR, h/rENT2 (*ei*) being NBMPR-insensitive. They also differ in sensitivity to inhibition by the vasoactive drugs dipyridamole, dilazep and drafazine (hENT1 > hENT2 > rENT1 = rENT2), and by the ability of h/rENT2 to interact with nucleobases as well as nucleosides. Structurally, the four proteins have a common predicted membrane topology of 11 potential transmembrane segments (TMs) with a large extracellular glycosylated loop between TMs 1 and 2, and a large intracellular loop between TMs 6 and 7.

In the present study, we have investigated uridine transport mediated by recombinant hENT1, rENT1, hENT2 and rENT2 in *Xenopus* oocytes following exposure to the impermeant thiol-reactive reagent *p*-chloromercuriphenyl sulphonate (PCMBs).

Abbreviations used: NT, nucleoside transporter; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; hENT, human ENT; rENT1, rat ENT1; NBMPR, nitrobenzylthioinosine {6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine}; PCMBs, *p*-chloromercuriphenyl sulphonate; TM, transmembrane segment; *c*, concentrative; *e*, equilibrative; *s* and *i*, sensitive and insensitive to inhibition by NBMPR, respectively; *f*, formycin B; *t*, thymidine; *g*, guanosine; *b*, broad selectivity.

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Only rENT2 was inhibited by PCMBS. A unique Cys residue (Cys¹⁴⁰) in rENT2 located in the outer half of TM4 and within (or close to) the substrate-translocation channel of the transporter was demonstrated to be responsible for transport inhibition. This TM is within a structural domain (TMs 3–6) that is also responsible for interactions with NBMPR, vasoactive drugs and nucleobases.

EXPERIMENTAL PROCEDURES

Construction of rENT2/C140S

cDNA for wild-type rENT2 (GenBankTM accession number AF015305) in the vector pGEM-T (Promega) was cloned as described previously [16]. Mutation of Cys¹⁴⁰ to Ser in TM4 of rENT2 to produce rENT2/C140S was performed by the megaprimer PCR mutagenesis method using *Pfu* DNA polymerase [18]. First-round amplification employed a sense primer corresponding to rENT2 nucleotide positions 562–590 with Cys¹⁴⁰ replaced by Ser (5'-CATCAATTCCTTCTCTGCAGTGCTTC-AAG-3') and an antisense primer for rENT2 nucleotide positions 812–832 (5'-CTTCTTGGTGAGGTAGTAACG-3'). The resulting megaprimer (size 270 bp) was gel-purified and used in a second round of PCR in conjunction with a primer corresponding to the SP6 site of pGEM-T. The mutation-containing PCR product was digested with *Spe*I and *Kpn*I, and ligated into *Spe*I- and *Kpn*I-digested wild-type rENT2. The rENT2/C140S cDNA was sequenced in both directions between the *Spe*I and *Kpn*I sites by *Taq* DyeDeoxy terminator cycle sequencing to ensure that the correct mutation had been introduced.

In vitro transcription and expression in *Xenopus* oocytes

Recombinant human and rat ENTs were produced in *Xenopus laevis* oocytes by standard procedures [14–16]. Plasmid cDNAs encoding hENT1 [14] and hENT2 [16] in the vector pBluescript II KS (+) were linearized with *Not*I, and transcribed with T3 RNA polymerase in the presence of cap analogue [^m7G(5')ppp(5')G] using the mMessage mMachineTM transcription system (Ambion). Plasmid cDNAs encoding rENT1 [15], rENT2 [15] and rENT2/C140S in the vector pGEM-T were linearized with *Sph*I and transcribed in the same system with SP6 RNA polymerase. Healthy defolliculated stage-VI oocytes of *X. laevis* were microinjected with 20 nl of water or 20 nl of water containing RNA transcript (1 ng/nl) and incubated in modified Barth's medium at 18 °C for 72 h prior to the assay of uridine-transport activity. We have established previously that oocytes lack endogenous uridine-transport processes [8,19].

Transport assays

Uptake of uridine, which is only slowly metabolized by oocytes [19], was traced with ¹⁴C-labelled uridine (Amersham; 1 µCi/ml). Assays using an incubation period of 2 min [14–16] to measure initial rates of transport (influx) were performed at 20 °C on groups of 10–12 oocytes in transport buffer (0.2 ml) containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes, pH 7.5. Unless otherwise indicated, the uridine concentration was 10 µM. In inhibition studies, oocytes were pretreated with PCMBS (0.1 mM, unless otherwise indicated) on ice for 30 min, and washed subsequently five times with ice-cold transport buffer to remove excess organomercurial before the assay of transport activity. To demonstrate substrate protection, various concentrations of unlabelled uridine (50 µM–10 mM) were included along with PCMBS during the ice-cold preincubation step. Reversal of PCMBS inhibition was achieved by a second preincubation (20 °C, 30 min) in the

presence of 1 mM dithiothreitol. After the 2 min assay period, extracellular radioactivity was removed by six rapid washes in ice-cold transport buffer. Individual oocytes were dissolved in 5% (w/v) SDS for quantification of radioactivity by liquid scintillation counting.

Results for transport experiments are given as means ± S.E.M. for 10–12 individual oocytes. Each experiment was performed at least twice on different batches of oocytes.

RESULTS AND DISCUSSION

Effects of PCMBS on recombinant human and rat ENT1 and ENT2

PCMBS is a membrane-impermeant thiol reagent that reacts selectively and reversibly with protein Cys residues. It acts as an inhibitor of *es* nucleoside-transport activity in erythrocytes from various species (human, rat, sheep), but only in unsealed membranes or inside-out vesicles where PCMBS has access to the internal membrane surface [20–22]. In intact cells and right-side-out membrane vesicles, the organomercurial has no effect. In contrast, *ei* nucleoside-transport activity in rat erythrocytes shows sensitivity to PCMBS inhibition in intact cells [22]. Loss of *ei* transport function is prevented by extracellular uridine, suggesting that PCMBS causes transport inhibition by reacting with exofacial Cys residue(s) within or closely adjacent to the substrate-translocation channel of the transporter.

Initial experiments compared the effects of PCMBS on recombinant human and rat ENT1 (*es*) and ENT2 (*ei*) produced individually in *Xenopus* oocytes. RNA transcripts from each transporter cDNA were synthesized by *in vitro* transcription and microinjected into oocytes, which were then assayed for nucleoside-transport activity (10 µM uridine influx, 20 °C) following preincubation with 0.1 mM PCMBS, as described in the Experimental procedures section. PCMBS treatment was performed at 1 °C to minimize diffusion of the organomercurial across the lipid bilayer [20–22]. Endogenous nucleoside uptake was measured in water-injected oocytes (typically ≈ 2% of that in RNA-transcript-injected oocytes). As shown by the representative experiment in Table 1, the four transporters exhibited similar functional activities in the range 0.9–1.8 pmol of uridine transported/oocyte per 2 min. In agreement with previous studies of the corresponding native erythrocytic NTs [21,22], PCMBS treatment had no significant effect on uridine transport by recombinant hENT1 or rENT1. Although the effect of PCMBS on the native human *ei* transport process has not been studied, Table 1 shows that recombinant hENT2 behaved like hENT1 and rENT1 and was also PCMBS-insensitive. In marked contrast,

Table 1 Effects of PCMBS on uridine uptake by hENT1, hENT2, rENT1 and rENT2

Oocytes producing recombinant hENT1, hENT2, rENT1 or rENT2 were incubated in transport buffer at 1 °C for 30 min in the absence or presence of 0.1 mM PCMBS, washed free of excess inhibitor with ice-cold transport buffer and assayed for uridine uptake (10 µM, 2 min flux) at 20 °C as described in the Experimental procedures section. Each value is the mean ± S.E.M. from 10–12 oocytes.

	Uridine influx (pmol/oocyte per 2 min)	
	Control	PCMBS-treated
rENT1	0.93 ± 0.08	0.85 ± 0.07
rENT2	1.24 ± 0.11	0.39 ± 0.08
hENT1	1.33 ± 0.09	1.15 ± 0.07
hENT2	1.78 ± 0.09	1.67 ± 0.06
Water-injected oocytes	0.03 ± 0.01	0.04 ± 0.01

Figure 1 Concentration-dependence of PCMBS inhibition of rENT2

Oocytes producing recombinant rENT2 were incubated in transport buffer at 1 °C for 30 min in the presence of varying concentrations of PCMBS (0–5 mM), washed free of excess inhibitor with ice-cold transport buffer, and assayed for uridine uptake (10 μM, 2 min flux) at 20 °C. Values are the means ± S.E.M. from 10–12 oocytes and are corrected for basal (non-mediated) uridine uptake in water-injected oocytes.

but consistent with earlier results [22] for the erythrocytic *ei* transport process, uridine uptake by recombinant rENT2 was inhibited markedly by PCMBS. Basal non-mediated uptake of uridine in water-injected oocytes was unaffected by PCMBS treatment.

PCMBS inhibition of recombinant rENT2

Subsequent experiments investigated the interaction of PCMBS with recombinant rENT2 in more detail. In previous studies of native *ei* transport activity in rat erythrocytes, the concentration of PCMBS required to cause 50% inhibition of transport activity was $\approx 20 \mu\text{M}$ [22]. The corresponding IC_{50} value for PCMBS inhibition of recombinant rENT2 in oocytes, obtained by linear regression analysis of an indirect Hill-plot transformation of the dose–response curve shown in Figure 1, was $27 \mu\text{M}$. The Hill coefficient was 1.1, consistent with a single site of PCMBS binding. In Figure 1, some uptake remained after exposure of cells to the highest concentration of PCMBS (5 mM). The magnitude of this residual flux was time-dependent and was decreased $\approx 50\%$ by extending the period of PCMBS treatment from 30 min to 1 h. More extensive exposure to PCMBS rendered the oocytes fragile. Incubation of PCMBS-treated oocytes with dithiothreitol resulted in full recovery of transport activity (Table 2A), establishing that PCMBS inhibited rENT2 transport activity by binding to a Cys residue. Dithiothreitol on its own has no effect on rENT2-mediated uridine transport in untreated oocytes (Table 2A).

The results presented in Table 2(B) demonstrate that nucleoside substrate (10 mM uridine) added to the external medium during PCMBS treatment protected the transporter against PCMBS inhibition. The concentration of uridine required to give 50% protection of transport activity (0.2 mM, Figure 2) corresponded closely to the rENT2 apparent K_m value for zero-*trans* uridine

Table 2 Reversal of PCMBS inhibition of rENT2 by dithiothreitol and protection by uridine

(A) Oocytes producing recombinant rENT2 were subjected to consecutive incubations at 1 °C for 30 min with 0.1 mM PCMBS, and 20 °C for 30 min with and without 1 mM dithiothreitol (DTT), after which uptake of uridine was determined (10 μM, 2 min flux at 20 °C) and compared with that in control rENT2 oocytes that had been incubated under similar conditions without PCMBS or dithiothreitol, or with dithiothreitol alone. (B) rENT2 oocytes were incubated with PCMBS (0.1 mM) at 1 °C for 30 min in the presence or absence of unlabelled uridine (10 mM), after which uptake of radiolabelled uridine was determined as described in (A) and compared with that in control rENT2 oocytes that were incubated without PCMBS or uridine. Oocytes in (A) and (B) were washed between incubations and before the assay of transport activity to remove excess reagents. Each value is the mean ± S.E.M. from 10–12 oocytes. NA, not applicable.

(a)

	Uridine influx (pmol/oocyte per 2 min)	
	rENT2	Water-injected oocytes
Control	1.15 ± 0.08	0.04 ± 0.01
DTT	1.10 ± 0.08	0.03 ± 0.01
PCMBS	0.35 ± 0.05	0.05 ± 0.02
PCMBS + DTT	0.97 ± 0.06	0.02 ± 0.01

(b)

	Uridine influx (pmol/oocyte per 2 min)	
	rENT2	Water-injected oocytes
Control	0.86 ± 0.09	0.01 ± 0.003
PCMBS	0.31 ± 0.04	NA
PCMBS + uridine	0.98 ± 0.09	NA

Figure 2 Concentration-dependence of uridine protection of rENT2

Oocytes producing recombinant rENT2 were incubated with PCMBS (0.1 mM) at 1 °C for 30 min in the presence or absence of increasing concentrations of unlabelled uridine, after which uptake of radiolabelled uridine was determined (10 μM, 2 min flux at 20 °C) and compared with that in control rENT2 oocytes that were incubated without PCMBS or uridine. Oocytes were washed before the assay of transport activity to remove excess inhibitor and substrate. Values are the means ± S.E.M. from 10–12 oocytes. Uridine influx is presented as a percentage of the control uninhibited flux corrected for basal (non-mediated) uridine uptake in water-injected cells.

Figure 3 Cysteine residues in hENT1, hENT2, rENT1 and rENT2 and topology model of rENT2

(Top panel) An alignment of the predicted amino acid sequences of hENT1 (GenBank accession number U81375), hENT2 (AF029358), rENT1 (AF015304) and rENT2 (AF015305). The positions of putative TMs are shown by boxes, and Cys residues are shown on black squares. Cys¹⁴⁰ of rENT2 is highlighted by *. (Bottom panel) The locations of Cys¹⁴⁰ and other rENT2 Cys residues in relation to the predicted membrane topology of the transporter. Potential TMs are numbered. Chimaeric studies have implicated the helix 3–6 structural domain in interactions with NBMPR, vasoactive drugs and nucleobases.

Table 3 Effects of PCMBS on uridine uptake by rENT1, rENT2 and rENT2/C140S

Oocytes producing recombinant rENT1, rENT2 or rENT2/C140S were incubated in transport buffer at 1 °C for 30 min in the absence or presence of 0.1 mM PCMBS, washed free of excess inhibitor with ice-cold transport buffer and assayed for uridine uptake (10 μ M, 2 min flux) at 20 °C as described in the Experimental procedures section. Each value is the mean $\pm$ S.E.M. from 10–12 oocytes.

	Uridine influx (pmol/oocyte per 2 min)	
	Control	PCMBS-treated
rENT1	1.52 $\pm$ 0.14	1.56 $\pm$ 0.15
rENT2	1.67 $\pm$ 0.06	0.47 $\pm$ 0.05
rENT2/C140S	1.34 $\pm$ 0.16	1.42 $\pm$ 0.14
Water-injected oocytes	0.09 $\pm$ 0.01	0.11 $\pm$ 0.02

influx in oocytes (0.3 mM, 20 °C) [15], and was similar to that determined previously for uridine protection of native *ei* transport activity in rat erythrocytes (0.4 mM) [22].

The $V_{\max}$ for rENT2-mediated entry of uridine into oocytes at the temperature used for PCMBS treatment (1 °C) was determined to be extremely low (< 5 pmol/oocyte per 30 min). Since 5 pmol/oocyte corresponds to an approximate intracellular concentration of 5 μ M, the protective effect of uridine against PCMBS inhibition occurred under conditions where there was little transport of nucleoside into the cell. Cellular uptake of PCMBS is also minimal at this temperature [23]. It can be concluded, therefore, that the Cys residue(s) in rENT2 that was sensitive to inhibition by PCMBS was located on the exterior surface of the membrane, a suggestion supported by the rapid onset of PCMBS inhibition of *ei* transport activity in erythrocytes [15]. Substrate protection by concentrations of uridine in the range of its apparent K_m for zero-*trans* influx suggests a location within or closely adjacent to the nucleoside-translocation channel.

Amino acid sequences and predicted membrane topology of human and rat ENT1 and ENT2 proteins

The hENT1 and rENT1 (*es*-type) and hENT2 and rENT2 (*ei*-type) NTs contain 456–457 amino acid residues and share 49–89% sequence identity and 58–91% sequence similarity (Figure 3, top panel). Hydropathy analyses suggest a common membrane architecture with 11 potential TMs connected by short (≤ 15 residues) hydrophilic regions, with the exceptions of larger loops linking TMs 1 and 2 and TMs 6 and 7, which are predicted to contain 27–41 and 66–80 residues, respectively. Only the first of these large loops is glycosylated [14–16,24–26], leading to the predicted topology shown for rENT2 in Figure 3 (bottom panel).

hENT1 and hENT2 each contain 10 Cys residues, whereas rENT1 and rENT2 contain 15 and 11, respectively (Figure 3, top panel). Of the 11 Cys residues in rENT2, only one (Cys¹⁴⁰) is not conserved in one or more of the other ENTs (Figure 3, top panel). This Cys, which is putatively located in the outer half of TM4 (Figure 3, bottom panel), is therefore the only candidate residue that could be responsible for the unique exofacial sensitivity of recombinant rENT2 to inhibition by PCMBS.

PCMBS sensitivity of rENT2/C140S

Involvement of Cys¹⁴⁰ in PCMBS inhibition of rENT2 was tested experimentally by site-directed mutagenesis. Conversion of rENT2 Cys¹⁴⁰ into Ser (the residue at this position in rENT1) gave

Figure 4 Kinetic properties of rENT2 and rENT2/C140S

The concentration-dependence of radiolabelled uridine uptake (2 min flux at 20 °C) was determined in oocytes injected with water alone (○) or with water containing RNA transcript (●) for rENT2 (A) or rENT2/C140S (B). Each value is the mean $\pm$ S.E.M. from 10–12 oocytes. Apparent K_m and $V_{\max}$ values for the mediated component of influx (uptake in RNA-injected oocytes minus uptake in water-injected oocytes), determined by non-linear regression analysis (ENZFITTER, Elsevier-Biosoft), are presented in the text.

a functional mutant protein (rENT2/C140S) with uridine-transport activity similar to that of wild-type recombinant rENT1 and rENT2. In the representative experiment presented in Table 3, mediated uridine uptake (10 μ M, 2 min) by rENT2/C140S, calculated as the difference in uptake by RNA- and water-injected oocytes, was 1.3 pmol/oocyte per 2 min, compared with 1.4 and 1.6 pmol/oocyte per 2 min for rENT1 and rENT2, respectively. Since the expressed transport activity was comparable with wild-type rENT2, Cys¹⁴⁰ was not essential for nucleoside-transport activity. Kinetic parameters for rENT2/C140S and wild-type rENT2 were indistinguishable, with apparent K_m values of 238 ± 37 and 244 ± 32 μ M, respectively, and $V_{\max}$ values of 30 ± 2 and 32 ± 2 pmol/oocyte per 2 min, respectively (Figure 4). As predicted by the sequence comparisons in Figure 3 (top panel), rENT2/C140S resembled rENT1 by

being unaffected by treatment with 0.1 mM PCMBS, whereas, in the same experiment with the same batch of oocytes, uridine uptake by rENT2 was inhibited by 77 % (Table 3). Higher concentrations of PCMBS were also without effect (results not shown).

Conclusions

We have identified in rENT2 a unique Cys residue (Cys¹⁴⁰) that is responsible for sensitivity to inhibition by extracellular PCMBS. This Cys residue is located in the outer half of putative TM4 (Figure 3, bottom panel) and is within a region of the transporter (TMs 3–6) implicated previously by hENT1/rENT1 chimaeric studies to be involved in transport inhibition of ENT proteins by dipyrindamole and other vasoactive drugs [27]. Studies with chimaeras produced with various rENT1/rENT2 and hENT1/rENT2 combinations have also identified TMs 3–6 as being involved in NBMPR binding ([28] and S. Y. M. Yao, M. Sundaram, R. J. Hyde, F. Abidi, M. Griffiths, E. G. Chomey, S. E. V. Phillips, C. E. Cass, S. A. Baldwin and J. D. Young, unpublished work). Mutation of Gly¹⁵⁴ in hENT1 to the corresponding residue in hENT2 converted the transporter from NBMPR-sensitivity into NBMPR-insensitivity [28]. The latter residue (Gly¹⁵⁴) occupies the same position in TM4 of hENT1 as Cys¹⁴⁰ in rENT2 (Figure 3, top panel).

The finding that binding of PCMBS to rENT2 Cys¹⁴⁰ was prevented by uridine suggests that this residue and the helix to which it belongs (TM 4) form part of the nucleoside-translocation channel of the transporter. The same rENT1/rENT2 chimaeras used to study NBMPR binding have also implicated TMs 3–6 in nucleobase transport (S. Y. M. Yao, M. Sundaram, R. J. Hyde, F. Abidi, M. Griffiths, E. G. Chomey, S. E. V. Phillips, C. E. Cass, S. A. Baldwin and J. D. Young, unpublished work). It has been found, for example, that insertion of TMs 3–6 of rENT2 (which transports nucleobases as well as nucleosides) into rENT1 (which does not transport nucleobases) results in acquisition of nucleobase-transport activity. This provides additional evidence that parts of the TMs 3–6 structural domain contribute to the translocation channel of the transporter, and argues against the possibility that uridine protects Cys¹⁴⁰ against PCMBS inhibition through long-range, substrate-induced conformational changes.

The conclusion, based on studies of recombinant ENTs, that parts of the nucleoside/nucleobase-transport machinery co-localize with the sites of NBMPR and dipyrindamole binding to the same region of the transporter, provides a structural basis to interpret previous functional and molecular studies of native erythrocytic *es* (hENT1) proteins. Kinetic and ligand-binding experiments have shown that NBMPR (a substrate analogue) and vasoactive drugs function as competitive inhibitors of zero-trans nucleoside influx and interact with the transporter at common or overlapping exofacial sites [1,2,29–31]. Previous photoaffinity labelling studies with the *es* transporter purified from human erythrocytes established that the site of covalent attachment of NBMPR was within 16 kDa of the site of N-linked glycosylation (Asn⁴⁸ in the loop between TMs 1 and 2) [24].

Since PCMBS had access only to the external membrane surface, and since uridine protection against PCMBS inhibition occurred under conditions where there was minimal intracellular accumulation of substrate, the identification of Cys¹⁴⁰ as the residue in rENT2 responsible for PCMBS inhibition validates the predicted membrane architecture of TM4 and adjacent helices in Figure 3 (bottom panel), and provides a basis for future structure/function and topological analyses.

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REFERENCES

- Baldwin, S. A., Mackey, J. R., Cass, C. E. and Young, J. D. (1999) Nucleoside transporters: molecular biology and implications for therapeutic development. *Mol. Med. Today* **5**, 216–224.
- Cass, C. E., Young, J. D., Baldwin, S. A., Cabrita, M. A., Graham, K. A., Griffiths, M., Jennings, L. L., Mackey, J. R., Ng, A. M. L., Ritzel, M. W. L., Vickers, M. F. and Yao, S. Y. M. (2000) Nucleoside transporters of mammalian cells. In *Membrane Transporters as Drug Targets* (Amidon, G. and Sadée, W., eds.), pp. 313–352, Plenum Publishing, New York.
- Perigaud, C., Gosselin, G. and Imbach, J. L. (1992) Nucleoside analogues as chemotherapeutic agents: a review. *Nucleosides Nucleotides* **11**, 903–945.
- Handschumacher, R. E. and Cheng, C. Y. (1993) Purine and pyrimidine antimetabolites. In *Cancer Metabolism* (Holland, E., Frei, E., Bast, R. C., Kufe, D. W., Morton, D. L. and Weichselbaum, R. R., eds.), pp. 712–732, Lea & Febiger, Philadelphia.
- Mackey, J. R., Baldwin, S. A., Young, J. D. and Cass, C. E. (1998) Nucleoside transport and its significance for anticancer drug resistance. *Drug Resistance Updates* **1**, 310–324.
- Fredholm, B. B. (1997) Adenosine and neuroprotection. *Int. Rev. Neurobiol.* **40**, 259–280.
- Shryock, J. C. and Belardinelli, L. (1997) Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. *Am. J. Cardiol.* **79**, 2–10.
- Huang, Q. Q., Yao, S. Y. M., Ritzel, M. W. L., Paterson, A. R. P., Cass, C. E. and Young, J. D. (1994) Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transport protein. *J. Biol. Chem.* **269**, 17757–17760.
- Che, M., Ortiz, D. F. and Arias, I. M. (1995) Primary structure and functional expression of a cDNA encoding the bile canalicular, purine-specific Na⁺(+)-nucleoside cotransporter. *J. Biol. Chem.* **270**, 13596–13599.
- Yao, S. Y. M., Ng, A. M. L., Ritzel, M. W. L., Gatl, W. P., Cass, C. E. and Young, J. D. (1996) Transport of adenosine by recombinant purine- and pyrimidine-selective sodium/nucleoside cotransporters from rat jejunum expressed in *Xenopus laevis* oocytes. *Mol. Pharmacol.* **50**, 1529–1535.
- Ritzel, M. W. L., Yao, S. Y. M., Huang, M. Y., Elliot, J. F., Cass, C. E. and Young, J. D. (1997) Molecular cloning and functional expression of cDNAs encoding a human Na⁺-nucleoside cotransporter (hCNT1). *Am. J. Physiol.* **272**, C707–C714.
- Wang, J., Su, S. F., Dresser, M. J., Schaner, M. E., Washington, C. B. and Giacomini, K. M. (1997) Na⁺(+)-dependent purine nucleoside transporter from human kidney: cloning and functional characterization. *Am. J. Physiol.* **273**, F1058–F1065.
- Ritzel, M. W. L., Yao, S. Y. M., Ng, A. M. L., Mackey, J. R., Cass, C. E. and Young, J. D. (1998) Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na⁺/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. *Mol. Membr. Biol.* **15**, 203–211.
- Griffiths, M., Beaumont, N., Yao, S. Y. M., Sundaram, M., Bouman, C. E., Davies, A., Kwong, F. Y. P., Coe, I. R., Cass, C. E., Young, J. D. and Baldwin, S. A. (1997) Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. *Nat. Med.* **3**, 89–93.
- Yao, S. Y. M., Ng, A. M. L., Muzyka, W. R., Griffiths, M., Cass, C. E., Baldwin, S. A. and Young, J. D. (1997) Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (*es*) and NBMPR-insensitive (*ei*) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues. *J. Biol. Chem.* **272**, 28423–28430.
- Griffiths, M., Yao, S. Y. M., Abidi, F., Phillips, S. E. V., Cass, C. E., Young, J. D. and Baldwin, S. A. (1997) Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (*ei*) equilibrative nucleoside transporter from human placenta. *Biochem. J.* **328**, 739–743.
- Crawford, C. R., Patel, D. H., Naeve, C. and Belt, J. A. (1998) Cloning of the human equilibrative, nitrobenzylmercaptopyrine riboside (NBMPR)-insensitive nucleoside transporter *ei* by functional expression in a transport-deficient cell line. *J. Biol. Chem.* **273**, 5288–5293.
- Sarkar, G. and Sommer, S. S. (1990) The “megaprimer” method of site-directed mutagenesis. *BioTechniques* **8**, 404–407.
- Huang, Q. Q., Harvey, C. M., Paterson, A. R. P., Cass, C. E. and Young, J. D. (1993) Functional expression of Na⁺(+)-dependent nucleoside transport systems of rat intestine in isolated oocytes of *Xenopus laevis*. Demonstration that rat jejunum expresses the purine-selective system N1 (*cif*) and a second, novel system N3 having broad specificity for purine and pyrimidine nucleosides. *J. Biol. Chem.* **268**, 20613–20619.

- 20 Jarvis, S. M. and Young, J. D. (1982) Nucleoside translocation in sheep reticulocytes and fetal erythrocytes: a proposed model for the nucleoside transporter. *J. Physiol. (London)* **324**, 47–66
- 21 Tse, C.-M., Wu, J.-S. R. and Young, J. D. (1985) Evidence for the asymmetrical binding of *p*-chloromercuriphenyl sulphonate to the human erythrocyte nucleoside transporter. *Biochim. Biophys. Acta* **818**, 316–324
- 22 Jarvis, S. M. and Young, J. D. (1986) Nucleoside transport in rat erythrocytes: two components with differences in sensitivity to inhibition by nitrobenzylthioinosine and *p*-chloromercuriphenyl sulfonate. *J. Membr. Biol.* **93**, 1–10
- 23 Rothstein, A. (1970) Sulfhydryl groups in membrane structure and function. *Curr. Top. Membr. Transp.* **1**, 135–176
- 24 Kwong, F. Y. P., Wu, J. S. R., Fincham, H. E., Davies, A., Henderson, P. J. F., Baldwin, S. A. and Young, J. D. (1993) Enzymic cleavage as a probe of the molecular structures of mammalian equilibrative nucleoside transporters. *J. Biol. Chem.* **268**, 22127–22134
- 25 Vickers, M. F., Mani, R. S., Sundaram, M., Hogue, D. L., Young, J. D., Baldwin, S. A. and Cass, C. E. (1999) Functional production and reconstitution of the human equilibrative nucleoside transporter (hENT1) in *Saccharomyces cerevisiae*. Interaction of inhibitors of nucleoside transport with recombinant hENT1 and a glycosylation-defective derivative (hENT1/N48Q). *Biochem. J.* **339**, 21–32
- 26 Kwong, F. Y. P., Fincham, H. E., Davies, A., Beaumont, N., Henderson, P. J. F., Young, J. D. and Baldwin, S. A. (1992) Mammalian nitrobenzylthioinosine-sensitive nucleoside transport proteins. Immunological evidence that transporters differing in size and inhibitor specificity share sequence homology. *J. Biol. Chem.* **267**, 21954–21960
- 27 Sundaram, M., Yao, S. Y. M., Ng, A. M. L., Griffiths, M., Cass, C. E., Baldwin, S. A. and Young, J. D. (1998) Chimeric constructs between human and rat equilibrative nucleoside transporters (hENT1 and rENT1) reveal hENT1 structural domains interacting with coronary vasoactive drugs. *J. Biol. Chem.* **273**, 21519–21525
- 28 Hyde, R. J., Abidi, F., Griffiths, M., Yao, S. Y. M., Sundaram, M., Phillips, S. E. V., Cass, C. E., Young, J. D. and Baldwin, S. A. (2000) Probing the structure/function relationships of human equilibrative nucleosides transporters using site-directed mutagenesis. *Drug Dev. Res.* **50**, 38
- 29 Jarvis, S. M., McBride, D. and Young, J. D. (1982) Erythrocyte nucleoside transport: asymmetrical binding of nitrobenzylthioinosine to nucleoside permeation sites. *J. Physiol. (London)* **324**, 31–46
- 30 Jarvis, S. M. (1986) Nitrobenzylthioinosine-sensitive nucleoside transport system: mechanism of inhibition by dipyrindamole. *Mol. Pharmacol.* **30**, 659–665
- 31 Agbanyo, F. R., Cass, C. E. and Paterson, A. R. P. (1988) External location of sites on pig erythrocyte membranes that bind nitrobenzylthioinosine. *Mol. Pharmacol.* **33**, 332–337

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