Crystal structure of a concentrative nucleoside transporter from Vibrio cholerae at 2.4 Å

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Nucleosides are required for DNA and RNA synthesis, and the nucleoside adenosine has a function in a variety of signalling processes1–3. Transport of nucleosides across cell membranes provides the major source of nucleosides in many cell types and is also responsible for the termination of adenosine signalling. As a result of their hydrophilic nature, nucleosides require a specialized class of integral membrane proteins, known as concentrative transporters (CNTs), for specific transport across cell membranes. In addition to nucleosides, NTs are important determinants for the transport of nucleoside-derived drugs across cell membranes4–9. A wide range of nucleoside-derived drugs, including anticancer drugs (such as Ara-C and gemcitabine) and antiviral drugs (such as zidovudine and ribavirin), have been shown to depend, at least in part, on NTs for transport across cell membranes4–11. Concentrative nucleoside transporters, members of the solute carrier transporter superfamily SLC28, use an ion gradient in the active transport of both nucleosides and nucleoside-derived drugs against their chemical gradients. The structural basis for selective ion-coupled nucleoside transport by concentrative nucleoside transporters is unknown. Here we present the crystal structure of a concentrative nucleoside transporter from Vibrio cholerae in complex with uridine at 2.4 Å. Our functional data show that, like its human orthologues, the transporter uses a sodium-ion gradient for nucleoside transport. The structure reveals the overall architecture of this class of transporter, unravels the molecular determinants for nucleoside and sodium binding, and provides a framework for understanding the mechanism of nucleoside and nucleoside drug transport across cell membranes.

Humans have three isoforms of concentrative nucleoside transporter (hCNT), and the substrate specificities and tissue distributions of these isoforms are different12–18. Knowledge of the mechanism of these transporters would help us not only to understand physiological processes associated with nucleosides but also to provide a framework for future drug design to improve nucleoside drug delivery. The major barrier to achieving a mechanistic understanding is the lack of atomic structures that reveal the origins of nucleoside specificity and the principles of function of CNTs. For structure determination and functional studies we chose a CNT homologue from Vibrio cholerae (vcCNT) because of its high sequence homology to hCNTs (39% identical to hCNT3; Supplementary Fig. 1) and optimal biochemical stability.

To test whether vcCNT can transport nucleosides, and if so what ion gradient vcCNT uses, we performed a radioactive nucleoside uptake assay using recombinant vcCNT–reconstituted liposomes. Studies have shown that the human CNTs use Na+ ions and the Escherichia coli CNT uses H+ for nucleoside transport17–19. Transport activity was measured by monitoring the uptake of [5,6-3H]uridine in both the presence and the absence of a Na+ gradient (Fig. 1a). We chose uridine because all of the CNTs that have been characterized so far transport uridine14–19. When a Na+ gradient was present, vesicles containing vcCNT accumulated radioactive uridine significantly more than the control empty vesicles. When a Na+ gradient was not present (Na+ was replaced with choline in the solution), uridine uptake was significantly decreased.

Figure 1 | vcCNT is a Na+-coupled nucleoside transporter with a trimeric architecture. a, Time course of the uptake of 2.4 μM [5,6-3H]uridine into vesicles containing vcCNT in the presence of a Na+ gradient (squares, NaCl), in the absence of a Na+ gradient (triangles, choline chloride), and the control empty vesicles in the presence of Na+ (diamonds, empty). Results are means ± s.d. (n = 3). b, Cartoon representation of the vcCNT trimer viewed from the cytoplasm. Individual protomers are coloured blue, red and green. c, Cartoon representation for the vcCNT trimer viewed parallel to the membrane. The putative membrane bilayer is indicated in c with grey boxes. The dimensions of the putative membrane bilayer, extracellular region, and intracellular region are shown.
Further characterization showed that uridine uptake was electrogenic and independent of pH gradients (Supplementary Fig. 2). These data clearly show that vcCNT, like human CNTs, uses a Na\(^+\) gradient to transport nucleosides.

We crystallized and solved the structure of vcCNT at 2.4 Å (Supplementary Table 1). Experimental phases to 3.5 Å were obtained by single anomalous dispersion (SAD) from a platinum-soaked crystal (see Methods). The final model contains a single protomer in the asymmetric unit and is of good quality with a free R factor of 22.8% (Supplementary Table 1).

vcCNT crystallizes as a homotrimer that is shaped like an inverted triangular basin with its mouth facing the intracellular side and a knob-like structure facing the extracellular side (Fig. 1b, c). The three-fold axis coincides with the crystallographic six-fold axis and is perpendicular to the membrane. When viewed from the intracellular side, each side of the triangle formed by the trimer is about 92 Å, and when viewed parallel to the membrane the trimer is about 57 Å in height (Fig. 1). The membrane-embedded region lies roughly in the middle of the transporter, judging from the positions of three amphipathic helices on each protomer. On the basis of the predicted location of the membrane bilayer, the mouth of the basin penetrates into the membrane plane (Fig. 1c and Supplementary Fig. 3). The presence of several polar amino acids on the basin surface probably allows bulk aqueous solution to reach deep into the membrane bilayer and access this surface of the transporter.

The structure reveals that each protomer contains eight transmembrane helices (TM1–TM8), two re-entrant helix-turn-helix hairpins (HP1 and HP2) with opposite orientations in the membrane, and three interfacial helices (IH1–IH3) that run parallel to the membrane (Fig. 2a). The orientation of the structure relative to the membrane is consistent with previous accessibility studies and conforms to the positive-inside rule (Supplementary Fig. 4). hCNTs are predicted to contain three more amino-terminal transmembrane helices than prokaryotic CNTs, suggesting an 11-TM topology for hCNTs\(^{20}\).

Each protomer can be grouped into two subdomains on the basis of their locations (at the outer and inner regions) relative to the centre of the protomer. TM1, TM2, IH1, EH (a short stretch of extracellular helices), TM3 and TM6 are located at the outer part of the transporter, and they seem to be important for maintaining the overall architecture of the transporter. These helices in the outer region form a scaffold for the transporter (hereafter termed the scaffold domain) (Fig. 2). Trimerization contacts are mediated by part of the scaffold domain: IH1, EH, TM3 and TM6 (Fig. 2b). IH1 is an amphipathic helix 40 Å long that is most probably situated at the water/membrane interface, and TM6 is about 60 Å long and tilted almost 60° with respect to the membrane normal. EH protrudes into the extracellular solution and is about 16 Å in length. Because IH1 is involved in trimerization, is constrained to be at the membrane/water interface and interacts with many TMs (TM2–TM5), it is most probably important in building and maintaining the overall structure of vcCNT.

Surrounded by the scaffold domain, many conserved amino acids implicated in nucleoside transport are localized at the inner domain (hereafter termed the transport domain). The transport domain is composed of two structural groups that are related by an internal two-fold pseudo-symmetry. The first group is composed of IH2, HP1, TM4a/b and TM5 (pink background in Fig. 2a), and the second group is composed of IH3, HP2, TM7a/b and TM8 (cyan background in Fig. 2a). These two groups, separated by TM6 in sequence, can be superimposed with a root mean squared deviation of 3.3 Å, with the two-fold symmetry operator running though the middle of TM6 and through 120°. Only TM1, TM6 and helices comprising the group of helices under the cyan triangular background are labelled. d, As in c but rotated through 120°. The scaffold domain has been removed to show the pseudo-two-fold relationship between the two groups of helices, coloured as pink and cyan triangular backgrounds. The two-fold symmetry axis runs through the nucleoside at the centre of the transporter.

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**Figure 2 | Topology and fold of the vcCNT protomer.** a, Schematic representation of vcCNT topology. The group of helices under the pink triangular background is related to the group of helices under the cyan triangular background by two-fold pseudo-symmetry, with the symmetry axis parallel to the membrane. b, Cartoon representation of the vcCNT protomer fold. Only helices comprising the scaffold domain and the group of helices under the pink triangular background are labelled. c, As in b but rotated through 120°. Only TM1, TM6 and helices comprising the group of helices under the cyan triangular background are labelled. d, As in c but rotated through 120°. The scaffold domain has been removed to show the pseudo-two-fold relationship between the two groups of helices, coloured as pink and cyan triangular backgrounds. The two-fold symmetry axis runs through the nucleoside at the centre of the transporter.
parallel to the membrane (Fig. 2d). There is no significant amino-acid sequence homology between these two groups (roughly 10% sequence identity). This two-fold symmetry relationship positions the tips of HP1, HP2 and unwound regions of TM4 and TM7 at the centre of the transport domain, which is located slightly below the middle of the membrane plane (Fig. 2d). Sequence alignment of hCNTs and vcCNT reveals high sequence conservation around HP1, HP2 and the two unwound helices (TM4 and TM7), indicating the functional importance of this region (Supplementary Fig. 1).

To the best of our knowledge, the overall fold of vcCNT is novel, although local structural elements such as helical hairpins and unwound helices have been observed previously.\(^{23,24}\)

The crystal structure suggests that vcCNT adopts a trimeric configuration. To test whether the stoichiometry of vcCNT is trimeric and further validate the physiological relevance of our crystal structure, we performed structure-guided disulphide bridge crosslinking experiments (Supplementary Fig. 5). Our cysteine mutants readily form disulphide-crosslinked trimers in both detergent micelles and cell membranes under oxidizing conditions; therefore our crystal structure reflects a physiologically relevant oligomerization state. Given the sequence conservation of most of the amino acids involved in trimerization, we propose that the stoichiometry of both eukaryotic and prokaryotic CNT family members is trimeric (Supplementary Fig. 1).

The structure contains three deep clefts (one per subunit) at the intracellular side facing the centre of the trimer (Fig. 3a). A simulated-annealing OMIT map clearly shows that the electron density in the cleft is that of uridine (Fig. 3b). The bound uridine in the cleft faces the intracellular basin of the transporter; however, it is not free to be released into the intracellular solution because TM6 and TM7b partly cover the binding site (Fig. 3a, b and Supplementary Fig. 6).

The nucleoside-binding site is located at the centre of the internal two-fold symmetry and lined by the tips of HP1 and HP2 and the unwound regions of TM4 and TM7 (Fig. 3b, c). Inspection of the interactions between uridine and the binding site shows that many polar or charged amino acids from HP1, HP2, TM4 and TM7 interact with the uracil base and ribose. HP1 and TM4b are responsible for interacting with the uracil base. The side chains of Glu 154, Thr 155 and Glu 156 from HP1 interact with the uracil base either directly (Glu 154) or indirectly through a water molecule (Thr 155 and Glu 156; Fig. 3c). Val 188 from TM4b interacts with the uracil base by means of van der Waals interactions. The involvement of these amino-acid residues in the interactions with the nucleoside is consistent with previous mutational studies:\(^{25–27}\); the residue corresponding to Gln 154, together with that corresponding to Val 188, is important for nucleoside specificity of hCNTs; Gln 154 is critical for Na\(^+\)-nucleoside coupled transport.

HP2 and TM7 are responsible for the interactions with ribose. The side chains of Glu 332 (HP2), Asn 368 (TM7) and Ser 371 (TM7) interact with the ribose either directly (Glu 332, Asn 368 and Ser 371) or indirectly through a water molecule (Asn 368). Mutation of the residue corresponding to Glu 332 in hCNTs has been shown to have significant functional effects on both nucleoside binding and the rate of transport.\(^{21,26}\)

Because vcCNT is a Na\(^+\)-coupled transporter, it must contain at least one Na\(^+\)-binding site. Initial hints regarding the location of the Na\(^+\)-binding site came from an \(F_o - F_c\) map that shows a strong peak

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**Figure 3 | Nucleoside-binding site and Na\(^+\)-binding site.**

- **a.** Cut-away surface representation of vcCNT viewed parallel to the membrane. The putative membrane bilayer is shown by horizontal lines. Uridine is shown as spheres. **b.** The vcCNT protomer viewed from the centre of the trimer. The scaffold domain is shown in ribbon representation; the transport domain is shown in cartoon representation. The blue mesh, covered by TM6 (green), is an \(F_o - F_c\), simulated annealing OMIT map, contoured at 4\(\sigma\), showing density for uridine.

- **c.** The nucleoside-binding site, showing HP1 (red), TM4b (orange), HP2 (blue) and TM7b (cyan). Hydrogen bonds are shown as dashed lines. **d.** The Na\(^+\)-binding site is located between HP1 (red) and the unwound region of TM4 (orange). The blue mesh is an \(F_o - F_c\) simulated annealing OMIT map, contoured at 6\(\sigma\), showing density for Na\(^+\). Coordination of the Na\(^+\) ion is shown as dashed lines. **e.** The Na\(^+\)-binding site is near the nucleoside-binding site.
vcCNT was crystallized in the presence of 100 mM CaCl₂, 37–42% PEG400 and 100 mM Tris-HCl pH 9.0 or 100 mM glycine pH 9.5. Platinum derivatives were prepared by soaking the crystals in K₃Pt(CNS)₆. A partial poly-Ala model was built with phases to 5.0 Å from single isomorphous replacement with anomalous scattering (SIRAS). A complete model was built by combining phases from single anomalous dispersion using a new platinum derivative with molecular replacement phases from the partial model (MR–SAD) to 3.5 Å. Structure refinement was then performed against the 2.4-Å native data. The final model is of good quality with good Ramachandran statistics (98% favoured and 2% allowed). The model contains residues 2–416 (residues 230–240 missing), a uridine, 5 decylmaltosides (1 full and 4 partial), a Na⁺ ion and 43 bound waters. Disordered residues Lys 226 and Glu 345 were modelled as Ala.

For the flux assay, protein was reconstituted into lipid vesicles and then vesicles were diluted into buffer containing either choline chloride or NaCl. Flux was initiated by the addition of 2.4 μM [5,6-H]uridine and 1 μM valinomycin.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

**METHODS SUMMARY**

vcCNT was expressed in E. coli C41 cells. Cells were lysed and protein was solubilized with dodecyl maltoside. Protein was purified by Co²⁺-affinity chromatography followed by gel-filtration chromatography in the presence of decyl maltoside and uridine.
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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions Z.J. expressed, purified and crystallized vcCNT. Z.J. performed radioactive flux and crosslinking experiments. C.-G.C. participated in part of the vcCNT crystallization and generated mutants for crystallization and functional studies. Z.J. and S.-Y.L. collected and processed the data, solved the structure, and wrote the paper. S.-Y.L. designed the study. All authors discussed the results and commented on the manuscript.

Author Information Atomic coordinates and structure factors for the reported crystal structure are deposited in the Protein Data Bank under accession code 3TIJ. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.-Y.L. (sylee@biochem.duke.edu).
METHODS

Expression and purification. The gene encoding vcCNT was cloned from Vibrio cholerae genomic DNA into a modified pET26 vector that contains a pelB leader sequence and a PreScission Protease cleavable HisN~8~maltose-binding protein fusion. The original vector was a gift from R. Dutzler (University of Zurich) and was further modified. The vector containing vcCNT was expressed in E. coli C41 (DE3). Cells were lysed with a homogenizer (Avestin) and protein was solubilized with 30 mM dodecyl maltoside (DDM) for 2 h at 4 °C. Solubilized lysates were spun down to remove the insoluble fraction, and supernatants were applied to Talon Co~6~ affinity resin. After binding, protein was eluted with imidazole and digested overnight with PreScission Protease. The digestion mixture was concentrated and applied to a Superdex 200 size-exclusion column in the presence of 5 mM decyl maltoside (DM), 1 mM uridine, 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 2 mM dithiothreitol. Peak fractions corresponding to vcCNT were collected for further experiments.

Crystallization. Initial crystallization conditions were obtained at the high-throughput crystallization-screening laboratory at the Hauptman-Woodward Institute~31. Protein was concentrated to about 10 mg mL~−1~ and mixed 1:1 with crystallization solution containing 100 mM CaCl~2~, 37–42% PEG400 and 100 mM buffer. Crystals grew over a wide pH range (5.6–9.5), but data for structure solution were collected on crystals grown at pH 9.0 (100 mM Tris-HCl) and pH 9.5 (100 mM glycine). Crystals were grown by using the microbatch-under-oil method. After 10–14 days, crystals were harvested, transferred to cryo solution containing 32.5% PEG400, and flash-frozen in liquid nitrogen. Platinum derivatives were prepared by soaking for 2–4 h in 2.5 mM K~3~Pt(CNS)~6~, and then transferring them to cryo solution and flash-freezing.

Structure determination. The data were collected on beamlines 22ID, 22BM and 24ID-C at the Advanced Photon Source. The data were processed with HKL2000 (ref. 32). Crystals of vcCNT diffract to 2.4 Å Bragg spacings and belong to the space group P6~3~22. Extensive screening of crystals was performed because roughly 70% of crystals are merohedrally twinned, with the twinning operator perpendicular to the crystallographic six-fold axis, leading to the apparent space group P6~3~22. We found that SeMet-substituted crystals were almost always twinned with significant twinning fractions (20–45%), which necessitated heavy-atom-soaked crystals as the choice for a de novo phasing method for the vcCNT structure. Initial phases to 5.0 Å were obtained by single isomorphous replacement with anomalous scattering (SIRAS) from a platinum-soaked crystal. Platinum sites were found by SHELXD~33; phasing was calculated to 5.0 Å Bragg spacings by using SOLVE~34; with the figure of merit 0.34, and the density was modified by solvent flattening with RESOLVE~35. A partial model was built by manually placing idealized poly-Ala helices into the solvent-flattened electron density map by using COOT~36~. After further extensive screening of platinum derivatives, we found a derivative that diffracted to 3.1 Å with significant anomalous signal but was non-isomorphous with any of our native data. Platinum sites were found from an anomalous difference Fourier map by using the partial model phases. Combined phases of single anomalous dispersion from the platinum derivative and molecular replacement from the partial model (MR–SAD) were calculated at 4.0 Å with the figure of merit 0.35 and extended to 3.5 Å by solvent flattening with PHASER~37~ and RESOLVE~35~ with the use of the PHENIX interface~38~. After iterative cycles of manual adjustment of poly-Ala helices and calculation of combined MR–SAD phases, the electron density map was of excellent quality and allowed us to place side chains into the partial model. We also collected native data to 2.4 Å at a long wavelength (1.6 Å) and identified sulphur sites by using an anomalous difference Fourier map, which helped guide the model building. After about 70% of manual model building was complete, molecular replacement was performed with the partial model against the 2.4 Å native data for further model building and refinement. Structure refinement was performed with PHENIX~39~. An anomalous difference Fourier map with the native data to 2.4 Å collected at a long wavelength (1.6 Å) identified six sulphur sites and helped guide the model building. The final model is of good quality with R~work~/R~free~ = 19.6/22.8% and good Ramachandran statistics (98% favoured and 2% allowed), and contains residues 2–416 (residues 230–240 missing), a uridine, 5 DMs (1 full and 4 partial), a Na~+~ ion and 43 bound waters.

Vesicle reconstitution and flux assay. Protein was reconstituted into lipid vesicles containing 10 mg mL~−1~ of 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) (3:1 ratio of POPE:POPG) at a mass ratio of 1:500 protein/lipid, as described previously~40~. Vesicles were reconstituted in the presence of 200 mM KCl, 20 mM HEPES pH 7.4, 100 mM choline chloride. Reconstituted vesicles were then flash-frozen and thawed three times, then extruded through a 1.0-μm filter with the use of the Avanti Mini-Extruder.

For the flux assay, vesicles were diluted 1:20 into buffer containing 200 mM KCl, 20 mM HEPES pH 7.4, and either 100 mM choline chloride or 100 mM NaCl. Flux was initiated by the addition of 2.4 μM [5,6-H]uridine and 1 μM valinomycin. All experiments were performed in triplicate at 30 °C. Vesicles were harvested on GF/B glass microfilter filters (Whatman) and counted by scintillation on the following day.

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