Structure of Prototypic Peptide Transporter DtpA from E. coli in Complex with Valganciclovir Provides Insights into Drug Binding of Human PepT1

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Supporting Information

ABSTRACT: Members of the solute carrier 15 family (SLC15) transport di- and tripeptides as well as peptidomimetic drugs across the cell membrane. Structures of bacterial homologues have provided valuable information on the binding and transport of their natural substrates, but many do not transport medically relevant drugs. In contrast, a homologue from Escherichia coli, DtpA (dipeptide and tripeptide permease), shows a high similarity to human PepT1 (SLC15A1) in terms of ligand selectivity and transports a similar set of drugs. Here, we present the crystal structure of DtpA in ligand-free form (at 3.30 Å resolution) and in complex with the antiviral prodrug valganciclovir (at 2.65 Å resolution) supported by biochemical data. We show that valganciclovir unexpectedly binds with the ganciclovir moiety mimicking the N-terminal residue of a canonical peptide substrate. On the basis of a homology model we argue that this binding mode also applies to the human PepT1 transporter. Our results provide new insights into the binding mode of prodrugs and will assist the rational design of drugs with improved absorption rates.

INTRODUCTION

The proton-dependent oligopeptide transporters (POTs) constitute a subfamily of the major facilitator superfamily (MFS), transporting di/tripeptides and peptide-like compounds coupled to cotransport of protons. Mammalian POTs are also referred to as solute carrier 15 transporters (SLC15s). Human PepT1 (SLC15A1) and PepT2 (SLC15A2) are of great pharmacological interest due to their roles in intestinal uptake and renal reabsorption of not only dietary peptides but also drugs such as β-lactam antibiotics (ceftibuten), angiotensin-converting enzyme inhibitors (enalapril), and antiviral prodrugs (valganciclovir and valacyclovir). The prodrugs valganciclovir and valacyclovir are L-valine derivatives of the guanosine analogs ganciclovir and acyclovir, used for treating cytomegalovirus and herpes simplex virus infections, respectively. Conjugation to valine makes these compounds more soluble and improves their uptake rates in humans by turning them into substrates for PepT1 and PepT2. PepT1 and PepT2 have been well characterized in terms of ligand binding, but their structures remain unknown. The bacterial homologue of PepT1, DtpA (dipeptide and tripeptide permease), provides an excellent prototype to understand the molecular mechanism of peptide and drug transport due to the high conservation of the binding site as well as the highly similar substrate specificity profile compared to PepT1. However, like for their human homologues, structural insights into relevant drug binding are missing for bacterial peptide transporters. Therefore, we combined biochemical, biophysical, and structural approaches to obtain molecular insights into binding and transport of peptides and drugs of the prototypic DtpA transporter, which provided the basis for structural modeling of the human PepT1 transporter.

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RESULTS AND DISCUSSION

To produce well-diffracting crystals of DtpA, we used nanobodies as crystallization chaperones, which can improve crystallization by increasing the thermal stability of the target protein, by locking it in a specific conformation, and by mediating crystal contacts. Four nanobodies were generated, which were confirmed to bind a conformational epitope of DtpA and increased its thermal stability by more than 10 °C (Supplementary Figure 1). Nanobody N00 was essential to interacting crystals. Three structures of the DtpA-N00 complex were determined (Table 1, Supplementary Figure 4).

The valganciclovir-bound DtpA-N00 structure is highly similar to the ligand-free structures, with an rmsd of 0.33 Å over 3809 atoms (glycine buffer condition) and 0.42 Å over 3869 atoms (MES buffer condition). Clear electron density could be observed for the bound valganciclovir molecule (Figure 2a). Valganciclovir has two chiral centers: one at the C1 position (the latter is highlighted with a red star in Figure 1b) and the other at the C2 position (the latter is highlighted with a red star in Figure 1b). It is synthesized as a mix of two diastereomers that only differ at the C2 position. Both diastereomers can be transported by hPepT1 (human peptide transporter 1). It was not possible to distinguish between the two diastereomers in the electron density map of DtpA (Figure 2b).

The valganciclovir-bound structure reveals an unexpected binding mode for the prodrug. The ligand-binding site of POTs has been previously characterized from structures of different transporters bound to di/tripeptides or the phosphonodipeptide alafosfalin. Since valganciclovir has a length similar to tripeptides, it has been suggested that the N-terminal valine residue of the prodrug would mimic the N-terminal residue of a di/tripeptide when bound to the transporter. However, in our DtpA-N00-valganciclovir structure, it is the guanine moiety and not the valine residue that occupies the position corresponding to the N-terminal residue of a di/tripeptide when bound to the transporter.

Table 1. Crystallographic Data Collection and Refinement Statistics for the Ligand-Free and Valganciclovir-Bound DtpA-N00 Structures

|                | DtpA-N00 in glycin e buffer, pdb id 6GS7 | DtpA-N00 in MES buffer, pdb id 6GS1 | DtpA-N00-Valganciclovir, pdb id 6GS4 |
|----------------|------------------------------------------|-------------------------------------|--------------------------------------|
| data collection|                                          |                                     |                                      |
| space group    | P2₁,2₁                                   | P2₁,2₁                              | P2₁,2₁                               |
| cell dimensions| a, b, c (Å)                              | 55.14, 120.53, 163.43               | 55.46, 120.72, 163.33                |
| α, β, γ (deg)  | 90, 90, 90                                | 90, 90, 90                          | 90, 90, 90                          |
| resolution (Å) | 19.94–3.30 (3.42–3.30)                   | 48.16–3.29 (3.41–3.29)              | 19.68–2.65 (2.74–2.65)              |
| Rmerge (%)     | 21.74 (217.7)                             | 26.73 (186.4)                       | 10.74 (207.7)                       |
| Rfree (%)      | 7.97 (74.81)                              | 11.14 (78.46)                       | 4.65 (86.77)                        |
| I/σ            | 7.04 (0.83)                               | 6.37 (1.06)                         | 10.74 (1.06)                        |
| CC 1/2 (%)     | 99.2 (49.4)                               | 99.4 (56.0)                         | 99.8 (46.3)                         |
| completeness (%)| 99.02 (99.70)                             | 99.34 (99.47)                       | 98.98 (97.29)                       |
| redundancy     | 8.5 (8.8)                                 | 6.7 (6.6)                           | 6.4 (6.6)                           |

Values in parentheses are for the highest-resolution shell.
The N-terminal valine residue of valganciclovir extends instead into a pocket formed by TM7 and an intrahelical loop in TM10 (I399-SGLG-L404). Here it forms van der Waals interactions with F289 from TM7 as well as with I399 and L402 from the TM10 loop (Figure 2c). In MFS transporters, it is not uncommon to find intrahelical loops in cavity-lining helices. However, among POTs, a loop within TM10 has previously only been observed in PepTSo2 (peptide transporter from bacterial species Shewanella oneidensis). Here TM7 and the loop within TM10 line the pocket that accommodates the side chains in position two of di- and tripeptides, i.e., “pocket 2” (P2). In PepTSt (peptide transporter from bacterial species Streptococcus thermophilus), which has an uninterrupted TM10, P2 is instead formed by TM2, TM7, and TM11 and is located in a partially overlapping position that is closer to the center of the cavity.

Compared to POT structures with an uninterrupted TM10, the periplasmic half of the helix superimposes well whereas the cytoplasmic half is shifted considerably, which in turn causes TM11 to be pushed away from the binding cavity (Figure 3). As a result, the binding cavity is significantly enlarged in POTs with a split TM10 (Figure 3b). Indeed, in comparison to other POT transporters with known structures, the binding site of DtpA is the largest (Figure 3b).

We conclude that DtpA binds valganciclovir in a particularly large binding cavity by accommodating its guanine base in a site that in other POTs generally engages the N-terminus of peptides and inserting its valine residue into a pocket, which is largely equivalent to the P2 peptide side chain binding pocket of PepTSo2.
with this, the increase of median values for the AK-AMCA competition is significantly higher for tripeptides (median = 68%) compared to dipeptides (median = 53%) (W = 37, n₁ = 10, n₂ = 18, P = 0.005, two tailed; Figure 4b, right). The significant difference of binding and transport between di- and tripeptides was further substantiated by a principal component analysis of the physicochemical properties of the tested peptide library. This analysis showed that the molar volume of a peptide ligand is indeed the most strongly contributing variable (Supplementary Figure 6). Finally, quantitative binding affinity measurements were carried out using microscale thermophoresis (MST) for a subset of these ligands, all showing binding affinities in the low to medium micromolar range (Supplementary Figure 7 and Supplementary Table 1). Notably, the highest binding affinity measured by MST was obtained for the tripeptide LLA (Kᵩ = 58 ± 10 μM; Figure 4c), which also induced the highest thermal stabilization, as determined by DSF, and the strongest inhibitory effect on AK-AMCA uptake of all tested ligands (Figure 4a and 4b and Supplementary Table 1). Taken together, our results indicate that DtpA preferably binds and likely transports tripeptides over dipeptides. This is in contrast to most other POTs with known structures, which show preferences toward dipeptides. However, PepT_so2,10 which shares the presence of an intrahelical loop in TM10 with DtpA, is an exception to this rule (Figure 3a), supporting the notion that a split TM10 and the associated enlargement of the binding cavity may be an adaptation for improving or enabling binding and transport of larger substrates.

From all drugs tested, only valacyclovir and valganciclovir showed a thermal stabilization effect on DtpA and prominent AK-AMCA competition (Figure 4a and 4b). Remarkably, these two drugs bind as tightly to the protein as the best binding tripeptides. The affinities measured using MST were 60 ± 13 μM for valacyclovir and 76 ± 16 μM for valganciclovir (Supplementary Table 1), similar to the Kᵩ of 49 ± 0.3 μM recently measured for the binding of valacyclovir to human PepT1.22

Next, we measured the effect of N00 on peptide uptake and affinity in vivo and in vitro. AK-AMCA uptake in cells overexpressing DtpA together with N00 was strongly reduced (Figure 4d). In addition, DtpA modified with a site-specific unnatural amino acid could be cross-linked to N00 upon UV irradiation (Supplementary Figure 8) in lysates, suggesting that N00 stabilizes the protein in the inward open conformation not only in the crystal and in detergent-solubilized form but also in the lipid membrane. Differences in binding affinity upon addition of N00 were typically small (about 2-fold or less), but an increase in affinity of 4–5-fold could be observed for two of the peptides, LA and AL (Supplementary Figure 7 and Supplementary Table 1), indicating that substrate binding affinities may in some cases vary between the inward and the outward open states.

To confirm the location of the ligand-binding site of DtpA by functional assays, five residues (Y38, Y71, K130, N160, and I399) were mutated to alanine. The correct folding of the mutants for selected di/tripeptides and the two drugs were strongly reduced relative to wild type (Supplementary Figure 9a). Binding affinities of these mutants for selected di/tripeptides and the two drugs were strongly reduced relative to wild type (Supplementary Figure 9b and Supplementary Table 2), and AK-AMCA uptake was abolished (Figure 4d). Two additional binding site mutants (Y156A and F289L) that were characterized...
previously were also found to abrogate AK-AMCA uptake.\textsuperscript{23} We therefore conclude that the mutational and structural data are in good agreement.

Taking advantage of the high sequence conservation in the binding site (Supplementary Figure 10), a homology model of human PepT1 (hPepT1) in complex with valganciclovir was built based on the DtpA structure, covering the whole sequence except for the extracellular domain (ECD) and the linker between the two MFS domains (Figure 5). All residues involved in valganciclovir binding in DtpA are observed in corresponding positions in hPepT1. Remarkably, the residues coordinating the guanine base of valganciclovir are fully conserved among DtpA (Y38, Y156, N160, E396) and hPepT1 (Y31, Y167, N171, E595) (Supplementary Figure 11). The residues interacting with the N-terminal valine residue form similar hydrophobic pockets in both proteins.

TM5, TM7, and TM10 of hPepT1 have previously been systematically studied by mutations. Here, Y167, N171, and S174 on TM5,\textsuperscript{24} F293 and F297 on TM7,\textsuperscript{25} and E595 and Y598 on TM10\textsuperscript{26} were suggested to play a role in ligand coordination (Supplementary Figure 12). Indeed, all of these residues form part of the ligand-binding site in our model, and most are in close proximity to valganciclovir. Furthermore, high solvent accessibility was observed for residues T601-E604,\textsuperscript{26} in line with these residues forming an intrahelical loop in TM10, as suggested by the hPepT1 model. Thus, this model explains substrate-binding similarities between hPepT1 and DtpA and, in the absence of a crystal structure of hPepT1, can serve as a model for analyzing di/tripeptide and drug interactions.

\section*{CONCLUDING REMARKS}

We determined the ligand-free and valganciclovir-bound structures of DtpA, a bacterial SLC15 homologue with high similarity to hPepT1, not only in terms of sequence but also regarding substrate specificity and functional properties.
relating to drug transport. We find that DtpA, in contrast to most characterized bacterial POTs to date, prefers to bind and transport tripeptides over dipeptides, which appears to relate to the presence of a characteristic intrahelical loop in TM10. The structure of DtpA in complex with valganciclovir shows that the prodrug binds in a similar position to di/tripeptides but with the guanine base instead of the valine residue mimicking the N-terminal residue of a di/tripeptide in the ligand-binding site. On the basis of homology modeling and a survey of published mutational data, we find that this binding mode most likely also applies to hPepT1. These novel insights into the structure and substrate binding of DtpA may facilitate future development of prodrugs with improved absorption rates for hPepT1, thereby lowering the pharmacologically effective dose and reducing side effects.

## METHODS

### Chemicals.

Terrific broth (TB) was from Melford, isopropyl-β-D-thiogalactopyranoside (IPTG) and 1,4-dithiothreitol (DTT) were from Roth, DNase I was from AppliChem, complete EDTA-free protease inhibitor cocktail was from Roche, n-dodecyl-β-D-maltopyranoside (DDM) was from Anatrace, and crystallization reagents were from Molecular Dimensions. Amino acids, di/tripeptides, and drugs were obtained from Sigma-Aldrich, Bachem, and Fluka. The unnatural amino acid H-p-Bz-Phe-OH (pBpF) was purchased from Bachem.

### Gene Construction, Protein Expression, and Purification of DtpA.

The full-length DtpA (Uniprot ID: P77304) gene was amplified from the Escherichia coli genome and cloned into the pET27 vector.27 The construct has an N- and C-terminal His-tag and a TEV cleavage site after the N-terminal tag. Point mutations were generated by the blunt-end PCR method. DtpA, wild type (WT) and mutants, were expressed and purified as previously described.15 Briefly, DtpA was overexpressed in E. coli C41(DE3) cells in TB medium. The cell pellet was resuspended in lysis buffer (20 mM NaF, pH 7.5, 300 mM NaCl, 5% glycerol, 15 mM imidazole) with 5 units/mL DNase I, 1 tablet of protease inhibitor/100 mL buffer, 1 mg/mL lysozyme, and 0.5 mM TCEP followed by cell lysis with an emulsifier (EmulsiFlex-C3, Avestin, three passages). The lysate was centrifuged 12 min at 10 000 g and the supernatant centrifuged for 50 min at 95 000g (Optima XL-90, Beckman Coulter). The pellet containing the membrane fraction was solubilized in 1% DDM (Anatrace, sol-grade) for 1 h. The sample was centrifuged for 50 min at 70 000g and the supernatant applied to Ni-IMAC beads (ThermoFischer). After 45 min incubation on a rotating wheel, the suspension was transferred to a gravity column. Following two wash steps with lysis buffer supplied with 0.5 mM TCEP, 0.03% DDM, and 40 mM imidazole, DtpA was eluted with lysis buffer containing 0.5 mM TCEP, 0.03% DDM, and 300 mM imidazole. TEV cleavage was performed overnight during dialysis in gel filtration buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM DTT, 0.5% glycerol, and 0.03% DDM), and the protein was further purified on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare). All steps following cell lysis were performed at 4 °C. The protein was concentrated to 11 mg/mL using a 50 kDa concentrator to 13 mg/mL, flash frozen, and stored at −80 °C until further use.

### Selection, Expression, and Purification of Nanobodies Against DtpA.

For the generation of DtpA-specific nanobodies, two noninbred llamas were injected six times at weekly intervals with a mixture of 94 different proteins (50 μg of each antigen weekly). After 6 weeks of immunization, two separate phage display libraries were constructed, one from each animal, in the pMESy2 vector, which is a derivative of pMESy4 that contain a C-terminal EPEA-tag for affinity purification. After pooling both libraries, nanobodies were selected against individual antigens in two rounds of parallel panning in 96-well plates containing one immobilized antigen in each well. After two selection rounds on DtpA, 70 clones were picked for sequence analysis, 22 clones encoded antigen-specific nanobodies as tested in ELISA, grouping them in 16 different sequence families. A nanobody family is defined as a group of nanobodies with a high similarity in their CDR3 sequence (identical length and >80% sequence identity). Nanobodies from the same family derive from the same B-cell lineage and likely bind to the same epitope on the target. Immunizations, library construction, selection by panning, and nanobody characterization were performed according to standard procedures. Four nanobodies were further characterized.

The nanobodies were expressed in E. coli WK6 cells and purified following standard procedures. Specifically, the cell pellet was resuspended in TES buffer (0.2 M TRIS, pH 8, 0.5 mM EDTA, 0.5 M sucrose) supplemented with one protease inhibitor tablet (Roche). Osmotic shock was performed by the addition of diluted TES buffer to release the periplasmic proteins. The solution was first centrifuged for 20 min at 10 000g and additionally for 30 min at 142 000g. The supernatant was applied to CaptureSelect beads (Thermo Fisher Scientific), which were equilibrated with wash buffer (20 mM NaP, pH 7.5, 20 mM NaCl). After three column volumes of washing, the nanobody was eluted with 20 mM HEPES, pH 7.5, 1.5 M MgCl2. The nanobodies were further purified on a HiLoad 16/600 Superdex 75 pg column in 20 mM HEPES, pH 7.5, 150 mM NaCl, 5% glycerol, concentrated with a 5 kDa cutoff concentrator to 13 mg/mL, flash frozen, and stored at −80 °C until further use.

### Analytical Gel Filtration Assay.

The DtpA-nanobody complex formation was evaluated with an analytical gel filtration chromatography setup using a Superdex 200 5/150 GL packed-column (GE Healthcare) attached to a 1260 Infinity liquid chromatography system (Agilent technologies). The size exclusion profiles of the samples containing both DtpA and nanobodies were compared to the separate profiles for DtpA and the nanobody (at the same concentrations). The samples were run in 20 mM NaP, pH 7.5, 150 mM NaCl, 5% glycerol, 0.5 mM TCEP, and 0.03% DDM. The concentration of DtpA was 0.2 mg/mL. All samples were run in duplicates at 10 °C following the UV absorption and the fluorescence (excitation at 280 nm and emission at 350 nm). The data analysis was performed in GraphPad Prism 5.0.

### Evaluation of Conformational or Linear Epitope Binding.

To determine whether the selected nanobodies are conformational or linear epitope binders, Western blot analysis was used (the principle of this approach is illustrated in Supplementary Figure 1a). SDS-PAGE using a 4–12% Bis-TRIS gel (ThermoFischer) under reducing conditions was performed with DtpA using a total amount of 0.3 and 0.03 μg per lane. On the same gel a positive control (N00, amount 15 μg) and a negative control (DtpC, another E. coli POT member with 0.3 and 0.03 μg) were included. The protein was transferred to a PVDF membrane (Biorad). One percent bovine serum albumin in TBS-T (TRIS buffer with Tween20) from Sigma was used for blocking. TBS-T was also used as washing buffer. The membrane was incubated for 1 h with one of the nanobodies N87, N89, N93, or N00 containing an EPEA tag (1 μg/mL nanobody diluted in TBS-T buffer). Anti-EPEA antibody coupled to biotin (ThermoFisher) was used as primary and streptavidin coupled to horseradish peroxidase (ThermoFisher) as secondary antibody. The blot was developed using Super Signal West Pico Substrate (ThermoFisher) and Super Signal West Femto Substrate (ThermoFisher) in a 1:10 ratio. The image was acquired with a ChemiDoc MP device (BioRad).

### Cross-Linking of DtpA and N00 in the Lipid Bilayer.

In a cross-linking experiment we investigated if N00 could bind to DtpA in the native membrane. For this experiment two mutants were generated with an UV-inducible cross-linker in different positions. One mutant (G53TAG) was at the proximity of the N00 binding site on the periplasmic side of the transporter, and the second mutant (F265TAG) was on the cytoplasmic side of the transporter as a control. The amber suppression plasmid pEvol-pBpF and the DtpA expression plasmid carrying the amber mutation were cotransformed into E. coli strain C41(DE3). The cross-linking protocol from Farrell et al. was followed.32 Shortly, DtpA mutants were overexpressed in medium containing 1 mM pBpF and the lysed cells were incubated with N00. Cross-linking was performed with UV light (365 nm, 60 min), and the DtpA mutant was purified as described above. Sodium dodecyl sulfate polyacrylamide gel electro...
phoresis (SDS-PAGE) using a 4–12% Bis-TRIS gel (ThermoFisher) was performed and transferred on a PVDF membrane (Biorad). Two percent bovine serum albumin in TBS-T (TRIS buffer with Tween20, from Sigma) was used for blocking, TBS-T was used as washing buffer. The membrane was incubated with HisProbe-HRP conjugate antibody (ThermoFisher) for 1 h. The blot was developed using Super Signal West Pico Substrate (ThermoFisher) and Super Signal West Femto Substrate (ThermoFisher) in a 1:10 ratio. The image was acquired with a ChemiDoc MP device (BioRad).

**Crystalization and Structure Determination.** DtpA and N00 were mixed in 1:1.2 molar ratio 1 h prior to crystalization and incubated at 4 °C. The final concentration of DtpA was 8.5 mg/mL. Crystalization plates were prepared with an automated liquid handler (mosquito, TTP Labtech) using the sitting drop vapor diffusion technique with a final drop volume of 300 nL (at 1:1, 1:2 and 2:1 (v/v) ratios of protein to precipitant). The initial crystals of the ligand-free structure were obtained from the MemGold2 crystallization screen (Molecular Dimensions) at room temperature. After several rounds of optimization, the best diffracting crystal grew in 0.2 M magnesium chloride, 0.1 M MES at pH 6.5, and 32% PEG 600.

The ligand binding was investigated by comparing the cavity volumes of all available POT tripeptides investigated in this study were obtained from the MemGold2 crystallization screen (Molecular Dimensions) at room temperature. After several rounds of optimization, the best diffracting crystal grew in 0.2 M magnesium chloride, 0.1 M MES at pH 6.5, and 32% PEG 600. Diffraction data were collected at the MASSIF-1 (ID30A-1) beamline at ESRF (Grenoble, France) using the fixed 0.896 Å wavelength at 100 K temperature. The data were processed using the XDS package. The space group was identified as P2_1_2_1, and the resolution limit was at 3.29 Å. Initial phases were obtained by molecular replacement using DTPD (PDB ID 4Q6S) and a natively obtained crystal structure as search models in Phaser33 as part of PHENIX. The resolution limit was at 3.29 Å. Initial phases were obtained by molecular replacement using DTPD (PDB ID 4Q6S) and a natively obtained crystal structure as search models in Phaser33 as part of PHENIX. The resolution limit was at 3.29 Å. Initial phases were obtained by molecular replacement using DTPD (PDB ID 4Q6S) and a natively obtained crystal structure as search models in Phaser33 as part of PHENIX.

The model was further built manually in Coot and refined in PHENIX in iterative cycles. The Ramachandran statistics for the final model are 97.3% for favored regions, 2.7% for allowed regions, and 0.0% for outliers. In the case of the DtpA-N00 structure bound to valganciclovir, the drug was added to a DtpA solution in powder form (estimated concentration of 20 mM) and N00 was added as described above. The workflow for structure determination of the drug-bound complex was the same as for the ligand-free form, but the best diffracting crystals grew in the following condition: 0.1 M glycine, pH 9.0, 35% PEG 400, 0.15 M CaCl_2, and 0.02% Anapoe-C12E10. The data set was collected at beamline P13 operated by EMBL Hamburg at the PETRA III storage ring (DESY, Hamburg, Germany) at 0.966 Å wavelength and 100 K temperature. The ligand-free structure was used as a search model for molecular replacement. The Ramachandran statistics for the final model are 96.6% for favored regions, 3.4% for allowed regions, and 0.0% for outliers. Structure figures were generated using PyMOL.

**Analysis of the Binding Site Volume.** The cavity introduced by the TM10 intrahelical loop and the shift of TM11 in DtpA was investigated by comparing the cavity volumes of all available POT structures. The volume calculation was performed with POVM 2.0 using the default parameters. All structures were overlaid, and the coordinates of the Cα atom of the valine moiety in valganciclovir were selected as the center. The volume of the binding site based on a 4 Å radius sphere was calculated for the following POT structures: DtpA (6G5S), PepT(D) (4LEP), DtpD (4Q6S), YePep(T (4W0V), GKPOT (4HKV), PepT(E) (6E13), PepT(E) (4UVM), PepT(S) (5OXX), and PepT(S) (6EXS). The results were analyzed in GraphPad Prism 5.0 (GraphPad Software, CA, USA).

**Binding Affinity Assay.** The binding affinity of DtpA and the di/tripeptides were measured with a label-free microscale thermophoresis device Monolith (NanoTemper Technologies). However, due to the intrinsic signal from valganciclovir and valacyclovir another approach was taken for these drugs. DtpA was labeled on the His6-tag with RED-tris-NTA dye (NanoTemper), and the binding affinity was measured with microscale thermophoresis following the RED-dye signal. To compare the results from unlabeled and labeled DtpA, the binding affinity of the tripeptide AFA was measured in both systems.

For the measurements on unlabeled DtpA, the protein was diluted to 0.5 μM with assay buffer (100 mM HEPEs, pH 7.5, 150 mM NaCl, 0.03% DDM). All ligand lyophilizates were dissovled in water to a final concentration of 50 mM. An 18 μL amount of DtpA at 0.25 mg/mL was mixed with 2 μL of 50 mM ligand/substrate (5 mM final concentration). In the case of the nanobodies, the same concentration of DtpA was mixed with a 1:1.2 molar ratio of the tested nanobody. Samples were incubated for 10 min at room temperature before loading them with standard capillaries into the Prometheus device. The excitation power was set between 15% and 25%, and the tested temperature range was from 20 to 85 °C. All measurements were done in triplicate, and the results were analyzed with Excel (Microsoft) and GraphPad Prism 5.0 (GraphPad Software, CA, USA).

**Thermal Stability Assay.** The thermal stability of DtpA and the di/tripeptides were measured with a label-free microscale thermophoresis device device Monolith (NanoTemper Technologies). However, due to the intrinsic signal from valganciclovir and valacyclovir another approach was taken for these drugs. DtpA was labeled on the His6-tag with RED-tris-NTA dye (NanoTemper), and the binding affinity was measured with microscale thermophoresis following the RED-dye signal. To compare the results from unlabeled and labeled DtpA, the binding affinity of the tripeptide AFA was measured in both systems.

For the measurements on unlabeled DtpA, the protein was diluted to 0.5 μM with assay buffer (100 mM HEPEs, pH 7.5, 150 mM NaCl, 0.03% DDM). A dilution series of the substrate was also prepared with the same buffer. DtpA was then mixed with the substrate dilution series and incubated for 10 min at room temperature before loading the samples in standard capillaries to the MST Label-Free device (NanoTemper Technologies, Munich, Germany). For the measurements with the DtpA-N00 complex, the concentration of DtpA was kept constant and a 1:1.2 molar ratio of N00 was mixed 1 h prior to the measurements. As a negative control, the binding affinity of N00 alone, at 2 μM concentration, to selected peptides was measured under the same conditions. The settings were 20% LED and 20% MST power. For the measurements with valganciclovir and valacyclovir, DtpA was diluted to 1.6 μM and labeled with RED-tris-NTA dye according to the supplier’s protocol but using the assay buffer in all steps. The MST NT.115 device (NanoTemper Technologies, Munich, Germany) was used with the standard capillaries and 20% LED and 20% MST power settings. All measurements were performed in duplicate, and the results were analyzed with GraphPad Prism 5.0.

**In Vivo Uptake and Competition Assay.** The in vivo uptake of the β-Ala-Lys peptide coupled to the fluorescent AMCA (7-amino-4-methylcoumarin-3-yl)acetic acid) group (AK-AMCA) was performed as previously described with minor modifications. E. coli C41(DE3) cells containing the DtpA plasmid were grown in 5 mL of LB medium supplemented with 100 μg/mL ampicillin to an OD600 nm of 0.7. DtpA expression was induced by the addition of 0.2 mM IPTG and followed by an incubation period of 3 h at 37 °C. The cell culture was then centrifuged for 3 min at 3000 rpm and resuspended to a final OD600 nm of 10 in the assay buffer (100 mM HEPEs, pH 7.5, 150 mM NaCl, 5 mM glucose). 40 μL of buffer, 40 μL of cells, 10 μL of AK-AMCA (final concentration 50 μM), and 10 μL of the competing ligand (final concentration 5 or 0.5 mM) were mixed in a 96-well plate and incubated for 20 min at 37 °C. The reaction was stopped by adding 200 μL of ice-cold assay buffer, and the cells were then washed twice with the same buffer. Finally, the cells were resuspended in 200 μL of assay buffer, and the fluorescence was measured in a M1000 microplate reader (TECAN) with excitation at 350 nm and emission at 450 nm. All experiments were performed in triplicate. The results were normalized by the fluorescence value of the control (cells overexpressing DtpA incubated with AK-AMCA) and plotted as AK-AMCA uptake rate percentage. The analysis was done in Excel and GraphPad Prism 5.0.

**Principal Component Analysis.** The physicochemical properties of di/tripeptides investigated in this study were obtained from the transition point of the fluorescence ratio from 330 to 350 nm corresponds to the melting temperature (T_m). The shift of T_m in the presence of a nanobody or a ligand is interpreted as potential binding. DtpA was diluted to 0.5 mg/mL with assay buffer (100 mM HEPEs, pH 7.5, 150 mM NaCl, 0.03% DDM). All ligand lyophilizates were dissolved in water to a final concentration of 50 mM. An 18 μL amount of DtpA at 0.5 mg/mL was mixed with 2 μL of 50 mM ligand/substrate (5 mM final concentration). In the case of the nanobodies, the same concentration of DtpA was mixed with a 1:1.2 molar ratio of the tested nanobody. Samples were incubated for 10 min at room temperature before loading them with standard capillaries into the Prometheus device. The excitation power was set between 15% and 25%, and the tested temperature range was from 20 to 85 °C. All measurements were done in triplicate, and the results were analyzed with Excel (Microsoft) and GraphPad Prism 5.0 (GraphPad Software, CA, USA).
LER43 based on the DtpA structure as the modeling template. The AK-AMCA competition rate and thermal stability for the groups of di- and tripeptides were compared to the experimentally determined biophysical parameters for PCA. Additionally, nonparametric statistical testing was performed to compare the experimentally determined biophysical parameters AK-AMCA competition rate and thermal stability that were not used together with the supporting experimentally determined parameters. A selection of the 10 most highly experimentally determined biophysical parameters AK-AMCA competition rate and thermal stability for the groups of di- and tripeptides.

**Model Building of Human PepT1 (hPepT1, SLC15A1) with Valganciclovir.** The homology model of hPepT1 (UniProt ID P46059) transmembrane domain was constructed using MODELLER based on the DtpA structure as the modeling template. The initial query-template alignment for the homology model was created with the HH-suite. The alignment was subsequently adjusted manually using the Swiss-PdbViewer according to predictions of secondary structure and membrane helices from GeneSilico MetaServer and to minimize distortions around loop regions. The regions including the ECD domain as well as the eukaryotic linker between TM6 and TM7 were excluded from modeling.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b11343.

Nanobody screening for DtpA binding; electron density maps of DtpA; DtpA-N00 binding site; stereoimage of the ligand-binding site with valganciclovir; AK-AMCA uptake and concentration-dependent competition assay with DtpA; principal component analysis of physicochemical data for di- and tripeptide ligands; binding curves derived from microscale thermophoresis experiment for DtpA and the DtpA-N00 complex; cross-linking of DtpA and N00 in a lipid environment; thermal stability and binding affinity analysis of DtpA mutants; sequence alignment of DtpA and hPepT1; ligand-binding site of DtpA and hPepT1 with valganciclovir; key residues for function in TM5, TM7, and TM10 highlighted in the hPepT1 homology model; thermal stability (T_m), binding affinity (K_J), and AK-AMCA uptake results for DtpA and DtpA-N00; binding affinity results for DtpA mutants; references (PDF)

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