Journal of Medicinal Chemistry

ARTICLE

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In Vitro Characterization of Human Peptide Transporter hPEPT1 Interactions and Passive Permeation Studies of Short Cationic Antimicrobial Peptides

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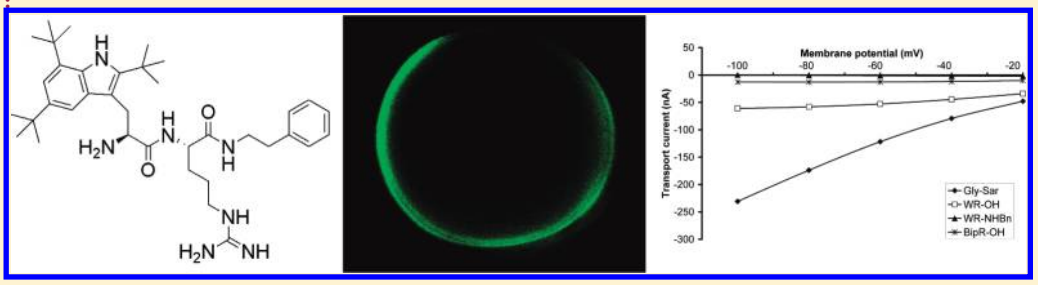
Supporting Information

ABSTRACT:



The present study assesses the permeation of cationic antimicrobial di- and tripeptides derived from lactoferricin via interaction with the human intestinal peptide transporter hPEPT1 and via passive routes. While some tested peptides displayed moderate affinity (0.6 and 2.7 mM) for interaction with hPEPT1, none served as substrate for hPEPT1 expressed by *Xenopus laevis* oocytes. It is shown that structural strategies employed to generate sufficient biological activity and metabolic stability such as introduction of large hydrophobic unnatural amino acids and different C-terminal modifications counteracted hPEPT1 mediated uptake. Most of the included peptides were nevertheless shown to permeate at rates suggesting moderate to excellent human oral absorption in the applied phospholipid vesicle-based passive permeation assay. Although the main factor governing passive permeation appears to be the hydrophobicity, peptide structure was also important and the overall permeation behavior was difficult to predict. Comparisons with a theoretical prediction model were also performed.

INTRODUCTION

The bioavailability of an orally administered drug depends on its solubility, the ability to cross the intestinal mucosa, and its metabolic stability. Most drugs access the circulatory system either via passive diffusion or through interactions with carrier proteins in the intestinal epithelium. While diffusion has historically been considered the main route of absorption for small drugs, recent studies suggest that selective transporters may play a prominent role in intestinal absorption.[1−3] Regardless of entry mode, the same biophysical forces, i.e., hydrogen bond formation and hydrophobic interactions, dictate the interaction with carrier proteins and/or membrane phospholipids. Lipinski's empirical "rule of 5" provides a means for predicting whether a compound is likely to display poor or good absorption via the passive diffusion route.[4] Several classes of drugs, however, mainly of natural origin, violate these rules.[1,5] Many orally bioavailable antibiotics belong to this group, and their modes of uptake are therefore difficult to predict. For the amino cephalosporin antibiotics it has been established that they manage to utilize the human intestinal di- and tripeptide transporter (hPEPT1) to gain entry into the circulatory system.[6] Although the physiological role of hPEPT1 is to transport di- and tripeptides formed during the luminal digestion, it also transports a variety of peptidomimetic drugs and prodrugs.[7−10] hPEPT1 has the unique feature to accept almost all naturally occurring di- and tripeptides accounting for more than 8000 substrates, yet each substrate displays different kinetic properties.[11−13]

Received: December 10, 2010
Published: March 14, 2011

dx.doi.org/10.1021/jm1015704 | *J. Med. Chem.* 2011, 54, 2422–2432

Truncated antimicrobial peptides based on bovine lactoferricin are emerging as a class of compounds with potential to treat infections caused by several types of drug resistant microorganisms.[14] Synthetic strategies such as truncation, capping, and introduction of unnatural amino acid residues have been employed to transform the original 25-mer into rapidly prepared druglike di- and tripeptides with high activity and selectivity.[15,16] From a drug design perspective they represent a class of antimicrobial peptides clearly within reach of pharmaceutical use with selected compounds currently in phase II clinical trials (Lytix Biopharma AS). It has recently been shown that several of these peptides can be designed with improved stability toward proteolytic degradation in the gastrointestinal tract.[17−19] Such increased half-life opens up for potential oral bioavailability which would further add to the attractiveness of these compounds.[20,21] Their small size in combination with the required pharmacophore suggests that they are potential candidates for either tertiary active transport via hPEPT1 or the passive permeation route.

To investigate if these compounds are substrates for transepithelial transport, a library of 17 antimicrobial di- and tripeptides was designed using synthetic strategies previously shown to generate peptides with increased antimicrobial effect and metabolic stability. hPEPT1 expressed in *Xenopus laevis* oocytes was used to investigate whether the peptides serve as substrates of the route via hPEPT1, while the passive diffusion of the peptides was studied using the phospholipid vesicle-based permeation assay (PVPA) as passive permeation model (Figure 1). Only a limited number of tested peptides were able to interact with hPEPT1 but in no case was electrogenic transport observed, demonstrating that the structural alterations used to generate improved antimicrobial potency prevent the peptides from acting as hPEPT1 substrates. Experiments to investigate passive diffusion, however, revealed that several synthetic strategies can be employed to generate potent peptides with excellent permeation properties via this route. The included peptides are all active membrane disruptors, and the corresponding challenges associated with the analysis of their transport properties are further discussed. A comparison with computationally generated theoretical parameters for oral uptake in humans is also included.

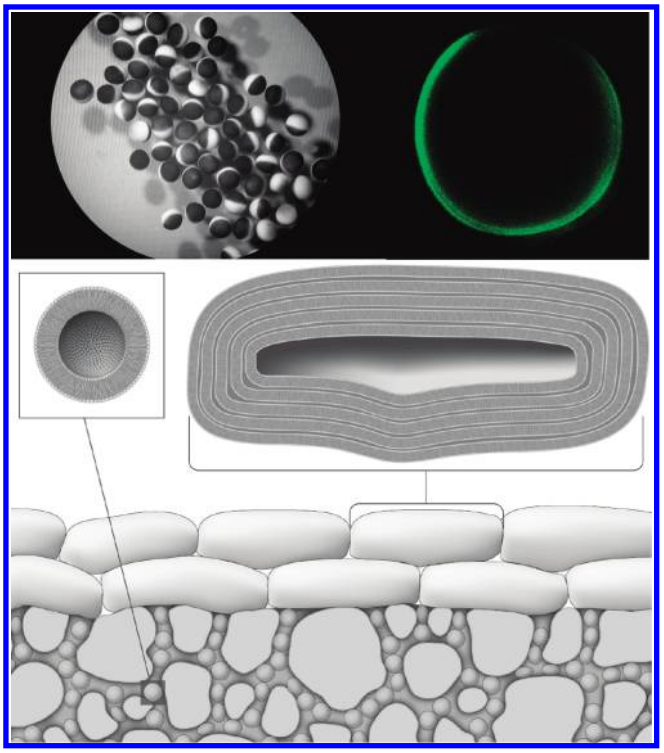


Figure 1. Representations of the biological and artificial systems used in the present study to investigate active and passive peptide uptake. Top left microscope image shows oocytes from *Xenopus laevis* with an average diameter of 1.1 mm. Top right illustrates the localization of cloned GFP coupled hPEPT1 in the oocyte membrane. The lower part (adapted from ref 51) illustrates a schematic bisection of the PVPA barrier showing large fused multilamellar liposomes deposited on top of a mixed cellulose ester filter with smaller liposomes filling the pores to generate a tight barrier.[51]

RESULTS

Selected Peptides. A combination of new (**4**, **7**, **9**, **13**, and the four reference peptides) and previously synthesized peptides from our extensive library was carefully chosen based on their properties to be included in the present study. Their structure and molecular properties are presented in Figure 2 and Table 1.

hPEPT1 Interactions. To evaluate a possible tertiary active transport, seven di- and tripeptides (**1**, **2**, **3**, **4**, **5**, **11**, and **14**) varying in structure and activity were selected. Inactive reference peptides WR-OH, WR-NHBn, and BipR-OH were synthesized and included to investigate the substrate preferences for hPEPT1. *Xenopus laevis* oocytes expressing hPEPT1 were used in a two-electrode voltage clamp (TEVC) experiment to assess whether the peptides can induce a transport current (concentration range 0.25−1.0 mM) or whether they can inhibit the transport current of the hPEPT1 substrate glycylsarcosine (Gly-Sar) (1 mM). Residual transport currents in the presence of the antimicrobial peptides are expressed as percent of the Gly-Sar current.

The results of the hPEPT1 experiments are summarized in Tables 2 and 3. The very active peptides **3** and **11** increased, even at very low concentration, the oocyte membrane conductance to extremely high values within seconds. However, no visible damage to the oocytes could be seen. Peptides **3** and **11** were therefore excluded from further analysis of hPEPT1-mediated transport. Peptide **5** displayed no affinity for binding to hPEPT1. The four remaining peptides displayed affinity for hPEPT1 with peptide **14** being the strongest inhibitor. Despite the obvious capability of these peptides to interact with the substrate binding domain of hPEPT1, none induced any significant currents and therefore they failed to show transport. The three inactive reference dipeptides were all transported via hPEPT1 as shown in Figure 3. A detailed analysis of the relations between structure and hPEPT1-mediated transport rate for these peptides can be found in the section Discussion.

Passive Permeation in the PVPA. The ability of the peptides to passively permeate across membranes was investigated using the PVPA method, which is a medium-throughput in vitro method for screening of passive diffusion of drugs across the intestinal epithelia. This model has proven to give strong coherence between its in vitro apparent permeability (P_{app}) and in vivo data on oral absorption of commercial drugs in humans. On the basis of the common classification system of absorption, drug compounds are classified as poorly absorbed (<30% absorbed in vivo) when displaying $P_{app} < 0.1 \times 10^{-6}$ cm/s, as moderately absorbed (30−70% absorbed in vivo) when the P_{app} values are between 0.1 and 0.9×10^{-6} cm/s, and to have excellent oral absorption (>70% in vivo absorption) with $P_{app} > 0.9 \times 10^{-6}$ cm/s.[22] The results of the permeability studies and the classifications are

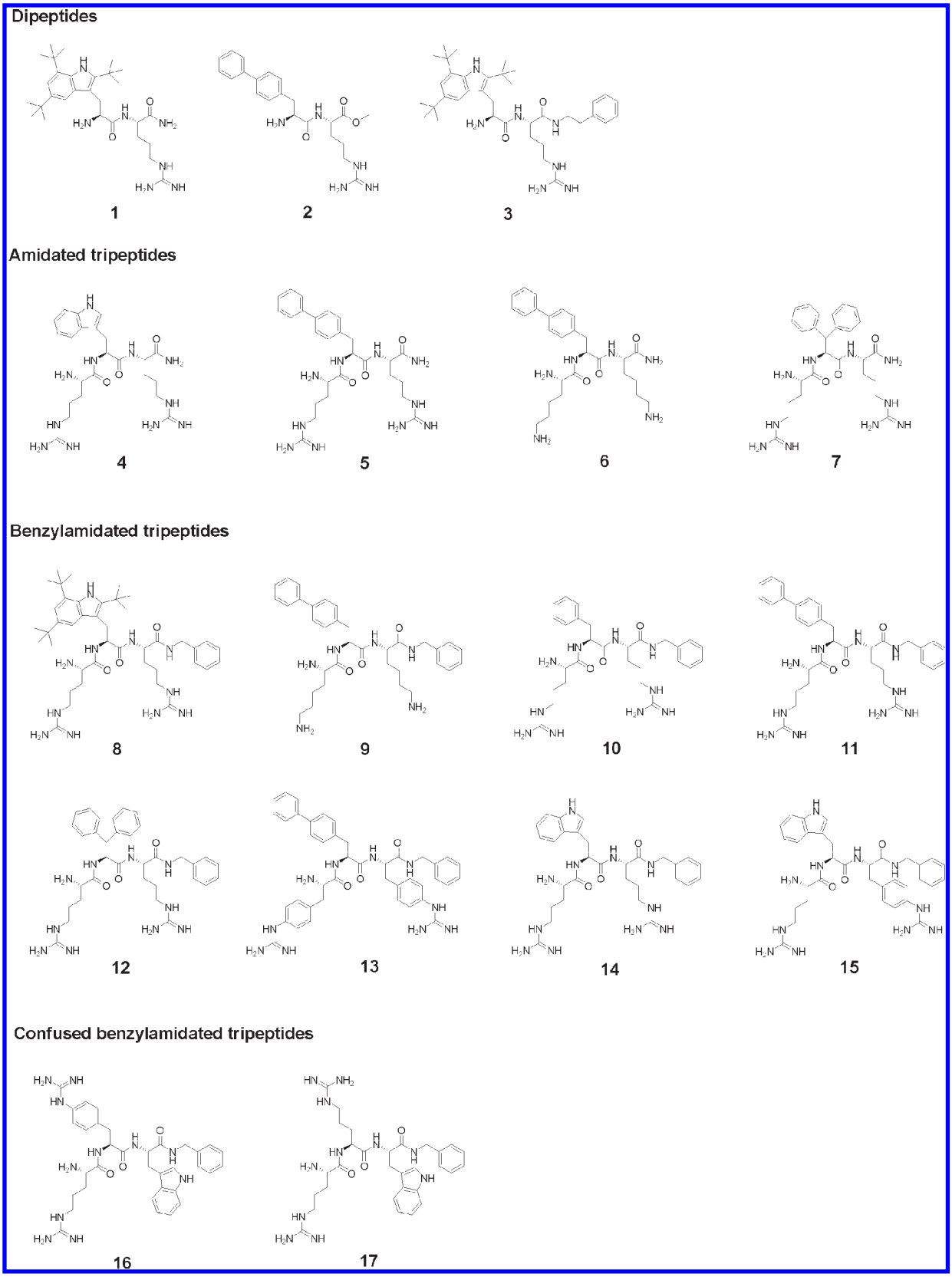


Figure 2. Structure of the 17 included antibacterial peptides. Four additional inactive reference peptides (not shown in figure) were also included.

shown in Table 4 and Figure 4. In addition, a correlation between the permeation data and the antimicrobial activity was clear as illustrated in Figure 5.

In contrast to the hPEPT1-mediated transport studies, most of the peptides were able to cross the phospholipid vesicle-based barrier at rates indicative of a moderate or excellent oral absorption.

dx.doi.org/10.1021/jm1015704 |*J. Med. Chem.* 2011, 54, 2422–2432

It was clear that the influence of the properties dictating the permeation across the membrane varied depending on the subtle design differences of the peptides. Peptide **8** proved difficult to work with and was excluded because of membrane disruption at the concentrations employed. The result from the computer generated oral uptake model is also presented in Table 4.

DISCUSSION

Selected Peptides. Antimicrobial peptides are produced by most living organisms as a first line of defense to slow microbial intruders before the onset of the adaptive immune system.[23] They display very favorable destructive properties and a mode of action that is little likely to cause bacterial resistance.[24,25] Nevertheless, their applicability as realistic future pharmaceuticals is currently somewhat limited by their size (generally between 10 and 50 amino acid residues) and low bioavailability. The heavily truncated lactoferricin based peptides used here represent an exception, and their pharmacophore is now actively used in rational drug design.[26,27] We describe for the first time the active and passive transport properties of such peptides as a basis for systemic drug delivery.

The current study employed pharmaceutically relevant peptides fulfilling, or nearly so, the pharmacophore, i.e., at least two units of hydrophobic bulk in conjunction with two positive charges (exemplified in Figure 6).[28] Both the bulk and the cationic charge are provided by a range of different natural and unnatural amino acid subunits. Several of the peptides display low MIC below 10 μg/mL and can be regarded as very active as shown in Table 1. Although structurally similar, the peptides range from inactive to highly active by design. This is further reflected in their differences in hydrophobicity as described by the theoretical ClogP values calculated using QikProp and their retention times on a C_{18} column which serves as an experimental estimate of their hydrophobicity. It has previously been shown that for some peptides there is a close correlation between antimicrobial activity and a certain degree of C_{18} retention, and the same is seen (see Supporting Information) for the tested peptides.[29]

The included peptides can be divided into three separate sublibraries: active dipeptides (**1**−**3**), amidated tripeptides (**4**−**7**), and highly active benzylamidated tripeptides (**8**−**17**). The last also contains peptides with confused sequences (**16** and **17**). Four structurally similar inactive di- and tripeptides were also included as references to provide positive controls.

hPEPT1 Interactions. As a member of the proton dependent oligopeptide transporter family the physiological role of hPEPT1 is to enable transepithelial transport of di- and tripeptides. The 708 amino acid residue protein is mainly expressed in the intestine, and several QSAR models have been developed to explain and predict the transport of the numerous substrates.[9,11,30−32] None of the compounds included in the present study have previously been evaluated as substrates for hPEPT1-mediated transport.

Table 1. Molecular Properties and Antibacterial Activities of the Included Peptides

					MIC (μg/mL)[c]		
peptide	sequence	mass	bulk[a]	charge[b]	*S. aureus*	*MRSA*	*E. coli*
1	TbtR-NH_2	527.4	1	+2	15	15	>150
2	BipR-OCH_3	411.2	1	+2	150	nd[d]	>200
3	TbtR-CH_2Bn	631.5	2	+2	2.5	2.5	10
4	RWR-NH_2	515.3	1	+3	>200	>200	>200
5	RBipR-NH_2	552.3	1	+3	200	50	nd[d]
6	KBipK-NH_2	496.3	1	+3	>200	>200	nd[d]
7	RDipR-NH_2	552.3	1	+3	100	>150	>150
8	RTbtR-NHBn	773.5	2	+3	2.5	2.5	7.5
9	KBipK-NHBn	586.4	2	+3	15	25	>150
10	RFR-NHBn	566.3	2	+3	150	75	>150
11	RBipR-NHBn	642.4	2	+3	5	5	100
12	RDipR-NHBn	642.4	2	+3	25	10	>150
13	GppBipGpp-NHBn	736.4	4	+3	<2.5	5	75
14	RWR-NHBn	605.4	2	+3	75	25	>150
15	RWGpp-NHBn	653.4	3	+3	50	10	>150
16	RGppW-NHBn	653.4	3	+3	50	15	>150
17	RRW-NHBn	605.4	2	+3	75	25	>150

[a] Number of isolated hydrophobic units. [b] At physiological pH. [c] Minimal inhibitory concentration determined by mico dilution experiments. [d] Not determined.

Table 3. Transport Generated by the Control Peptides (All Concentrations 1 mM)

sequence	flux rate,[a] Gly-Sar	flux rate,[a] test substance	flux rate[b] (%) (n)
WR-OH		14.5	43.5 ± 2.2 (3)
WR-NHBn	33.4	0.3	1.2 ± 1.0 (3)
BipR-OH		3.6	10.7 ± 1.5 (3)

[a] Flux rates expressed in pmol/(s·cm^2) were calculated from the transport-associated currents measured at −60 mV membrane potential. For the calculation, the surface area of an oocyte was estimated at 3.80 mm^2 (corresponding to a diameter of 1.1 mm). [b] Compared to the transport of Gly-Sar.

Table 2. Inhibition of Dipeptide-Induced Current

peptide	sequence	flux rate,[a] Gly-Sar 1 mM	flux rate,[a] Gly-Sar 1 mM + test substance	inhibition (%) (n)	IC_{50}[b] (mM)
1	TbtR-NH_2	43.8	34.8	20.3 ± 6.3 (4)	0.6
2	BipR-OMe	64.0	57.2	10.4 ± 2.3 (5)	nc[c]
4	RWR-NH_2	72.2	61.8	15.1 ± 6.1 (4)	2.7
5	RBipR-NH_2	56.4	56.7	−3.3 ± 8.3 (4)	nc[c]
14	RWR-NHBn	27.4	3.8	87.6 ± 0.5 (4)	nc[c]

[a] Flux rates expressed in pmol/(s·cm^2) were calculated from the transport-associated currents measured at −60 mV membrane potential. For the calculation, the surface area of an oocyte was estimated at 3.80 mm^2 (corresponding to a diameter of 1.1 mm). Differences were due to various surface densities of hPEPT1. [b] The IC_{50} values were calculated from the mean inhibitions for those substrates where data could reasonably be fitted to the Michaelis−Menten kinetics. [c] Not calculated because of poor fit.

dx.doi.org/10.1021/jm1015704 |*J. Med. Chem.* 2011, 54, 2422–2432

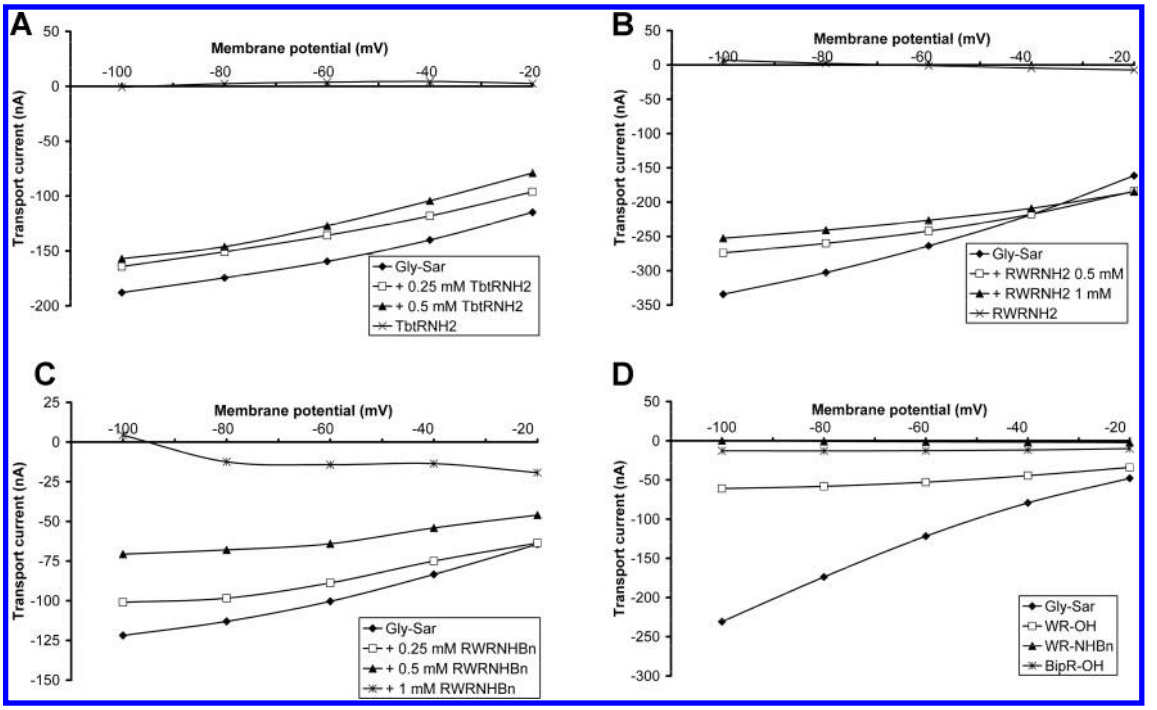


Figure 3. (A−C) Current−voltage relations of Gly-Sar (1 mM, pH 6.5) in the absence and presence of increasing concentrations of RWR-NH_2 (**4**), TbtR-NH_2 (**1**), and RWR-NHBn (**14**), respectively, and of the inhibitors without added Gly-Sar (in A and B). The values presented are the mean of four independent measurements. The data shown in Table 2 were calculated from the current values recorded at −60 mV membrane potential. (D) Current−voltage relations of the substrates WR-OH, WR-NHBn, and BipR-OH in comparison with the transport of Gly-Sar, a known substrate of hPEPT1. All compounds were analyzed at 1 mM at pH 6.5. The values presented are the mean of three independent measurements. The data shown in Table 3 were calculated from the current values recorded at −60 mV membrane potential.

Such a study does not therefore only answer important questions about the likely absorption via hPEPT1 but also provides insight into the mechanism of transport and substrate specificity of the peptide transporter, as the peptides contain numerous unnatural elements. The TEVC technique provides a direct, fast, and sensitive measurement of the functional properties of this class of transporters, allowing measurements to be carried out under well-defined voltage conditions.[33]

It has earlier been shown that affinity to hPEPT1 does not necessarily translate into transport behavior but can also reflect inhibitory activity.[34] Such behavior is seen in the present study where four of the tested peptides display binding to hPEPT1 with modest affinities but are not transported as shown in Table 2. The promising dipeptide **3** and tripeptide **11** could not be analyzed because of their membrane disruptive activity at all concentrations employed. Both peptides **3** and **11** are highly bioactive, and using them at concentrationsof >200 × their MIC is impossible with this technique. Of the five remaining peptides fulfilling the pharmacophore, only peptide **5** did not display any binding to hPEPT1. Out of the remaining four compounds, peptide **14** was the only one that produced a considerable inhibition of the dipeptide-induced current. However, this substance inhibited to a somewhat lower degree also the current induced by the glucose analogue α-methyl D-glucopyranoside (αMDG) via the sodium-coupled glucose transporter SGLT1 used as selectivity control. This suggests that peptide **14** can also impair membrane protein functions in an unspecific manner. Moreover, the degree of inhibition as a function of concentration of peptide **14** was nearly linear; i.e., it did not seem to comply with the expected protein-mediated kinetics (data not shown).

The remaining three peptides (**1**, **2**, and **4**) all inhibited the dipeptide transport by 10−20% when employed at 1 mM. Only peptides **1** and **4** allowed for proper IC_{50} calculations and revealed values of 0.6 and 2.7 mM, respectively. They may therefore be classified as inhibitors with medium affinity.[35] None of the peptides could, however, induce a transport current, indicating that they do not serve as substrates and subsequently cannot be taken up into the enterocytes via hPEPT1.

The three reference peptides all displayed electrogenic transport but at different rates as presented in Table 3 and Figure 3. The natural dipeptide WR-OH yielded transport currents but at lower rates than Gly-Sar, possibly because of a lower affinity (inhibition studies were not performed). Addition of a C-terminal benzylamide (WR-NHBn), which is a common strategy for increasing the antibacterial activity, nearly abolished transport. In contrast, the replacement of the tryptophan of WR-OH with the unnatural amino acid biphenylalanine (BipR-OH) retained a low residual transport activity. The biphenyl side chain of Bip is similar in accessible areas (625 $Å^2$ compared to 578 $Å^2$) to tryptophan but is significantly longer and more rigid. It is also more hydrophobic, and those two factors may explain the difference in residual transport. Biphenylalanine has previously been included as N-terminal amino acid in BipL-OH, a peptide that was shown to be transported at a low rate but displayed a high affinity, illustrating that the Bip placement within the sequence could also be a contributing factor.[36] To our knowledge the most similar peptide previously investigated is KWK-OH by Andersen et al. That peptide displayed an affinity at 3.7 ± 0.9 mM for hPEPT1.[9] Only very few studies on peptides with C-terminal arginines have been performed, and DIR-OH and

dx.doi.org/10.1021/jm1015704 |J. Med. Chem. 2011, 54, 2422–2432

Table 4. Theoretical and Experimental Peptide Hydrophobicity Together with Experimental Apparent Passive Permeability and a Prediction of Oral Absorption in Humans Based on the Experimental Data and Theoretical Calculations

					predicted oral absorption	
peptide	sequence	ClogP[a]	t_R[b] (min)	P_{app} (10^{-6} cm/s)	experimental[d]	theoretical[a]
1	TbtR-NH_2	1.597	16.3	0.43 ± 0.05	moderate	7.2
2	BipR-OCH_3	1.304	13.7	1.06 ± 0.17	excellent	24.2
3	TbtR-CH_2Bn	4.188	18.1	5.27 ± 1.83	excellent	53.0
4	RWR-NH_2	−2.995	3.15	2.37 ± 0.55	excellent	0.0
5	RBipR-NH_2	−1.548	15.6	0.01 ± 0.01	poor	0.0
6	KBipK-NH_2	−0.852	13.8	0.06 ± 0.03	poor	0.0
7	RDipR-NH_2	−2.035	7.6	0.83 ± 0.13	moderate	0.0
8	RTbtR-NHBn	2.590	19.0	nd[c]	nd[c]	5.0
9	KBipK-NHBn	1.600	11.0	0.26 ± 0.07	moderate	0.0
10	RFR-NHBn	−0.816	8.9	2.31 ± 0.71	excellent	0.0
11	RBipR-NHBn	0.944	11.3	0.01 ± 0.01	poor	0.0
12	RDipR-NHBn	0.389	10.6	0.42 ± 0.10	moderate	0.0
13	GppBipGpp-NHBn	1.647	11.7	0.90 ± 0.27	excellent	0.0
14	RWR-NHBn	−0.904	9.2	0.77 ± 0.28	moderate	0.0
15	RWGpp-NHBn	1.525	9.5	0.47 ± 0.11	moderate	0.0
16	RGppW-NHBn	−0.012	9.3	0.38 ± 0.17	moderate	0.0
17	RRW-NHBn	−0.941	9.3	0.96 ± 0.25	excellent	0.0
	reference drugs					
	metoprolol	1.884		3.23 ± 0.78[e]	excellent	89.5
	terbutaline	0.094		0.40 ± 0.05[e]	moderate	63.0
	chlorothiazide	−0.421		0.03 ± 0.01[e]	poor	51.5

[a] Calculated using QikProp. [b] Retention time on C_{18} column. The compound differs too much in retention to allow isocratic elution, and different gradients have been applied to the separate libraries. The retention times should thus only be compared within the libraries [c] Not determined. Peptide disrupted membrane barrier [d] According to definition in ref 22. [e] From ref 22.

DRR-OH recently investigated by Omkvist et al. displayed no or very low affinities (21 mM).[37] Previous work (unpublished) from the Freising laboratory also demonstrated a low affinity or even lack of binding for peptides carrying a cationic C-terminus. Furthermore, the data of Vig and co-workers show a nearly complete shutdown of transport activity upon C-terminal amidation both for the investigated tripeptide GGG-NH_2 and for several dipeptides such as GF-NH_2, YG-NH_2, and YY-NH_2.[34] FA-NH_2 was the only amidated peptide out of eight in that study to display a weak affinity for hPEPT1. Thus, both the C-terminal arginine and its amidation seem to counteract transport via hPEPT1. The fact that the incorporation of an unnatural hydrophobic alternative to W also had a negative effect on transport strongly indicates that active peptides fulfilling the pharmacophore for antibacterial activity, schematically illustrated in Figure 6, do not represent viable substrates for active transport by hPEPT1.

Passive Permeability. No or low active uptake does not prevent a drug candidate from being orally active. Since the short peptides are all adhering reasonably to Lipinki's "rule of 5" with regard to size and polarity, efficient permeation appears feasible.[4] To assess this, a passive diffusion study of the entire peptide library was subsequently initiated using the PVPA method. PVPA is a recently developed robust model that has been shown to provide permeability data that correlate well with in vivo data on fraction absorbed in humans, to the same extent as the Caco-2 cell model and even better than PAMPA models.[22] In addition to the analysis of drugs, PVPA has also be been successfully used to investigate formulations of poorly soluble drugs containing surfactants.[38] The peptides employed here are all relatively hydrophobic, and a transcellular uptake is the anticipated passive route. The passive uptake behavior of larger water-soluble pharmaceutical peptides through the intestinal epithelium via the paracellular route has recently been reported, and an alternative paracellular transport via the anionic tight junctions cannot be ruled out for the most polar of the included peptides in an in vivo situation.[39,40]

The permeation data were also compared with theoretical values for uptake generated by QikProp.[41,42] Both Qikprop and the assay are able to predict classification of small drugs according to in vivo oral absorption. The potential secondary structures of peptides can, however, have an unexpected impact on the permeability that is difficult to anticipate as has been seen in several transport studies.[43,44] To verify good compliance with in vivo data, the passive transport of three commercial drugs metoprolol (excellent absorption), terbutaline (moderate absorption), and chlorothiazide (poor absorption) was investigated using the two methods (Table 4). Both methods showed good correlation with a slightly too high theoretical uptake for chlorothiazide compared to 13−25% in vivo absorption earlier reported.[22]

The three active dipeptides (**1**−**3**) all crossed the phospholipid vesicle-based barrier at substantial rates. Peptide **3** was efficiently transported across the barrier at 5.27 × 10^{-6} cm/s, predicting a ranking as excellently absorbed in humans. The predicted absorption of peptide **2** was also classified as "excellent" according to the earlier definition, while peptide **1** only displayed moderate transport. This pattern is also suggested by QikProp albeit with slightly lower absorption values. These three peptides are all rather hydrophobic, as evident by both their C_{18}

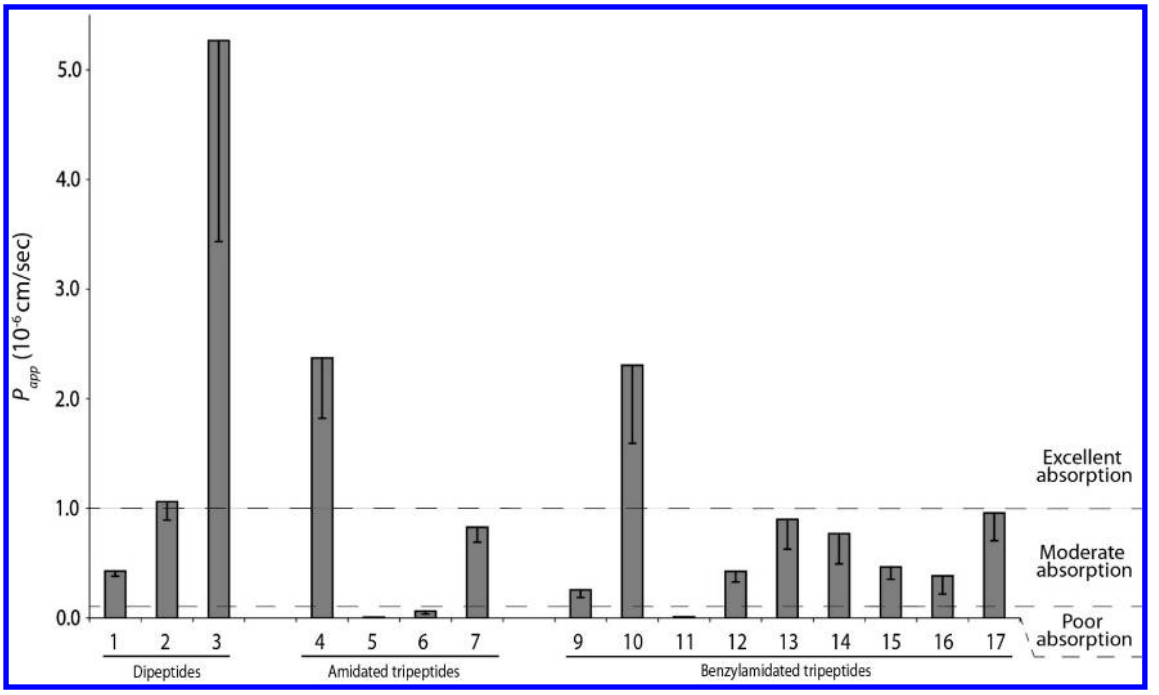


Figure 4. Graphical representation of the apparent passive permeation rates of the peptides using the PVPA.

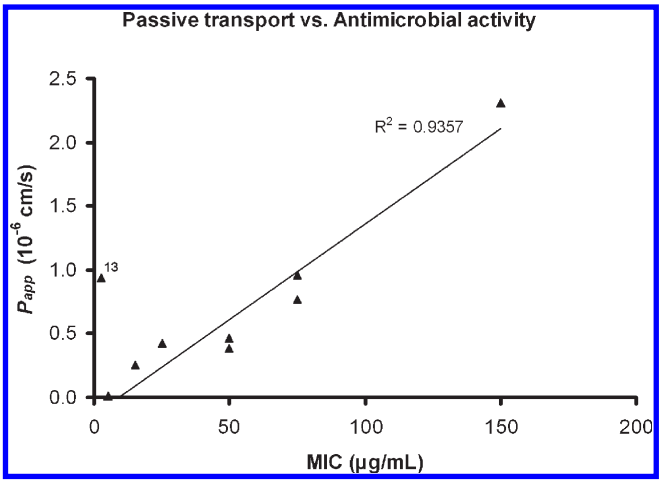


Figure 5. Passive transport as a function of the antimicrobial effect of the peptides from library 3 against *Staphylococcus aureus* ATCC 25923. Peptide **13** is omitted from the presented regression analysis. R^2 with peptide **13** included is 0.73.

retention times and their ClogP values, and it seems that the very bulky Tbt unit of peptide **3** is providing the extra hydrophobic push, setting that peptide apart from the other ones.

Tripeptides **4**−**7** lack a bulky C-terminal modification and are thus less hydrophobic than the other tripeptides. This is clearly reflected in their lower antimicrobial activity as shown in Table 1. The transport properties of these four peptides are distinctly different from the dipeptides with the most polar peptide **4** displaying the highest diffusion rate. In fact the most hydrophobic peptides **5** and **6** are hardly diffusing through the liposome barrier at all. To further investigate this unforeseen trend, an inactive highly polar tripeptide (RAR-NH_2, ClogP = −4.705) was prepared (not included in Table 4) and investigated. With an apparent permeability value of 12.0 × 10^{-6} cm/s this control peptide was permeating significantly faster than any other tested compound across the barrier. Peptides high in arginine are known to transverse membranes, and several studies have been devoted to develop polyarginine transmembrane carriers for challenging drug molecules.[45,46] The mechanism behind this effect is not clear, and suggestions ranging from active transport, through facilitated carrier mediated transport, to passive transcellular transport have been proposed.[45−47] Although the optimal polyarginine chains are four to eight units,[48] it seems that a similar effect is seen for our short peptides containing two arginines together with a free N-terminus and little hydrophobic bulk. Clearly two or more mechanisms are simultaneously acting to decide whether permeation across a membrane of these peptides is favored or not. The theoretical model did not anticipate a transport for any of these tripeptides.

The third library is composed of the type of modified tripeptides known for yielding the most efficient antibacterials with compounds very similar to those in phase II clinical trials.[14] With the exception of peptide **11**, remaining peptides within library 3 were either moderately or excellently transported. Peptide **8** disrupted the integrity of the barriers and could not be analyzed in high enough concentrations (200 × MIC). This effect has previously been seen for an active peptide in our preliminary study.[16] When plotting the retention time against permeation for the nine peptides (peptide **8** not included) investigated in library 3, a trend of increasing permeability with decreasing retention time could be seen (Figure 7). The only peptide not adhering to this trend is **13** with its two unnatural 4-guanidinophenyl side chains as arginine mimics. The confused sequences **16** and **17** displayed near to identical permeation values compared with their "native" counterparts **14** and **15**, suggesting that the overall hydrophobicity and not sequence is the main factor for these peptides. Again, the theoretical model was not able to predict the transport seen experimentally for this library. The dependence on hydrophobicity for activity also results in a close inverse correlation between the passive permeability and the

dx.doi.org/10.1021/jm1015704 |*J. Med. Chem.* 2011, 54, 2422–2432

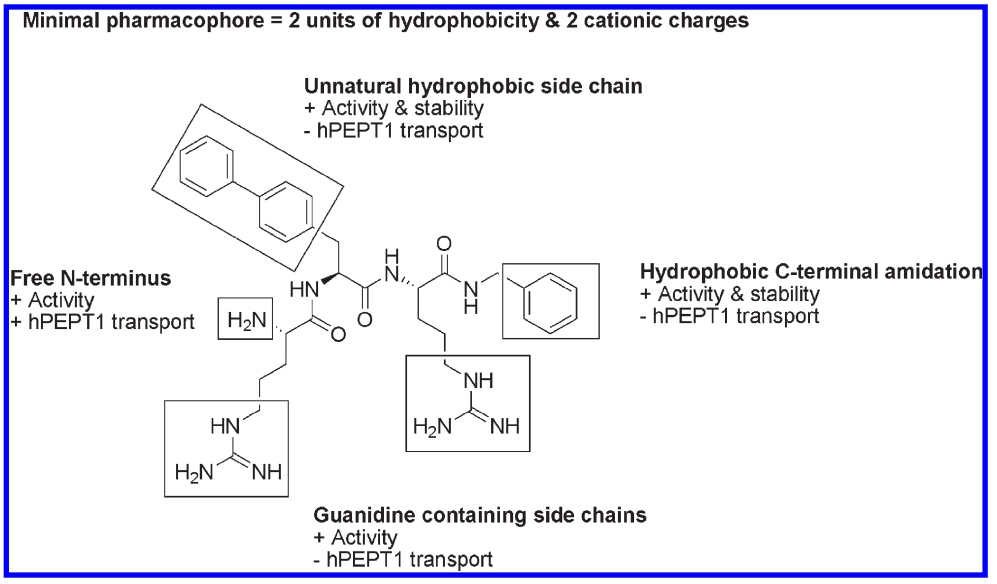


Figure 6. Schematic representation of the structural features employed to influence the properties of the included peptides, here exemplified with peptide **11**.

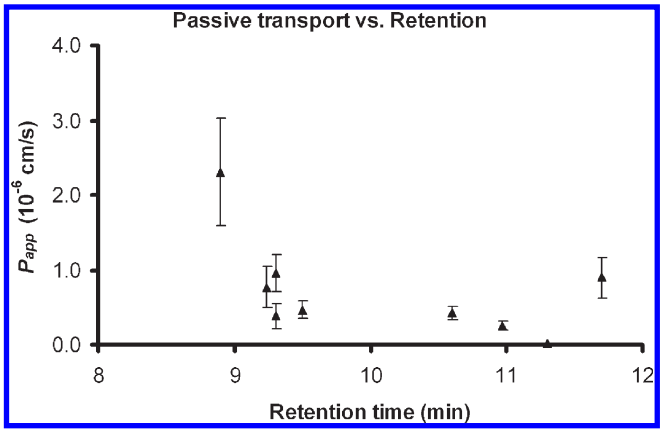


Figure 7. Passive transport (P_{app}) correlated with experimental C_{18} column retention. The transport of the more polar peptides is favored with the notable exception of peptide **13** incorporating Gpp instead of Arg as cationic amino acids.

antimicrobial activity of the active benzylamidated tripeptides illustrated in Figure 5.

The fact that the polar peptides are more rapidly crossing the barrier may indicate that they are less likely to dwell inside the barrier matrix compared to the more hydrophobic active ones. Such behavior may allow an insight into the mode of action of this class of peptides which remain elusive and still under debate. A low P_{app}, seen for the more bioactive peptides, would, if they first enter the membrane, permit them to reside longer inside a biological membrane and could be a contributing factor enabling or causing the membrane destabilization believed to be the main route to cell lysis. It is also important to realize that such a trend may prevent an efficient uptake of the most antibacterial drug candidates, although several of the active peptides displayed a significant uptake. The fact that peptides **3** and **13** display both a high uptake and a high antibacterial effect shows that such a combination is possible.

Collectively it is clear that the generality of the observations is low even for such a homogeneous group of peptides. The physicochemical properties responsible for barrier permeation are exerting their effect to different degrees depending on the peptide type, and the large differences between the different groups make interlibrary comparisons problematic. Hydrophobicity was shown to be the main determinant, while no correlation between molecular mass and transport was evident (see Supporting Information). It cannot be ruled out that the ability of the peptides to adopt different configurations in solution and in a membrane may have a large impact on the passive transport and also the bioactivity. It has previously been shown that secondary structure exerts a considerable influence on the paracellular transport of more polar peptides.[39] Such an effect would increase with peptide size and could explain the unpredicted results and the fact that QikProp did not anticipate any uptake of the tripeptides while the smaller reference drugs and dipeptides fitted well with the predictions. What is nevertheless clear is that most of the analyzed short cationic antimicrobial peptides in fact display a significant passive transport correlating to a moderate or excellent oral absorption and, based on those observations, may be suited for oral administration.

This is the first reported comprehensive study of the uptake of short membrane active cationic peptides, and while it has generated detailed insights into their transport modes it also highlights challenges associated with the methods employed. The first one is peptide solubility, and the second one is lysis at the concentrations employed. The peptides are designed to be selective against anionic membranes but also display lytic properties against eukaryotic and neutral membranes albeit at significantly higher concentrations. Such a general cytotoxic effect is important to consider even though these and similar peptides have been shown to display large selectivity factors.[14,19] This effect is apparent in the hPEPT1 study where both peptides **3** and **11** had to be excluded because of oocyte membrane damage at even the lowest employed concentration. The PVPA was more tolerant and allowed for analysis of peptides **3** and **11**, while peptide **8** influenced the barrier integrity. An uptake of peptide **8** cannot be ruled out at lower concentrations, although it is unlikely if it adheres to the trend of the other peptides in library 3. Even though the peptides included represent particularly challenging molecules to evaluate because of their membrane activity, only one had to be excluded, which indicates that the PVPA is a robust

dx.doi.org/10.1021/jm1015704 |*J. Med. Chem.* 2011, 54, 2422–2432

screening assay. QikProp was used to assess if the theoretical properties predicted could be generally applicable to these unusual druglike compounds. Even though the theoretical ClogP values are not absolute descriptors of hydrophobicity for ionizable compounds, they still correlated reasonably well with the experimental hydrophobicity determined chromatographically (see Supporting Information). The peptides ranged significantly in retention, and each library had to be investigated using a separate linear gradient. QikProp predicted the oral absorption of the three reference drugs with some accuracy and was also able to reasonably predict well the absorption, according to the in vitro passive permeability, of the active dipeptides **1**−**3**. For libraries 2 and 3, however, no oral absorption was anticipated. This is in clear contrast to the experimental data and implies that the general applicability of QikProp is limited, at least with regard to this compound class where secondary structure may also play a decisive role.

CONCLUSION

The goal of the present study was to assess whether short cationic antimicrobial peptides are able to be transported by hPEPT1 which mediates efficient uptake of di- and tripetides in the mammalian intestine and whether they permeate passively across cell membranes. It is shown that the strategies employed to generate peptides with high biological activity, such as the introduction of large hydrophobic unnatural amino acids and C-terminal amidation, counteract the transport capability for hPEPT1-mediated uptake. While some of the included peptides displayed moderate affinity for the transporter, none of them revealed significant transport by it. On the other hand, the PVPA study aimed at investigating the passive permeation properties revealed high permeation rates, suggesting a moderate to excellent oral absorption for most peptides. The factor mainly governing the permeation rate was the hydrophobicity but the structural features of the compounds are also important, making predictions difficult. It is thus clear that while the promising compounds are no proper substrates for hPEPT1-mediated uptake, they do offer, next to a high bioactivity, the potential for oral absorption via passive transcellular mechanisms. Those findings, in combination with their significant metabolic stabilities, make them attractive targets to pursue. A correlation between membrane permeation rates and biological activity may also provide insight into the mechanisms by which this class of compounds acts as antimicrobials that are efficient against bacteria resistant to conventional antibiotics.

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine (lipoid E-80) was obtained from Lipoid, Ludwigshafen, Germany. Filter inserts (Transwell, d = 6.5 mm) and plates were purchased from Corning Inc., Lowell, MA, U.S., and the mixed cellulose ester filters (0.65 μm pore size) from Millipore, Billerica, MA, U.S.. *Xenopus laevis* frogs were supplied by the African Xenopus Facility, Knysna, R.S.A. Standard chemicals and solvents were supplied by Sigma-Aldrich.

Peptide Preparation. Peptides (**4**, **7**, **9**, **13**, and the four reference peptides) were prepared as described earlier.[16,19] See Supporting Information for detailed description on chemicals used, preparation, purification, characterization, and antimicrobial testing of all included peptides.

Oocyte Preparation. *Xenopus laevis* oocytes were collected under anesthesia (immersion in a solution of 0.7 g/L 3-aminobenzoic acid ethyl ester) from frogs that were killed with an anesthetic overdose after the final oocyte collection. Oocytes were treated with 2.5 mg/mL collagenase for 70 min and were separated manually thereafter. They were incubated in Barth's solution containing (in mM) NaCl 88, KCl 1, $MgSO_4$ 0.8, $CaCl_2$ 0.4, $Ca(NO_3)_2$ 0.3, $NaHCO_3$ 2.4, HEPES 10 (pH 7.5) at 17 °C overnight. Next day stage V/VI oocytes were injected with about 25 ng of hPEPT1 cRNA in 18−27 nL and incubated for another 3−5 days at 17 °C.[49]

Electrophysiology. TEVC experiments were performed as described previously.[33] Briefly, the oocyte was placed in an open chamber (~0.5 mL total volume) and continuously superfused (~3 mL/min) at room temperature with Barth's solution in which HEPES was replaced by MES (pH 6.5). Oocytes were voltage-clamped at −60 mV using a TEC-05 amplifier (NPI Electronic, Tamm, Germany) and current−voltage (I/V) relations were measured using short (100 ms) pulses separated by 200 ms pauses in the potential range of −100 to −20 mV. I/V measurements were made immediately before and 20−30 s after substrate application when current flow reached steady state. The current evoked at a given membrane potential was calculated as the difference between the currents measured in the presence and the absence of substrate. Positive currents denote positive charges flowing out of the oocyte. The inhibitory effect of the selected substances was tested by comparison of currents generated by the peptide transporter substrate Gly-Sar at 1 mM in the absence and presence of the putative inhibitor.

Preparation of the PVPA Barriers. The phospholipid vesicle-based barriers were prepared and stored according to earlier reported procedures.[22,50] In brief, liposome dispersions extruded through filters with pore sizes of 800 nm and 800 + 400 nm, respectively, were deposited on a filter support by use of centrifugation. The liposomes were added in consecutive steps, first the smaller liposomes allowing them to deposit inside the pores of the filter and then the larger ones to layer on top of the filter. Freeze−thaw cycling was then performed to promote fusion of the liposomes and hence produce a tight permeation barrier.

Permeability Experiments. Permeability experiments were performed at room temperature without agitation. Inserts containing the different peptides dissolved in phosphate buffer, pH 7.4, were moved at certain time intervals to wells containing equal quantity of fresh phosphate buffer, pH 7.4.[50] UV absorbance (Spectramax 190; Molecular devices, Molecular Device Corporation, Sunnyvale, CA, U.S.) at wavelengths suitable for the different peptides was used to quantify the amount of peptides in the acceptor compartments. The electrical resistance across the lipid barriers was measured (Millicell-ERS, Millipore, Billerica, MA, U.S.) immediately after completion of the permeation studies to control the integrity of the barriers. Barriers displaying a resistance below 1000 Ω were excluded from the study. Each peptide was tested in at least eight parallels to ensure reliable data.

Theoretical Predictions. QikProp (Schrödinger, New York, NY) was used to calculate the ClogP values for the peptides and their theoretical oral absorption.[41] All the peptides were within the structural limits of the program.

ASSOCIATED CONTENT

Supporting Information. Synthetic procedures, purity data, HPLC traces, ^{1}H NMR spectra for all the tested peptides, plots of ClogP vs C_{18} retention, antimicrobial activity vs C_{18} retention, and molecular weight vs P_{app}. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

Professor Georg Nagel at the University of Würzburg is gratefully acknowledged for providing *Xenopus* oocyte images. The Norwegian Structural Biology Centre (NorStruct) is supported by the Functional Genomics Program (FUGE) of the Research Council of Norway.

■ ABBREVIATIONS USED

hPEPT1, human intestinal peptide transporter 1; PVPA, phospholipid vesicle-based permeation assay; TEVC, two-electrode voltage clamp; αMDG, α-methyl D-glucopyranoside; Gly-Sar, glycylsarcosine; PAMPA, parallel artificial membrane permeability assay; Bn, benzyl; GFP, green fluorescent protein; MIC, minimal inhibitory concentration; RP-HPLC, reversed phase high performance liquid chromatography; Bip, biphenylalanine; Dip, diphenylalanine; Gpp, L-2-amino-3-(4-guanidinophenyl)propanoic acid; ATCC, American Type Culture Collection; P_{app}, apparent permeability

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