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Application of Enzymatically Stable Dipeptides for Enhancement of Intestinal Permeability. Synthesis and In Vitro Evaluation of Dipeptide-Coupled Compounds

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Abstract—Transport across the intestinal barrier of compounds with low permeability may be facilitated by targeting the human oligopeptide transporter, hPepT1. A flexible synthetic pathway for attaching compounds to dipeptides through ester or amide bonds was developed. Furthermore, a synthetic approach to functionalize model drugs from one key intermediate was generated and applied to a glucose-6-phosphatase active model drug. The model drug was coupled to D-Glu-Ala through various linkers, and the G-6-Pase activity as well as the aqueous solubility and transport properties of these prodrugs, as compared to those of the parent drugs, were examined. None of the peptide-coupled compounds seemed to be transported by hPepT1, though one of the peptide-coupled compounds had affinity for hPepT1. Interestingly, in one case the parent drug was actively effluxed, while the corresponding peptide-coupled prodrug was not. The low aqueous solubility of the parent compounds was not increased after attachment to a dipeptide. This suggests that only compounds with a certain intrinsic aqueous solubility should be targeted to hPepT1 by attachment to a dipeptide. Important information about the design of peptide-coupled drugs targeted for hPepT1 is presented.

Introduction

The human oligopeptide transporter, hPepT1, situated in the small intestine, is involved in the absorption of nutrient oligopeptides and transports numerous di- and tripeptides and peptidomimetics.[1,2] Previous studies have shown that model drugs such as benzyl alcohols can be coupled to an enzymatically stable dipeptide which is recognized by hPepT1 and transported across the intestine via hPepT1.[3,4] Benzyl alcohol has been attached to the side-chain carboxylic acid of dipeptides such as D-Asp-Ala and D-Glu-Ala, and these model prodrugs were shown to be relatively enzymatically stable and cleaved during or after transport across the intestinal epithelium by pH-dependent hydrolysis.[5,6] To our knowledge, no model drugs significantly larger than benzyl alcohol have been attached to enzymatically stable dipeptides with the purpose of targeting hPepT1.

The overall purpose of the present work was to examine if the above strategy to increase the permeability across the intestine could be applied to larger biologically active molecules with low intestinal permeability. By attaching the target compound to a dipeptide, which is recognized by hPepT1, the compound may be transported by carrier-mediated mechanisms across the intestinal epithelium. Furthermore, attachment of a dipeptide may lead to increased aqueous solubility of the target compound, thus contributing to an overall increase in absorption. As model compounds we selected glucose-6-phosphatase (G-6-Pase) inhibitors of the 4-(4-chlorophenyl)-4,5,6,7-tetrahydrothieno3,2-cpyridine-5-yl-phenylmethanone type.[7] These compounds were found to be active as inhibitors of G-6-Pase in in vitro assays using disrupted microsomes as well as hepatocytes, but they lacked in vivo activity. The catalytic site of the G-6-Pase enzyme is situated inside the microsomes, meaning that the compounds have to pass two membranes to reach their target. Investigations of the model drugs in the Caco-2 cell line revealed that the compounds had very low or no permeability. This may

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be due to the inherent poor aqueous solubility of the compounds or other physicochemical characteristics, which prevent the compounds from permeating cell membranes and undergo passsive transcellular transport. Consequently, it seemed worthwhile to investigate if linkage to a dipeptide could increase permeability as seen with peptide-coupled benzyl alcohol.

We therefore developed a flexible method for introduction of various linkers into one key structure of the model drug. The functionalized model drug was then attached to the enzymatically stable dipeptide D-Glu-Ala through ester or amide bonds, and the G-6-Pase activity as well as the aqueous solubility and transport properties of these prodrugs, as compared to those of the parent drugs, were examined.

Results and Discussion

Synthesis of D-Glu-Ala

Based on previous studies, D-Asp-Ala was the first dipeptide chosen for attachment to the model drug.[3] However, D-Asp-Ala gave rise to problems during synthesis of the prodrugs, probably due to formation of an intramolecular cyclic imide.[8] Extending the side-chain in Asp with one methylene group minimized cyclization, and therefore D-Asp-Ala was replaced by D-Glu-Ala. This substitution seemed to exert a general decrease in affinity for hPepT1, but affinity was still retained in the 5 mM range.[4]

D-Glu-Ala was synthesized from Boc-D-Glu(OFm)-OH and H-Ala-*O*-*t*-Bu·HCl as illustrated in Scheme 1. After HOBt and EDAC catalyzed coupling, the Fm-group protecting the side-chain carboxylic acid could be removed selectively with triethylamine.

Boc-D-Glu-Ala-*O*-*t*-Bu was purified by ion exchange and coupled to the target model drug. The product was finally deprotected by trifluoroacetic acid in dichloromethane to give the trifluoroacetate salts. This synthetic approach provides a flexible method for attachment of compounds to the side-chain carboxylic acid of a dipeptide through ester or amide bonds.

Introduction of linkers in the model drug

Compounds of the [4-(4-chlorophenyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine-5-yl]-phenylmethanone type were prepared with the purpose of controlling the glucose output from the liver in diabetic patients.[7] A general structure is presented in Scheme 2. Structure–activity experiments had previously mapped the area least sensitive to structural changes with respect to the pharmacological action, and this site, the *para* position in the phenyl group attached to carbonyl, was used for functionalization. The 4-iodo key intermediate **2** was synthesized from **1** as outlined in Scheme 2.

Using **2** as a starting point, various linkers were inserted by halogen–metal exchange, followed by addition of an electrophile or cross-coupling protocols. Halogen–metal exchange was first attempted using the corresponding 4-bromo derivative, but lithiation and addition of electrophile gave a complex mixture. In contrast, the 4-iodo derivative **2** was converted smoothly to the formyl derivative **3** via a magnesium intermediate,[9] and further reactions provided a series of functionalized compounds originating from one key structure (**2**) (Scheme 3).

From the formyl derivative **3** two different compounds were prepared, to which subsequent coupling to the dipeptide was possible: A compound with a hydroxymethyl linker (**4**) and a compound with an aminomethyl linker (**7**). A derivative with an amino linker (**9**) was synthesized from the acetamino derivative **8**, which was obtained in a similar way as the 4-iodo derivative **2**.

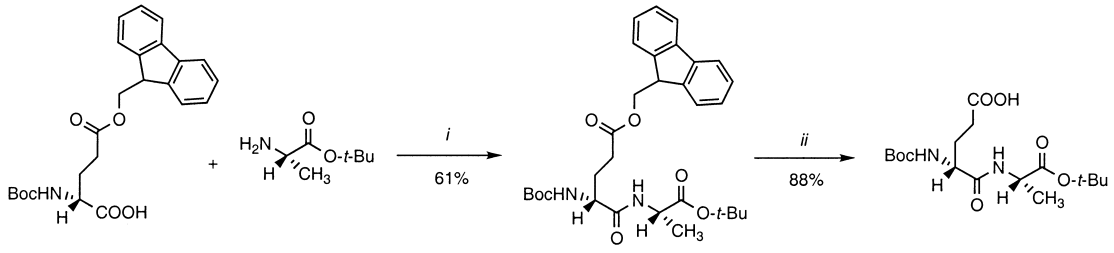
Synthesis of model prodrugs

The functionalized derivatives with a hydroxymethyl, an aminomethyl or an amino linker were attached to D-Glu-Ala through amide or ester bonds, respectively. To minimize the risk of racemization, the coupling reactions were performed without addition of base. The synthesis of the model prodrugs is outlined in Scheme 4.

Glucose-6-phosphatase activity and transport properties

Since the linkers introduced in key structure **2** were not removable, the functionalized compounds, as well as the model prodrugs, were tested with respect to G-6-Pase inhibitory activity according to previously described procedures.[7] The hydroxymethyl linker compound **4** showed the highest G-6-Pase inhibitory activity, followed by the amino linker compound **9**. The aminomethyl linker compound **7** did not show significant activity (Fig. 1).

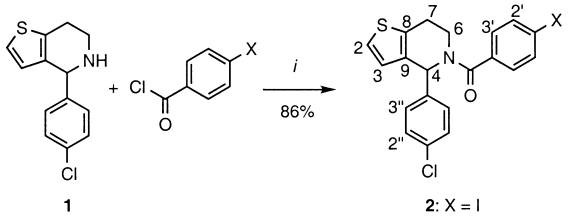
As expected, the corresponding prodrugs **13**, **14,** and **15** all showed decreased G-6-Pase activity, presumably due



Scheme 1. Synthesis of Boc-D-Glu-Ala-*O*-*t*-Bu. (i) HOBt, EDAC, DIPEA, DMF, rt, 3 days; (ii) TEA, CH_2Cl_2, rt, 3 days.

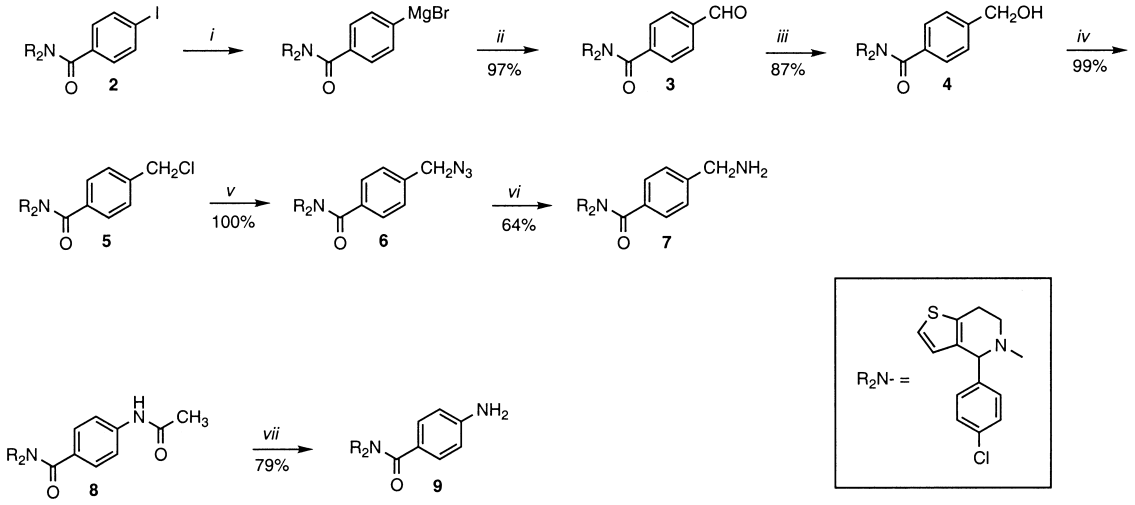
to the bulkiness of the peptide-coupled compounds, which are no longer recognized by the G-6-Pase enzyme. Based on previous observations of cleavage of this kind of prodrugs after transport, the active model drug is expected to be released by pH-dependent hydrolysis after transport to the active site.[3–5]

The transport properties of the six compounds were measured in Caco-2 cells, and the permeability is presented in Figure 2. Verapamil was used as a control for monitoring the function of P-glycoprotein. The presence of P-glycoprotein confirms that the monolayers express transporter characteristics of a properly matured Caco-2 phenotype.

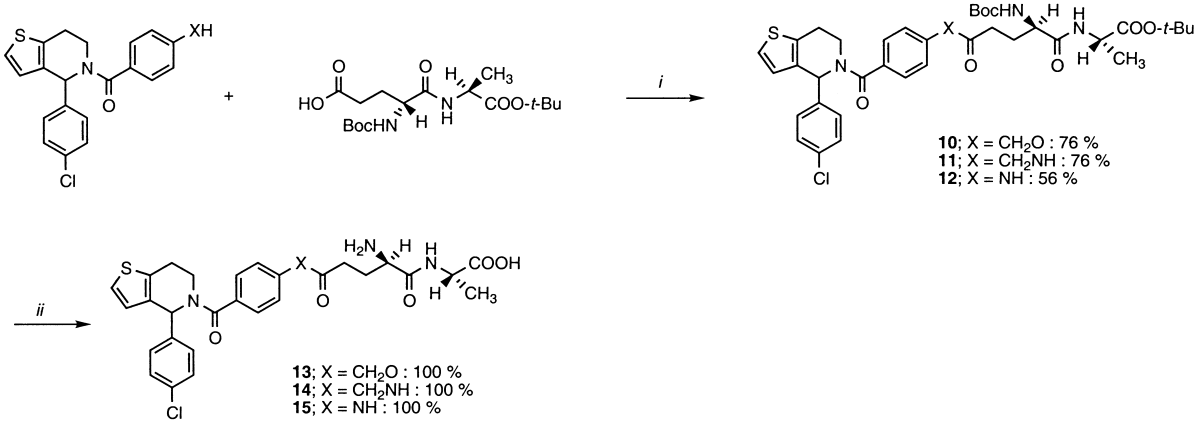


Scheme 2. Synthesis of the 4-iodo key intermediate **2**. (i) TEA, CH_2Cl_2, 0 °C, 10 min then R.T., 3 h.

The apical to basolateral transport was not significantly larger than the basolateral to apical transport in any of the compounds. This suggests that none of the compounds are transported into the cell by carrier-mediated mechanisms. Interestingly, the parent compound **4** with the hydroxymethyl linker was substrate for an efflux transporter such as P-glycoprotein, while the peptide-coupled compound **13** was not (efflux transporter score for compound **4** = 3.3 and for prodrug **13** = 1.5). This suggests that active efflux can be reduced by attachment to a dipeptide. A similar observation was made with the antiherpes drug Acyclovir, which is actively effluxed from the cell. Attachment of valine to Acyclovir results in a hPepT1 substrate, Valacyclovir, which is no longer effluxed from the cell.[10] The two other prodrugs, the aminomethyl linked prodrug **14** (not shown) and the amino linked prodrug **15** had low permeability in the Caco-2 cells, probably due to the poor aqueous solubility of these compounds. Compound **9** with the amino linker was transported, but was also actively effluxed as seen with compound **4**. The aqueous solubility of compound **7** with the aminomethyl linker was too low to perform the transport study.



Scheme 3. Synthetic pathway for functionalised compounds **4** and **7** derived from **2** and for the amino derivative **9**. (i) *i*-PrMgBr, −15 °C, 1 h; (ii) DMF, −15 °C, 3 h; (iii) $NaBH_4$, 0 °C to rt; (iv) $SOCl_2$, 0 °C, 1 h; (v) NaN_3, rt, 24 h; (vi) Pd/C, rt, 3 h; (vii) 2 M HCl, 80 °C, 15 h.



Scheme 4. Synthesis of model prodrugs. (i) HOBt, EDAC, DMF, rt, 15 h; (ii) TFA, CH_2Cl_2, rt, 3 h.

Compound	IC_{50} (μM)
4	0.51
13	> 100
7	> 100
14	> 100
9	25
15	> 100

Figure 1. Glucose-6-phosphatase activity (IC_{50}).

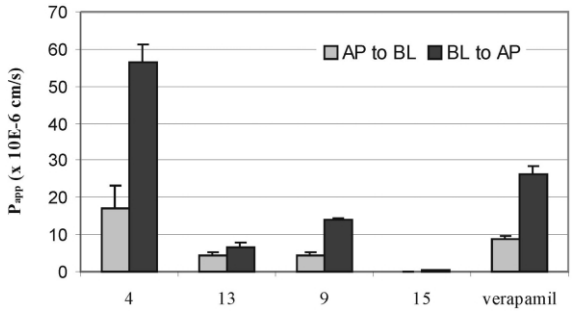


Figure 2. Permeability of compounds **4** and **9** and the corresponding model prodrugs **13** and **15** (all 50 mM). AP: Apical side, BL: Basolateral side.

The hPepT1-affinity of prodrug **13** was tested in the presence of Gly-Sar to clarify if the compound was a hPepT1 substrate. Due to the limited aqueous solubility, concentrations above 0.1 mM were not obtained, and it was not possible to calculate the K_i value. Some inhibition of hPepT1 with respect to Gly-Sar uptake at the Caco-2 monolayers was observed, though. Recently, several compounds have been reported to interact with hPepT1 without being transported.[11,12] Since further transport studies could not be performed due to low aqueous solubility, no conclusion about hPepT1-mediated transport of **13** could be made.

The turbidimetric solubility of the three functionalized compounds and the corresponding prodrugs was measured by nephrelometry, which revealed that the aqueous solubility of the parent compounds and the prodrugs was very low (from 40–100 μg/mL). Thus, coupling to D-Glu-Ala did not increase the aqueous solubility significantly.

Conclusions

In conclusion, this study presents the first attempt to transport dipeptide-coupled active drug substances via hPepT1. A general synthetic pathway for attaching compounds to dipeptides through ester or amide bonds was developed. Furthermore, a synthetic approach to functionalize model drugs from one key intermediate was generated. None of the peptide-coupled compounds were transported by hPepT1. One of three peptide-coupled compounds seemed to have a certain affinity for hPepT1, though. Interestingly, in one case the parent compound was actively effluxed from the cell, while the corresponding peptide-coupled compound was not. This suggests the possibility to reduce active efflux by attachment to dipeptides. Since the aqueous solubility of the parent compounds was not improved significantly when a dipeptide was attached, this did not contribute to an overall increase in permeability.

This study implies restrictions towards the size of compounds that can be transported via hPepT1 by attachment to D-Glu-Ala. Furthermore, the compounds should possess a certain intrinsic solubility to be able to explore the oligopeptide transporter by the dipeptide prodrug approach. Thus, important information about rational design of dipeptide-coupled prodrugs targeted for hPepT1 was obtained.

Experimental

Chemistry

Materials. All reactions involving air-sensitive reagents were performed under a nitrogen atmosphere using syringe-septum cap techniques, and glassware was flame-dried prior to use. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) was handled under a nitrogen atmosphere. Flash chromatography was performed using silica gel (Merck 60, 70–230 mesh). Melting points are uncorrected. NMR spectra were recorded on a 300 MHz instrument (Varian Gemini). NMR signals were assigned using APT spectra and comparing with the spectrum of the general structure shown in Scheme 2 (X=H), which was throughoutly assigned using C-H correlated and HMBC spectra. Elemental analyses were performed by Microanalytical Laboratory, Department of Physical Chemistry, University of Vienna, Austria.

Boc-D-Glu(OFm)-OH and H-Ala-*O*-*t*-Bu·HCl were purchased from Bachem, Bubendorf, Switzerland. 2-Thienylethyl amine was purchased from Avocado Research Chemicals Ltd, Karlsruhe, Germany. All other reagents and solvents were obtained from Fluka or Aldrich and used without further purification except dimethyl formamide (DMF) and triethylamine (TEA), which were dried and stored over 3Å molecular sieves. *i*-PrMgBr was titrated (1.42 M in THF) prior to use.[13]

[4-(4-Chlorophenyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine-5-yl]-(4-iodophenyl)-methanone (2). To a stirred solution of **1** (1.00 g, 4.03 mmol) in dichloromethane (50 mL) was added dry TEA (1.1 mL, 8.06 mmol). The solution was cooled to 0 °C, 4-iodobenzoyl chloride (1.60 g, 6.05 mmol) was added, and stirring was con-

tinued for 10 min at 0 °C, The mixture was then allowed to warm up to rt and stirred for additional 3 h. After addition of saturated aqueous $NaHCO_3$ (50 mL), the solution was stirred overnight at rt. The organic phase was separated and washed with HCl (1 M, 2×50 mL), NaOH (2 M, 2×50 mL), and water (2×50 mL). Residues of benzoic acid anhydride were converted to benzamide by addition of NH_3 (25% in water, 25 mL) to a dichloromethane solution. After stirring for 1 h at rt, the water phase was removed, and the organic phase was evaporated in vacuo. Flash chromatography (CH_2Cl_2) yielded 3.30 g (86%) as colorless crystals of 2; R_f = 0.79 (EtOAc 1:1); mp 220–221 °C; ^{1}H NMR ($CDCl_3$) δ 7.77 (2H, d, J = 8.4 Hz, 2×H-2′), 7.30 (4H, m, 2×H-2″ + 2×H-3″), 7.20 (1H, d, J = 5.1 Hz, H-2), 7.11 (2H, d, J = 8.4 Hz, 2×H-3′), 6.95 (1H, broad s, H-4), 6.72 (1H, d, J = 5.1 Hz, H-3), 3.71 (1H, m, H-6^A), 3.28 (1H, m, H-6^B), 2.96 (1H, m, H-7^A), 2.85 (1H, m, H-7^B). Anal. calcd for $C_{20}H_{15}NOSClI$: C, 50.07; H, 3.15; N, 2.92. Found: C, 49.81; H, 3.29; N, 3.13. The racemate was used in the study.

[4-(4-Chlorophenyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine-5-yl]-(4-formylphenyl)-methanone (3). **2** (1.00 g, 2.09 mmol) was dissolved in dry THF (20 mL) by heating. The solution was cooled to −15 °C, then *i*-PrMgBr (1.42 M) (1.76 mL, 2.50 mmol) was added over a 2 min period. Stirring was continued for 1 h at −15 °C, DMF (800 μL, 10.4 mmol) was added, and the solution was stirred for further 3 h at −15 °C. After addition of HCl (1 M, 50 mL), the solution was allowed to warm up to rt in 1 h. A colorless precipitate appeared, which was isolated by filtration and dried in vacuo. This gave 779 mg (97%) of **3**; mp 190–191 °C; ^{1}H NMR ($CDCl_3$) δ 10.06 (1H, s, CHO), 7.95 (2H, d, J = 8.4 Hz, 2×H-2′), 7.53 (2H, d, J = 8.1 Hz, 2×H-3′), 7.32 (4H, m, 2×H-2″ + 2×H-3″), 7.22 (1H, d, J = 5.1 Hz, H-2), 6.98 (1H, broad s, H-4), 6.75 (1H, d, J = 5.1 Hz, H-3), 3.63 (1H, m, H-6^A), 3.31 (1H, m, H-6^B), 2.98 (1H, m, H-7^A), 2.86 (1H, m, H-7^B). Anal. calcd for $C_{21}H_{16}O_2NSCl$: C, 66.05; H, 4.22; N, 3.67. Found: C, 65.78; H, 4.48; N, 3.53. The compound should be stored under a nitrogen atmosphere at −18 °C.

[4-(4-Chlorophenyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine-5-yl]-(4-hydroxymethyl-phenyl)-methanone (4). **3** (500 mg, 1.31 mmol) was suspended in methanol (10 mL) and cooled to 0 °C. To this solution was added $NaBH_4$ (198 mg, 5.24 mmol) over a 5 min period. Stirring was continued for 20 min while the solution was allowed to warm up to rt. The crystals formed were washed with water (10 mL, acidified with 4 M HCl) and with further 2×10 mL water until neutral. Drying ($MgSO_4$) gave 503 mg (100%) of the crude product. Recrystallization from ethyl acetate gave 440 mg (87%) as colorless crystals of **4**; mp 122.5–123.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.44–7.26 (8H, m, 2×H-2′ + 2×H-3′ + 2×H-2″ + 2×H-3″), 7.20 (1H, d, J = 5.4 Hz, H-2), 6.98 (1H, broad s, H-4), 6.74 (1H, d, J = 5.4 Hz, H-3), 4.73 (2H, d, J = 5.4 Hz, CH_2O), 3.75 (1H, m, H-6^A), 3.27 (1H, m, H-6^B), 2.98 (1H, m, H-7^A), 2.83 (1H, m, H-7^B), 1.93 (1H, t, J = 5.7 Hz, OH). Anal. calcd for $C_{21}H_{18}NO_2SCl$: C, 65.70; H, 4.73; N, 3.65. Found: C, 65.44; H, 4.91; N, 3.53.

[4-(4-Chlorophenyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine-5-yl]-(4-chloromethyl-phenyl)-methanone (5). A solution of **4** (100 mg, 0.26 mmol) in dichloromethane (3 mL) was treated with thionyl chloride (0.12 mL, 1.62 mmol) and stirred for 1 h at 0 °C. The mixture was concentrated, co-evaporated three times with toluene and dried in vacuo to give 104 mg (99%) of **5** as a colorless solid, which was used without further purification. For analysis, the compound was purified by flash chromatography (CH_2Cl_2); mp 129–131 °C; R_f = 0.72 (EtOAc 1:1); ^{1}H NMR ($CDCl_3$) δ 7.44 (2H, d, J = 8.4 Hz, 2×H-3′), 7.37 (2H, d, J = 8.4 Hz, 2×H-2′), 7.34–7.27 (m, 2×H-2″ + 2×H-3″), 7.21 (1H, d, J = 5.1 Hz, H-2), 6.97 (1H, broad s, H-4), 6.73 (1H, d, J = 4.5 Hz, H-3), 4.60 (2H, s, CH_2Cl), 3.75 (1H, m, H-6^A), 3.28 (1H, m, H-6^B), 2.98 (1H, m, H-7^A), 2.84 (1H, m, H-7^B). Anal. calcd for $C_{21}H_{17}NOSCl_2$: C, 62.69; H, 4.26; N, 3.48. Found: C, 62.92; H, 4.36; N, 3.52.

(4-Aminomethyl-phenyl)-4-(4-chloro-phenyl)-6,7-dihydro-4*H*-thieno[3,2-c]pyridine-5-yl-methanone (7). Sodium azide (162 mg, 2.49 mmol), DMF (10 mL), and **5** (1000 mg, 2.49 mmol) were stirred for 24 h at rt. After addition of water (50 mL), the product was extracted with dichloromethane (3×50 mL). The solvent was removed in vacuo, and the product was dissolved in ethyl acetate (20 mL) and washed with saturated aqueous $CaCl_2$ (3×10 mL) to remove DMF. Removal of the solvent in vacuo gave 1.02 g (100%) of the azide **6** as a colorless solid.

The azide **6** (965 mg) was dissolved in MeOH (50 mL) and cooled to −78 °C. This solution was added to Pd/C (10%) (50 mg) and stirred under hydrogen for 3 h at rt. After filtration through Celite, the solvent was evaporated in vacuo. Flash chromatography (CH_2Cl_2-MeOH 9:1) gave 583 mg (64%) as colorless crystals of **7**; mp 68–72 °C; ^{1}H NMR ($CDCl_3$) δ 7.40–7.26 (8H, m, aromatic protons), 7.20 (1H, d, J = 5.1 Hz, H-2), 6.97 (1H, broad s, H-4), 6.73 (1H, d, J = 5.1 Hz, H-3), 3.91 (2H, s, CH_2NH_2), 3.76 (1H, m, H-6^A), 3.26 (1H, m, H-6^B), 2.98 (1H, m, H-7^A), 2.82 (1H, m, H-7^B), 1.95 (2H, s, NH_2). Anal. calcd for $C_{21}H_{19}N_2OSCl$ + 1.4 mol H_2O: C, 61.80; H, 5.38; N, 6.86. Found: C, 61.69; H, 5.01; N, 6.72.

(4-Acetamino-phenyl)-[4-(4-chloro-phenyl)-6,7-dihydro-4*H*-thieno[3,2-*c*]pyridine-5-yl]-methanone (8). 1-Hydroxybenzotriazole hydrate (649 mg, 4.81 mmol) was added to a stirred solution of 4-acetaminobenzoic acid (717 mg, 4.00 mmol) in dry DMF (5 mL) under nitrogen atmosphere at rt. After addition of EDAC (921 mg, 4.81 mmol), the solution was stirred for 30 min, then **1** (1000 mg, 4.00 mmol) was added. The solution was stirred overnight at rt. Addition of water (30 mL) gave a colorless precipitate. The solution was filtered, and the residue was dissolved in dichloromethane (30 mL). The organic solution was washed with HCl (1 N, 10 mL) and NaOH (1 N, 3×10 mL), then dried ($MgSO_4$), filtered, and evaporated in vacuo. Flash chromatography (EtOAc) yielded 1490 mg (91%) of **8** as colorless crystals; R_f = 0.50 (EtOAc); mp 162–163 °C; ^{1}H NMR ($CDCl_3$) δ 7.86 (1H, s, NH), 7.48 (2H, d, J = 8.4 Hz, H-2′), 7.30 (4H, m, 2×H-2″ + 2×H-3″), 7.28 (1H, d,

$J=8.4$ Hz, 2×H-3′), 7.20 (1H, d, $J=5.1$ Hz, H-2), 6.94 (1H, broad s, H-4), 6.71 (1H, d, $J=4.5$ Hz, H-3), 3.80 (1H, m, H-6^A), 3.27 (1H, m, H-6^B), 2.99 (1H, m, H-7^A), 2.84 (1H, m, H-7^B), 2.15 (3H, s, CH_3).

(4-Amino-phenyl)-[4-(4-chloro-phenyl)-6,7-dihydro-4*H*-thieno[3,2-*c*]pyridine-5-yl]-methanone (9). 8 (600 mg, 1.46 mmol) was dissolved in HCl (2 M, aq) (7.3 mL, 14.6 mmol) and MeOH (50 mL). The solution was stirred overnight at 80 °C, evaporated in vacuo to dryness, and co-evaporated twice with methanol (2×10 mL) to remove HCl. After addition of water (10 mL), the product was extracted with dichloromethane (5×20 mL). Evaporation in vacuo afforded 536 mg (99%) of **9** as colorless crystals. Recrystallization from 2-propanol gave one batch containing **9** as the hydrochloride (249 mg, 46%, mp 235–238 °C) and one batch containing the free amine (269 mg, 33%); mp 169–170 °C; $R_f=0.26$ (EtOAc 1:1); ^{1}H NMR ($CDCl_3$) δ 7.30–7.25 (4H, m, 2×H-2″ + 2×H-3″), 7.23 (2H, dt, $J=8.7$ Hz, $J=2.4$ Hz, 2×H-3′), 7.18 (1H, d, $J=5.4$ Hz, H-2), 6.71 (1H, d, $J=5.4$ Hz, H-3), 6.66 (2H, dt, $J=8.4$ Hz, $J=2.40$ Hz, 2×H-2′), 3.85 (2H, s, NH_2), 3.25 (1H, m, H-6^A), 3.03 (1H, m, H-6^B), 2.85 (1H, m, H-7^A), 2.80 (1H, m, H-7^B). Anal. calcd for $C_{20}H_{17}N_2OSCl$: C, 65.12; H, 4.64; N, 7.59; Cl, 9.61. Found: C, 64.83; H, 4.74; N, 7.34; Cl, 9.65.

Boc-D-Glu(OFm)-Ala-*O*-*t*-Bu. Boc-D-Glu(OFm)-OH (5.00 g, 11.8 mmol) and 1-hydroxybenzotriazole hydrate (1.80 g, 11.8 mmol) were dissolved in dry DMF (100 mL) under nitrogen atmosphere. After 2–3 min, *N*-ethyldiisopropylamine (1.90 mL, 12.9 mmol) was added. H-Ala-*O*-*t*-BuHCl (1.71 g, 11.8 mmol) and EDAC (3.38 g, 17.6 mmol) were added, and the solution was stirred under nitrogen atmosphere for 3 days at rt. The solution was evaporated to dryness in vacuo, and the residue was dissolved in ethyl acetate (100 mL). The organic solution was washed with saturated aqueous $NaHCO_3$ (3×20 mL) and 5% aqueous acetic acid (3×30 mL). Drying ($MgSO_4$), filtration, and removal of EtOAc in vacuo afforded the crude product as a yellow oil. Flash chromatography (CH_2Cl_2→CH_2Cl_2-MeOH 9:1) yielded 3.96 g (61%) as colorless crystals; mp 52–54 °C; $R_f=0.60$ (CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 7.80–7.28 (8H, m, aromatic Fm-protons), 6.69 (1H, d, $J=6.0$ Hz, NH), 5.25 (1H, d, $J=6.3$ Hz, NH), 4.50–4.32 (3H, m, CH in Glu + CH in Ala + CH in Fm-group), 4.22 (2H, t, $J=6.9$ Hz, CH_2 in Fm-group), 2.65–2.40 (2H, m, CH_2 in Glu), 2.25–1.85 (2H, m, CH_2 in Glu), 1.46 (9H, s, Boc), 1.43 (9H, s, COO-*t*-Bu), 1.37 (3H, d, $J=7.2$ Hz, CH_3). Anal. calcd for $C_{31}H_{40}N_2O_7$: C, 67.37; H, 7.30; N, 5.07. Found: C, 67.44; H, 7.27; N, 5.01.

Boc-D-Glu-Ala-*O*-*t*-Bu. Boc-D-Glu(OFm)-Ala-*O*-*t*-Bu (4.00 g) was dissolved in dichloromethane (30 mL), dry TEA (5 mL) was added, and the solution was stirred for 3 days at rt. The solvent was removed in vacuo, and the residue was dissolved in water (50 mL). The solution was filtered, and the residue was extracted with further 3×10 mL of water. MeOH (80 mL) was added to the combined aqueous solutions. This solution was passed through a column with ion exchanger (Amberlite IR-120 (50 mL) at the H^+-form. The column was eluted with further 50 mL of MeOH-water [1:1]. Removal of the solvent in vacuo gave 2.39 g (88%) of Boc-D-Glu-Ala-*O*-*t*-Bu as colorless crystals; mp 135–136 °C; ^{1}H NMR ($CDCl_3$) δ 7.20 (1H, d, $J=6.9$ Hz, NH), 5.49 (1H, d, $J=7.8$ Hz, NH), 4.60–4.24 (2H, m, CH in Glu + CH in Ala), 2.60–1.80 (4H, m, 2×CH_2 in Glu), 1.44 (9H, s, Boc), 1.42 (9H, s, COO-*t*-Bu), 1.37 (3H, d, $J=7.2$ Hz, CH_3 in Ala); ^{13}C NMR (DMSO) δ 176.3 (COOH), 172.2/171.5 (COO-*t*-Bu), 156.1 (CONHR), 82.1/80.4 ($\underline{C}(CH_3)_3$), 53.2 (CH), 48.7 (CH), 29.8 ($\underline{C}H_2COOH$, $\underline{C}H_2CH$), 18.1 (CH_3).

4-*tert*-Butoxycarbonylamino-4-(1-*tert*-butoxycarbonyl-ethylcarbamoyl)-butyric acid 4-[4-(4-chloro-phenyl)-6,7-dihydro-4*H*-thieno[3,2-*c*]pyridine-5-carbonyl]-benzyl ester (10), Standard procedure. To a stirred solution of Boc-D-Glu-Ala-*O*-*t*-Bu (300 mg, 0.80 mmol) in dry DMF (5 mL) was added 1-hydroxybenzotriazole hydrate (162 mg, 1.20 mmol) under a nitrogen atmosphere at rt. After addition of EDAC (338 mg, 1.76 mmol), the solution was stirred for 30 min, then **4** (615 mg, 1.60 mmol) was added. The solution was stirred for 15 h at rt. The solvent was evaporated in vacuo, and the residue was dissolved in dichloromethane (30 mL), washed with water (2×10 mL) and saturated aqueous $NaHCO_3$ (4×10 mL), dried ($MgSO_4$), filtered, and evaporated in vacuo. Flash chromatography (EtOAc 2:1→EtOAc 1:1) yielded 452 mg (76%) of the protected compound **10** as a colorless solid with a broad melting point; $R_f=0.37$ (P.E.′EtOAc [1:1]); ^{1}H NMR (DMSO) δ 7.44–7.26 (8H, m, 2×H-2′ + 2×H-3′ + 2×H-2″ + 2×H-3″), 7.21 (1H, d, $J=4.8$ Hz, H-2), 6.98 (1H, broad s, H-4), 6.73 (1H, d, $J=4.8$ Hz, H-3), 6.67 (1H, d, $J=6.9$ Hz, NH), 5.20 (1H, m, NH), 5.16 (2H, s, CH_2O), 4.42 (1H, m, CH in Ala), 4.20 (1H, m, CH in Glu), 3.76 (1H, m, H-6^A), 3.28 (1H, m, H-6^B), 3.00 (1H, m, H-7^A), 2.84 (1H, m, H-7^B), 2.52 (2H, m, CH_2 in Glu), 2.26–1.86 (2H, m, CH_2 in Glu), 1.46 (9H, s, Boc), 1.44 (9H, s, COO-*t*-Bu), 1.37 (3H, d, $J=6.9$ Hz, CH_3).

1-(1-Carboxy-ethylcarbamoyl)-3-{4-[4-(4-chlorophenyl)-4,5,6,7-tetrahydro-4*H*-thieno[3,2-*c*]pyridine-5-carbonyl]-benzyloxycarbonyl}-propyl-ammonium trifluoroacetate (13), Standard procedure. The protected conjugate **10** (452 mg) was dissolved in dichloromethane (20 mL). After addition of TFA (10 mL), the solution was stirred for 3 h at rt. The solvent was removed by evaporation in vacuo, and TFA was co-evaporated with ether twice. This produced 426 mg (100%) of **13** as a colorless solid with a broad melting point (60–75 °C); ^{1}H NMR (DMSO) δ 8.81 (1H, d, $J=7.5$ Hz, NH), 8.19 (3H, broad s, NH_3), 7.50–7.20 (9H, m, aromatic protons + H-2), 6.85 (1H, d, $J=5.1$ Hz, H-3), 6.76 (1H, broad s, H-4), 5.13 (2H, s, CH_2O), 4.25 (1H, m, CH in Ala), 3.84 (1H, m, CH in Glu), 3.61 (1H, m, H-6^A), 3.22 (1H, m, H-6^B), 2.97 (1H, m, H-7^A), 2.86 (1H, m, H-7^B), 2.5 (2H, m, CH_2 in Glu), 2.02 (2H, m, CH_2 in Glu), 1.28 (3H, 2×d, $J=7.5$ Hz, CH_3); ^{13}C NMR (DMSO) δ 174.1 (COOH), 172.1 ($COOCH_2$), 169.8 ($CONR_2$), 168.2 (CONHR), 158.9 (q, $J=36$ Hz, CF_3CO), 140.6 (C-4″), 138.1 (C-4′), 136.2 (C-8), 134.9 (C-1′), 133.6 (C-1″), 132.9 (C-9), 130.4 (C-3″), 129.0 (C-2′), 128.5 (C-2″), 127.1 (C-3′), 127.0 (C-3), 124.7 (C-2), 116.3 (q,

$J = 293$ Hz, CF_3), 66.1 ($PhCH_2O$), 53.1 (C-4), 51.7 (CH), 48.2 (CH), 41.4 (C-6), 29.1 (CH_2CO), 26.6 (CH_2CH), 25.4 (C-7), 17.6 (CH_3); MS (EI) 584 (M^+), calcd 584 (M).

2-(2-*tert*-Butoxycarbonylamino-4-{4-[4-(4-chloro-phenyl)-6,7-dihydro-4*H*-thieno[3,2-*c*]pyridine-5-carbonyl]-benzylcarbamoyl}-butyrylamino-propionic acid *tert*-butyl ester (11). The standard procedure yielded 76% of the protected compound **11** as a colorless solid with a broad melting point; $R_f = 0.47$ (EtOAc); ^{1}H NMR (DMSO) δ 7.37–7.27 (8H, m, aromatic protons), 7.20 (1H, d, $J = 5.4$ Hz, H-2), 6.95 (1H, broad s, H-4), 6.74 (1H, d, $J = 5.7$ Hz, H-3), 6.74 (1H, NH), 5.61 (1H, d, $J = 6.0$ Hz, NH), 4.49 (2H, m, CH_2Ph), 4.40 (1H, m, CH in Ala), 4.13 (1H, m, CH in Glu), 3.75 (1H, m, H-6^A), 3.26 (1H, m, H-6^B), 2.97 (1H, m, H-7^A), 2.83 (1H, m, H-7^B), 2.35 (2H, m, CH_2 in Glu), 2.20–1.92 (2H, m, CH_2 in Glu), 1.44 (9H, s, Boc), 1.42 (9H, s, COO-*t*-Bu), 1.32 (3H, d, $J = 7.2$ Hz, CH_3).

1-(1-Carboxy-ethylcarbamoyl)-3-{4-[4-(4-chlorophenyl)-4,5,6,7-tetrahydro-4*H*-thieno[3,2-*c*]pyridine-5-carbonyl]-benzylcarbamoyl}-propyl-ammonium trifluoroacetate (14). The standard procedure yielded 100% of **14** as a yellow solid with a broad melting point (100–120 °C); ^{1}H NMR (DMSO) δ 8.80 (1H, d, $J = 7.5$ Hz, NH), 8.54 (1H, t, $J = 6.0$ Hz, NH in linker), 8.18 (3H, broad s, NH_3), 7.47–7.22 (9H, m, H-2 + aromatic protons), 6.85 (1H, d, $J = 5.1$ Hz, H-3), 6.76 (1H, broad s, H-4), 4.30 (2H, m, CH_2Ph), 4.28 (1H, m, CH in Ala), 3.84 (1H, m, CH in Glu), 3.63 (1H, m, H-6^A), 3.20 (1H, m, H-6^B), 2.98 (1H, m, H-7^A), 2.86 (1H, m, H-7^B), 2.26 (2H, m, CH_2 in Glu), 1.97 (2H, m, CH_2 in Glu), 1.31 (3H, d, $J = 7.2$ Hz, CH_3); ^{13}C NMR (DMSO) δ 174.1 (COOH), 171.6 (CONHR), 170.0 ($CONR_2$), 168.4 (CONHR), 158.7 (q, $J = 34$ Hz, $CF_3\underline{C}O$), 141.6 (C-4″), 140.6 (C-4′), 135.1 (C-8), 134.9 (C-1′), 133.6 (C-1″), 132.9 (C-9), 130.4 (C-3″), 129.0 (C-2′), 127.9 (C-2″), 127.5 (C-3), 127.0 (C-3′), 124.7 (C-2), 116.8 (q, $J = 294$ Hz, CF_3), 52.1 (CH), 48.1 (CH), 42.2 (CH_2NH), 41.4 (C-6), 30.7 ($\underline{C}H_2CO$), 27.4 ($\underline{C}H_2CH$), 25.4 (C-7), 17.7 (CH_3); MS (EI) 583 (M^+), calcd 583 (M).

2-(2-*tert*-Butoxycarbonylamino-4-{4-[4-(4-chloro-phenyl)-6,7-dihydro-4*H*-thieno[3,2-*c*]pyridine-5-carbonyl]-phenylcarbamoyl}-butyrylamino-propionic acid *tert*-butyl ester (12). The standard procedure yielded 56% of the protected compound **12** as a colorless solid with a broad melting point; $R_f = 0.63$ (EtOAc); ^{1}H NMR ($CDCl_3$) δ 9.11 (1H, broad s, NH), 7.65 (2H, d, $J = 8.4$ Hz, 2×H-3′), 7.36 (2H, d, $J = 8.4$ Hz, 2×H-2′), 7.30 (4H, broad s, 2×H-2″ + 2×H-3″), 7.20 (1H, d, $J = 5.1$ Hz, H-2), 6.95 (1H, broad s, H-4), 6.73 (1H, d, $J = 5.1$ Hz, H-3), 6.56 (1H, d, $J = 6.9$ Hz, NH), 5.51 (1H, s, NH), 4.43 (1H, m, CH in Ala), 4.19 (1H, m, CH in Glu), 3.82 (1H, m, H-6^A), 3.28 (1H, m, H-6^B), 3.00 (1H, m, H-7^A), 2.85 (1H, m, H-7^B), 2.47 (2H, t, $J = 6.3$ Hz, CH_2 in Glu), 2.24–1.96 (2H, m, CH_2 in Glu), 1.46 (9H, s, Boc), 1.44 (9H, s, COO-*t*-Bu), 1.35 (3H, d, $J = 7.2$ Hz, CH_3).

1-(1-Carboxy-ethylcarbamoyl)-3-{4-[4-(4-chlorophenyl)-4,5,6,7-tetrahydro-4*H*-thieno[3,2-*c*]pyridine-5-carbonyl]-phenylcarbamoyl}-propyl-ammonium trifluoroacetate (15). The standard procedure yielded 100% of **15** as a colorless solid with a broad melting point (115–135 °C); ^{1}H NMR (DMSO) δ 10.22 (1H, s, NH), 8.83 (1H, d, $J = 7.5$ Hz, NH), 8.20 (3H, d, $J = 4.2$ Hz, NH_3), 7.65 (2H, d, $J = 8.7$ Hz, 2×H-2′), 7.41 (2H, d, $J = 8.7$ Hz, 2×H-3′), 7.46–7.20 (5H, m, 2×H-2″ + 2×H-3″ + H-2), 6.83 (1H, d, $J = 5.1$ Hz, H-3), 6.73 (1H, broad s, H-4), 4.28 (1H, m, CH in Ala), 3.87 (1H, m, CH in Glu), 3.70 (1H, m, H-6^A), 3.20 (1H, m, H-6^B), 2.99 (1H, m, H-7^A), 2.87 (1H, m, H-7^B), 2.43 (2H, m, CH_2 in Glu), 2.03 (2H, m, CH_2 in Glu), 1.31 (3H, d, $J = 7.2$ Hz, CH_3); ^{13}C NMR (DMSO) δ 174.1 (COOH), 170.7 (CONHR), 170.0 ($CONR_2$), 168.4 (CONHR), 158.7 (q, $J = 40$ Hz, $CF_3\underline{C}O$), 140.9 (C-4″), 140.6 (C-4′), 135.0 (C-8), 133.7 (C-1″), 132.8 (C-1′), 130.9 (C-9), 130.4 (C-3″), 129.0 (C-2′), 128.0 (C-2″), 127.0 (C-3), 124.7 (C-2), 119.2 (C-3′), 116.7 (q, $J = 295$ Hz, CF_3), 53.0 (C-4), 51.9 (CH), 48.2 (CH), 40.7 (C-6), 31.5 ($\underline{C}H_2CO$), 26.9 ($\underline{C}H_2CH$), 25.2 (C-7), 17.7 (CH_3); MS (EI) 569 (M^+), calcd 569 (M).

Transport assay

Materials

Caco-2 human colon carcinoma cells were obtained from the ATCC (Rockville, MD, USA). [^{3}H]Glycylsarcosine ([^{3}H]Gly-Sar, 2.1 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). 2-(*N*-Morpholino)-ethanesulfonic acid (Mes) was purchased from Sigma (St. Louis, MO, USA) and *N*-2-hydroxyethylpiprazine-*N*′-2-ethanesulfonic acid (Hepes) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). All analytical grade solvents used for HPLC analysis were obtained from Merck (Darmstadt, Germany), and Ultima Gold scintillation fluid was purchased from Packard (Groningen, The Netherlands).

Cell culture and pretreatment

Caco-2 cell culture was maintained according to a previously published method;[3,13] cells were used between passages 62 and 70. Transwell® monolayers reached a transepithelial electrical resistance (TEER) of between 600–800 ohms×cm^2, and the total amount of protein on each confluent Transwell® filter was calculated to be 0.42 mg/cm^2 using the Lowry method.

IC_{50} Determination experiments

[^{3}H]Gly-Sar displacement experiments were performed as has been described previously.[3,13] Briefly, Caco-2 monolayers were first rinsed and then incubated with HBSS (apical media = 10 mM Mes, pH 6.0; basal media = 10 mM Hepes, pH. 7.4) for 15 min at 37 °C under a 5% CO_2 atmosphere in order to equilibrate the cells to the change in pH gradient. Next, [^{3}H]Gly-Sar (0.25 μCi), and in certain wells, dipeptide-modified compounds of various concentrations were added concomitantly to the apical media of the Caco-2 Transwells. Following a 15-min incubation period, buffer was removed from both the apical and basal chambers and the cells were washed four times with ice-cold HBSS, pH 7. Following this

washing step, the entire polycarbonate membrane was cut from the Transwell support and placed into a scintillation vial, scintillation fluid was added, and the cell-associated radioactivity was counted via liquid scintillation spectrometry.

Transport of test compounds and Gly-Sar across Caco-2 monolayers

Transport via the apical oligopeptide transporter was performed as has been described previously.[13] Briefly, the apical-to-basal transport of each compound was measured hourly over the course of a 2-h experiment. For all experiments, confluent Caco-2 monolayers were pretreated as described in 'Cell culture and pretreatment', then either the dipeptide-modified prodrug (50 μM) or [^{3}H]Gly-Sar was added to the apical media. The TEER was monitored periodically throughout the course of the experiment. Samples taken from the basal compartment were prepared for HPLC analysis as has been described previously.[14]

The apparent permeability coefficients were calculated using eq (1):

$$P_{app} = V \times dC/A \times Co \times dt \quad (1)$$

where $V \times (dC/dt)$ represents the steady-state rate of transport of the dipeptide from the apical to the basolateral chamber following initial lag time; Co represents the initial concentration of the dipeptide in the apical chamber; and A represents the area of the Transwell membrane used for these experiments (1 cm^2).

The efflux transporter score was calculated using eq (2):

$$P_{app}(BL\ toAP)\ : P_{app}(AP\ toBL), \quad (2)$$

where AP is apical side and BL is basolateral side.

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