Supporting Information

Exploration and Pharmacokinetic Profiling of Phenylalanine Based Carbamates as Novel Substance P 1-7 Analogues

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1 Chemistry

Preparative RP-HPLC was performed on a system equipped with a Zorbax SB-C8 column (150 × 21.2 mm) or a 10 µm Vydac C18 column (250 × 22 mm), in both cases with UV detection at 230 nm. Analytical RP-HPLC-MS was preformed on a Gilson-Finnigan ThermoQuest AQA system (Onyx monolithic C18 column, 50 × 4.6 mm; MeCN/H_2O gradient with 0.05% HCOOH) in ESI mode, using UV (214 and 254 nm) and MS detection. The purity of each of the peptides was determined by RP-HPLC using the columns: ACE 5 C18 (50 × 4.6 mm) and ACE 5 Phenyl (50 x 4.6 mm) or Thermo Hypersil Fluophase RP (50 x 4.6 mm) with a H_2O/MeCN gradient with 0.1% TFA and UV detection at 220 nm. NMR spectra were recorded on a Varian Mercury plus spectrometer (^{1}H at 399.8 MHz and ^{13}C at 100.5 MHz or ^{1}H at 399.9 MHz and ^{13}C at 100.6 MHz) at ambient temperature. Chemical shifts (δ) are reported in ppm referenced indirectly to TMS via the solvent residual signal. Exact molecular masses were determined on a Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source at the Department of Pharmaceutical Biosciences, Uppsala University, Sweden. Amino acid analyses were performed at the Department of Biochemistry and Organic Chemistry, Uppsala University, Sweden. All other chemicals and solvents were of analytical grade from commercial sources.

2 Compound 4

Z-Phe-OSu (450 mg, 1.135 mmol) was weight into a rund bottle flask and flushed with N_2. THF (20 mL) was added and the solution was cooled to -0 ºC. NH_3 (g) was bubbled into the flask which resulted in precipitation of a white solid. After 50 minutes the NH_3 (g) was turned of and the reaction was stirred at room temperature over night. The precipitate was filtered of and dried under vacuum. The filtrate was evaporated to yield a white solid. An aliquote of the crude compound was purified on RP-HPLC to give **13** (15 mg, 54%) as a white solid. HPLC purity: C18-column > 99%, Phenyl > 99%. ^{1}H NMR (CD_3CN) δ 2.83 (dd, *J* = 9.4, 14.0 Hz, 1H), 3.14 (dd, *J* = 4.9, 14.0 Hz, 1H) 4.31 (ddd, *J* = 4.9, 8.4, 9.4 Hz, 1H), 4.97 (d, *J* = 12.7 Hz, 1H), 5.03 (d, *J* = 12.7 Hz, 1H) 5.84 (br s, 1H) 5.89 (d, *J* = 8.4 Hz, 1H) 6.42 (br s, 1H) 7.29 (m, 10H). ^{13}C NMR (CD_3CN) δ 38.7, 57.0, 67.0, 127.6, 128.6, 128.9, 129.3, 129.4, 130.3, 138.2, 138.7, 156.9, 174.3. Anal. Calcd for ($C_{17}H_{18}N_2O_3$): C, 68.44; H, 6.08; N, 9.39. Found: C, 68.35; H, 6.11; N, 9.17.

3 Compound 5

A mixture of Z-Phe-OH (150 mg, 0.5 mmol), cyanamide (79.8 mg, 1.9 mmol) and HATU (237 mg, 0.625 mmol) was weight into a reaction tube, evacuated and then flushed with N_2. DIEA (435 µL, 2.5 mmol) and dry DMF(14 mL) was added and the reaction mixture was stirred at 40 ºC under N_2 atmosphere for three days. The reaction mixture was diluted with EtOAc and washed with NaOAc buffer (pH 4), 10 % aqueous $NaHCO_3$ and brine. The organic layer was evaporated and purified on RP-HPLC to give **14** (15 mg, 15%) as a white solid. HPLC purity: C18 94.4%, Fluophase 93.7%. ^{1}H-NMR (MeOD) δ 3.18 (dd, *J* = 2.4, 14.0, 1H), 3.31 (dd, *J* = 5.7, 14.0, 1H), 4.63 (dd, *J* = 2.4, 5.7, 1H), 5.32 (d, *J* = 11.7, 1H), 5.52 (d, *J* = 11.7, 1H), 6.78-6.84 (m, 2H), 7.07-7.18 (m, 3H), 7.40-7.50 (m, 3H), 7.55-7.60 (m, 2H). ^{13}C-NMR (MeOD) δ 36.2, 64.3, 70.5, 116.9, 128.3, 129.4, 130.0, 130.3, 130.5, 130.6, 135.4, 136.1, 152.4, 185.5. HRMS ($M + H^+$): 324.1351, $C_{18}H_{18}N_3O_3$ requires: 324.1348

4 Compound 6

The equipment and the solid chemicals were dried over night before use. The Z-Phe-OH (150 mg, 0.5 mmol) and CDI (162 mg, 1 mmol) were weight into a round bottom flask, sealed with a septum and flushed with N_2. Dry THF (8 mL) was added and the mixture was heated in an oil bath at 60 ºC for 1.5 h. The methylsulfonamide, dissolved in dry THF (3 mL), and 1.8-diazabicyclo[5.5.0]undec-7ene (DBU) (224 µL, 1.5 mmol) were added to the reaction mixture whereafter it was stirred at room temperature for 3 h. The reaction mixture was evaporated and the crude was dissolved in DCM and washed with 5 % citric acid and brine. The organic layer was evaporated and a aliqoute (24 mg (10%) of the crude) was purified on RP-HPLC to give **15** (9.6 mg, 50%) as a white solid. HPLC purity: C18 > 99%, Fluophase >

99%. $[\alpha]^D$ = +8 (c = 0.1, MeOH), ^{1}H-NMR (MeOD) δ 2.91 (dd, *J* = 8.9, 13.7, 1H), 3.10 (dd, *J* = 6.1, 13.7, 1H), 3.15 (s, 3H), 4.37 (dd, *J* = 6.1, 8.9, 1H), 5.02 (d, *J* = 12.5, 1H), 5.06 (d, *J* = 12.5, 1H), 7.20-7.35 (m, 10H). ^{13}C-NMR (MeOD) δ 38.6, 41.2, 58.0, 67.7, 128.0, 128.7, 129.0, 129.5, 129.6, 130.5, 137.6, 138.1, 158.3, 173.6. HRMS (M + H^+): 377.1173, $C1_8H_{21}N_2O_5S$ requires 377.1171.

5 Compound 7

The Z-Phe-OH (150 mg, 0.5 mmol) was dissolved in dry THF (6 mL) and flushed with N_2. N-methylmorpholine (56 µL, 0.5 mmol) was added and the reaction mixture was cooled to -15 ºC before the addition of isobutylchloroformate (69 µL, 0.54 mmol). Hydroxylamine hydrochloride (42 mg, 0.6 mmol) in dry DMF (3mL) and triethylamine (84 69 µL, 0.6 mmol) were thereafter added and the mixture was stirred for 2 h at -15 ºC. The mixture was diluted with H_2O and extracted with EtOAc. The organic layer was washed with brine, evaporated and a aliqoute (29 mg (20%) of the crude) was purified on RP-HPLC to give **16** (9.5 mg, 30 %) as a white solid. HPLC purity: C18 97.4%, Fluophase 97.1%. ^{1}H-NMR (MeOD) δ 2.88 (dd, *J* = 8.5, 13.6, 1H), 3.07 (dd, *J* = 6.5, 13.6, 1H), 4.26 (dd, *J* = 6.5, 8.5, 1H), 4.99 (d, *J* = 12.5, 1H), 5.04 (d, *J* = 12.5, 1H), 7.14-7.35 (m, 10H). ^{13}C-NMR (MeOD) δ 39.3, 55.7, 67.6, 127.8, 128.7, 128.9, 129.4, 129.5, 130.4, 138.2, 138.3, 158.1, 170.6. HRMS (M + H^+): 315.1348, $C_{17}H_{19}N_2O_4$ requires 315.1345.

6 Compound 8

A mixture of Z-Phe-OH (171 mg, 0.572 mmol), benzhydrazide (117 mg, 0.859 mmol), HATU (261 mg, 0.686 mmol), DCM (6 mL) and DIEA (399 µL, 2.29 mmol) was stirred at room temperature over night. The mixture was diluted with EtOAc and washed with NaOAc buffer (pH 4), 5% aq. $NaHCO_3$ and brine. The organic layer was evaporated and a aliquote (20 mg (6%) of the crude) was purified on RP-HPLC to give **17** (14 mg, 99%) as a white solid. HPLC purity: C18 > 99%, Fluophase > 99%. ^{1}H-NMR (MeOD) δ 2.94 (dd, *J* = 9.8, 14.0, 1H), 3.31 (dd, *J* = 4.7, 14.0, 1H), 4.55 (dd, *J* = 4.7, 9.8, 1H), 4.97 (d, *J* = 12.5, 1H), 5.04 (d, *J* = 12.5, 1H), 7.18-7.33 (m, 10H), 7.45-7.51 (m, 2H), 7.54-7.60 (m, 1H), 7.86-7.90 (m, 2H). ^{13}C-NMR (MeOD) δ 39.2, 56.5, 67.5, 127.7, 128.61, 128.64, 128.9 129.39, 129.40, 129.6, 130.4, 133.3, 133.6, 138.1, 138.4, 158.2, 169.0, 173.3. HRMS (M + H^+): 418.1771, $C_{24}H_{24}N_3O_4$ requires 418.1767.

7 Compound 9

Compound **17** was used without any further purification. All the equipment was dried before use. A mixture of **17** (83 mg, 0.247 mmol), (methoxycarbonylsulfamoyl)-triethylammonium hydroxide inner salt (Buregess reagent) (145 mg, 0.608 mmol) and dry THF (4 mL) was stirred at 70 ºC under N_2 atmosphere in a sealed reaction tube for 3 h. The reaction mixture was evaporated and a aliquote (100 mg (47%) of the crude) was purified on RP-HPLC to give **18** (30 mg, 65%) as a white solid. HPLC purity: C18 89.6 %, Fluophase 89.4%. ^{1}H-NMR (MeOD) δ 3.25 (dd, *J* = 9.2, 13.8, 1H) 3.39 (dd, *J* = 6.4, 13.8, 1H), 5.05-5.06 (m, 2H), 5.28 (dd, *J* = 6.4, 9.2, 1H), 7.17-7.34 (m, 10H), 7.51-7.63 (m, 3H), 7.93-7.98 (m, 2H). ^{13}C-NMR (MeOD) δ 39.6, 50.6, 67.7, 124.6, 127.9, 128.1, 128.7, 129.0, 129.5, 129.6, 130.37, 130.38, 133.3, 137.7, 138.1, 158.1, 166.5, 168.1. HRMS (M + H^+): 400.1660, $C_{24}H_{22}N_3O_3$ requires 400.1661.

8 [2,4-DehydroPro]SP_{1-7}

The precursor peptide for tritium-labeling [2,4-DehydroPro]SP_{1-7} was prepared by standard solid-phase peptide synthesis techniques using Fmoc/*t*-butyl protection and purified as described above. Tritium labeling of the precursor was performed by Amersham Biosciences (Cardiff, UK) and resulted in 370 MBq (10 mCi) of [^{3}H]-SP_{1-7} with a specific activity of 3.11 TBq/mmol (84 Ci/mmol).

9 Animal experiment and membrane preparation

The preparations of receptor membranes were conducted using spinal cords from male Sprague–Dawley rats. The rats (Alab AB, Sollentuna, Sweden), weighing 200–250 g, were housed in groups of four in air-conditioned rooms (22–23 °C and a humidity of 50–60%) under an artificial light–dark cycle and had

free access to food and water. Prior to tissue sampling the rats were allowed to adapt to the laboratory environment for 1 week. The animals (n=15) were killed by decapitation and the spinal cords were rapidly removed and quickly frozen. Tissues were then kept at −80 °C until analyzed. The animal experiment was approved by the local ethical committee in Uppsala, Sweden.

The frozen spinal cords weighing approximately 250 - 300 mg/animal, were thawed and placed on ice before being homogenized for 30 s in 30 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4), containing 5 mM KCl and 120 mM NaCl, using a Polytron homogenizer. The homogenate was then centrifuged at 40,000 × g for 20 min at 4 °C and the supernatant was discarded. The resulting pellet was re-suspended in 30 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4), containing 300 mM KCl and 10 mM EDTA. Following incubation on ice for 30 min the sample was centrifuged at 40,000 × g for 20 min at 4 °C. The pellet obtained was diluted and homogenized in 30 volumes of ice-cold 50 mM Tris–HCl, containing 0.02% BSA, 5 mM, EDTA, 3 mM $MnCl_2$ and 40 μg bacitracin and re-centrifuged twice as described above. The final pellet was re-suspended and homogenized in 5 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4) and immediately frozen at −80 °C until used in binding studies. The protein concentration of the membrane suspension was determined according to the method of Lowry[1] using bovine serum albumin as protein standard.

10 Radioligand Binding assay (Compound 2–9)

Assessment of the binding affinity for the various compounds analyzed in this study was carried out using the analogue [^{3}H]-$SP_{1–7}$ as tracer. Assays were performed in tubes containing 50 μl of spinal cord membrane suspension (200 μg protein) and 0.9 nM of [^{3}H]-$SP_{1–7}$ (specific activity: 3.11 TBq/mmol (84 Ci/mmol)) in a final volume of 0.5 ml 50 mM Tris binding buffer (pH 7.4), containing, 3 mM $MnCl_2$, 0.2% BSA and peptidase inhibitors (40 μg/ml bacitracin, 4 μg/ml leupeptin, 2 μg/ml aprotinin and 4 μg/ml phosphoramidon). The amount of total and unspecific binding in percent of the total radioactivity added were approximately 7% and 1.7%, respectively. The competition experiments were determined at six different concentrations varying between 0.01 nM and 1 μM of unlabeled compounds. Non-specific binding was determined in the presence of 1 μM $SP_{1–7}$. Samples were incubated for 60 min at 4 °C before being terminated by rapid filtration under vacuum with a Brandel 24-sample cell harvester through Whatman GF/C glass fiber filters treated with a solution containing 50 mM Tris (pH 7.4), 0.3% polyethylenimine (PEI) and 0.5% Triton X-100 at 4 °C overnight. Filters were washed twice with 3 ml of cold 50 mM Tris–HCl (pH 7.4) complemented with 0.1 mg/ml BSA and 3 mM $MnCl_2$. The filters were air dried for about 60 min before the bound radioactivity was determined using a liquid scintillation counter (Beckman LS 6000IC) at 63% efficiency in 5 ml of counting scintillant. The specific binding was determined as the difference between total and unspecific binding. All assays were run in triplicates. Data and statistics from the competition experiments were analyzed in the GraFit program (Erithacus Software, UK).

11 Pharmacokinetic Profiling (Compound 2–4)

These studies were performed at AstraZeneca, Södertälje according to literature.[2-6] All animal studies were approved by the Stockholm Södra Animal Research Ethical Board. All standard chemicals used for in vitro studies were purchased from reputable companies and of standard purity. CYP substrates used in the inhibition experiments were from Sigma-Aldrich. A brief description of the permeability, CYP inhibition and the solubility studies performed are presented below:

<u>Permeability experiments</u>

Caco-2 cells (ATCC, American Type Culture Collection, ATCC No. HTB-37) were maintained with DMEM and passed weekly at a confluence at 80-90%. After trypsinization, the cells were seeded on polycarbonate filter inserts in the apical well to obtain the desired cell concentration of 227 000 cells/cm^2 and maintained at culture conditions (37°C with 5% CO_2 and 95% humidity). The medium was changed every second day. Cells with passage number between 25-50 and grown for 14-25 days on

filter inserts were used for permeability experiments. The permeability experiments were carried out in transportbuffer (Hank's buffered salt solution supplemented with 25 mM HEPES, pH 7.4). Compounds dissolved in DMSO were diluted to 10 µM in transportbuffer to give a final DMSO concentration of less than 1%. Each compound was assayed in duplicates for both directions. The experiments were performed using a Tecan Genesis RSP 200 robot. In short: The filter-grown cell monolayers were equilibrated in transportbuffer and 37 °C for 10 minutes in a shaking incubator. The basolateral wells contain 0.80 mL and the apical wells contain 0.20 ml. Transportbuffer was then removed from both sides. Test compounds were added to the donor side and fresh buffer to the receiver side before returning to the incubator. The assay was run for 60 min for the AB direction and 30 min for the BA direction. Aliquots of samples were taken from the donor side in the beginning and at the end of experiment. Receiver samples were taken from the basolateral side at the end of experiment. The integrity of the epithelial cell monolayer was monitored by measuring the passive transmembrane diffusion of radiolabeled [^{14}C]mannitol. Concentrations of compounds in donor and receiver samples were analyzed by LC/MS/MS[4]. Liquid scintillation was used for analysis of [^{14}C]mannitol. The apparent permeability coefficient (P_{app}) and mass balances were calculations as described.[6]

CYP Inhibition

The CYP inhibition incubations consisted of 0.15 mg/mL human microsomal protein (mix of 7 individuals (Caucasians; five men and two women)), 20 or 2 µM test compound, cocktail solution containing the final concentrations of 8 µM Phenacetin (CYP1A2), 4 µM Diclofenac (CYP2C9), 4 µM Bufuralol (CYP2D6) and 10 µM Midazolam (CYP3A4) in 50 mM potassium phosphate buffer pH 7.4. Following 10 minutes preincubation at 37°C, the reaction was initiated by addition of 1 mM NADPH. The final incubation volume was 100 µL. The samples were incubated for 8 minutes at 37°C. The reaction was stopped by the addition of 100 µL ice-cold acetonitrile and 20 µL of internal standard (400 nM warfarin) in acetonitrile was added. The samples were centrifuged and the supernatants were transferred to glass vial 96-plate and analysis of parent molecules as well as specific substrate metabolites were conducted by LC/MS/MS.[4] The specific substrate metabolites were Acetaminophen (CYP1A2), 4-OH-diclofenac (CYP2C9), 1-OH-bufuralol (CYP2D6) and 1-OH-midazolam (CYP3A4). As a background control, the baseline formation rate of metabolites in the presence of 0.2% DMSO was used. In addition, the positive controls Furafylline (CYP1A2), Sulfaphenazole (CYP2C9), Quinidine (CYP2D6) and Ketoconazole (CYP3A4) were included in the assay.

Solubility experiment

Aqueous solubility was determined with screening method producing crystalline-like solubility values starting from 10 mM solutions in a 96-plate formate, called Dried DMSO Solubility test.[2]
The Dried DMSO Solubility test determines the amount of test compound dissolved in buffer solution by HPLC-UV/MS or UPLC-UV/MS after incubation of known amount of test compounds concentrated from DMSO solution in 0.1 M sodium phosphate buffer, pH 7.4 for 24 hours at a fixed temperature of 22 °C with mixing on a shaking bed (500 rpm) with StirStix and following separation of a sample solution from not dissolved particles. In the present protocol version, a pre-incubation for at least 4 hours at a fixed temperature of 1°C with StirStix at 500-rpm prior addition of buffer was implemented to generate the most stable solid form. The sample is quantified against the standard of the test compound dissolved in DMSO. The upper limit for the measurement is 400 µM. To ensure that the estimated solubility data are valid, sample identity and purity are determined also by examining mass spectral data in positive and negative ionization mode respectively chromatographic data, monitored at 220 and 254 nm.

Plasma Protein and Brain Tissue Binding

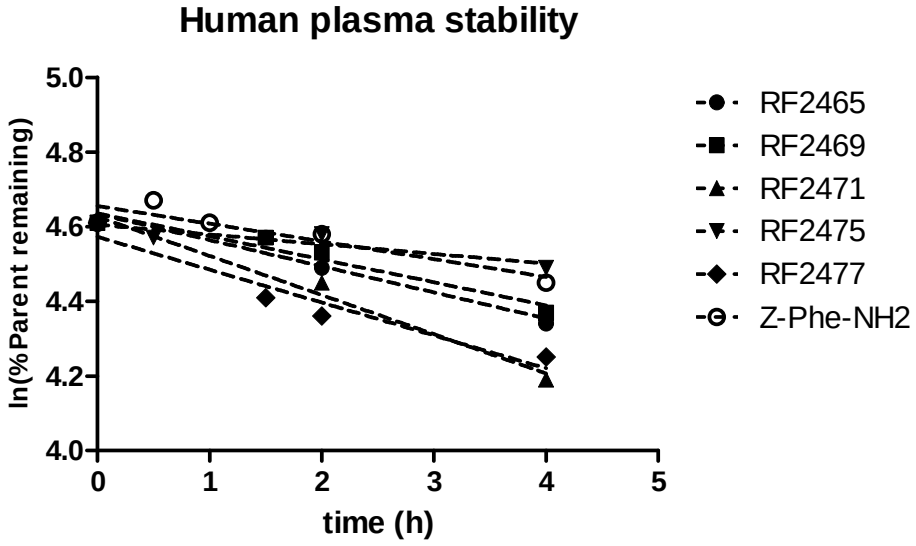
The plasma protein binding was measured using the equilibrium dialysis method reported by Sohlenius-Sternbeck, A.K et al. 2010.[3] The brain tissue binding was measured using a brain slice method described by Fridén, M. et al. 2009.[5]

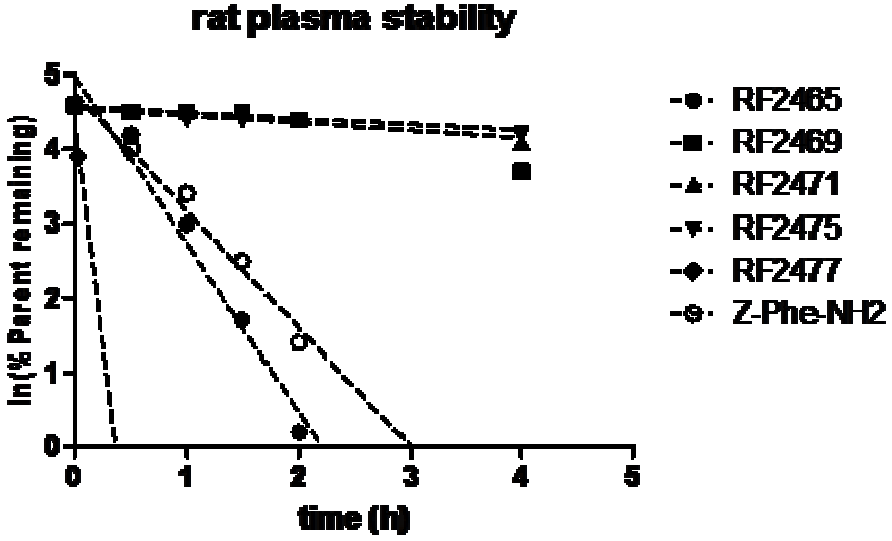
12 Metabolic Stability (Compound 5–9)

Compounds (1 µM) were pre-incubated for 15 min at 37°C with pooled human liver microsomes (0.5 mg/mL; Xenotech, Kansas, KS) in 0.1M potassium phosphate buffer pH 7.4 prior to the addition of NADPH (1 mM) to initiate the reaction. Reactions were then incubated for 0, 5, 20 and 40 min and at each time point the reaction was stopped by the addition of acetonitrile. Plates were centrifuged at 3,500 rpm for 20 min at 4°C, and the supernatants were subjected to liquid chromatography/mass spectrometry analysis. The natural logarithm of the analytical peak area ratio (relative to 0 min sample which was considered as 100%) was plotted against time and analyzed by linear regression. In vitro half-life time (t½, min) and in vitro intrinsic clearance (Cl_{int}, µl/min/mg) were calculated on the basis of first-order reaction kinetics of the percentage of remaining compound. Dextromethorphan (3 µM) and midazolam (5 µM) were used as positive controls for cytochrome P450 enzymes (CYP) isoforms CYP2D6 and CYP3A4, respectively.

13 Plasma stability (compound 4–9)

Plasma stability of RF2465 (**8**), RF2469 (**9**), RF2471 (**5**), RF2475 (**6**), RF2477 (**7**) and Z-Phe-NH_2 (**4**) was performed using pooled human plasma (two male + two female, nonsmoker) from Uppsala Academic Hospital and Wistar rat plasma from Innovative Research (MI, USA). Compound (from 1 mM DMSO stock) was spiked in 0.7 ml plasma in HPLC vials to a final concentration of 1 µM and incubated (37˚C) for 4 h. Immediately after compound introduction and rapid mixing an aliquot (50 µl) was taken and precipitated in 150 µl ice-cold methanol containing Warfarin (IS) on a 96-well plate. This sample was considered as the 0 min control value. Additionally a QC sample was prepared which introduced compound in pre-precipitated plasma and this sample was used as the 0 min control for RF2477 in rat plasma. Sample aliquots were taken at 0, 0.5, 1, 1.5, 2 and 4 h of incubation. After the incubation the plate was centrifuged and frozen until LC-MS/MS analysis. The results are presented relative to the 0 min control (see graphs below).





14 Cell Culture

Used in the transport and uptake studies of compound **5**–**9**

Caco-2 cells (obtained from American Tissue Collection, Rockville, MD) were maintained in an atmosphere of 90% air and 10% CO_2 as described previously.[5] For transport experiments, 3.0×10^5 cells (passages 98 to 102) were seeded on polycarbonate filter inserts (12 mm diameter; pore size 0.4 µm; Costar, Cambridge, MA) and allowed to grow and differentiate for 21–24 days. The monolayers integrity was assessed by measuring the paracellular marker [^{14}C]-Mannitol (1.0 µCi/mL 57.3 mCi/mmol; Perkin-Elmer Life Sciences, Boston, MA) transport and the transepithelial electrical resistance (TEER) before and after the experiments.

CHO-K1 and CHO-PepT1 cells (control and stably transfected cells, respectively, were kind gifts from Dr. Anna-Lena Ungell, AstraZeneca Mölndal) and were maintained at 37°C in an atmosphere of 90% air and 10% CO_2, with DMEM containing 4.5 g/L glucose, 10 % fetal bovine serum, 1% non essential amino acids and 50 µg/mL of gentamicin sulphate (Invitrogen, Carlsbad, CA). For the uptake experiments, 1.0×10^5 cells/well were seeded in 24-well plates and allowed to grow in antibiotic-free media for 2 days.

15 Transcellular Transport and Uptake Experiments (Compound 5–9)

Stock solutions (10 mM) of the peptides were prepared in dimethylsulfoxide (DMSO) and diluted to 100 µM (final DMSO concentration of 1%) in Hank's balanced salt solution (HBSS) containing 10 mM MES at pH 6.0 (HBSS pH 6.0) or containing 10 mM HEPES at pH 7.4 (HBSS pH 7.4). Compound **5** and **7**-**9** were further diluted to 25 µM due to poor aqueous solubility. In all experiments, [^{14}C]-Glycyl-sarcosine ([^{14}C]-GlySar; 1.82 µM, 0.1 µCi/mL; 55 mCi/mmol; ARC, St. Louis, MO) was used as a PepT1 substrate control. For inhibition controls, an excess of unlabeled competitor (10mM; Sigma-Aldrich, St. Louis, MO) was used.

The intestinal epithelial permeability was determined from transport rates across Caco-2 cell monolayers. The cell monolayers were gently rinsed with HBSS pH 6.0 and left to equilibrate in the same solution for 30min at 37°C. The transport experiments were run for 2 h at 37°C, and were started by the application of the compound solution to the donor side, which was the apical chamber in the apical to basolateral (a–b) experiments, and the basolateral chamber in the basolateral to apical (b–a) experiments. Filter inserts were continuously stirred at 500 rpm on IKA-Schüttler MTS4 to obtain data that were unbiased by the aqueous boundary layer. The receiver chambers were sampled in suitable time points and the samples were replaced with equal volumes of pre-heated receiver solution.

For transport studies performed under sink conditions, where less than 10% of the compound was transported across the Caco-2 cell monolayers, the apparent permeability coefficients (P_{app}) were calculated from the equation

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{AC_0}$$

where $\Delta Q/\Delta t$ is the steady-state flux (mol/s), C_0 is the initial concentration in the donor chamber at each time interval (mol/mL), and A is the surface area of the filter (cm^2). If the fraction transported exceeded 10% the P_{app} coefficients were calculated applying non-sink conditions from the equation:

$$C_R(t) = \frac{M}{V_D + V_R} + \left(C_{R,0} - \frac{M}{V_D + V_R} \right) e^{-P_{app}A(1/V_D+V_R)t}$$

where $C_R(t)$ is the time-dependent drug concentration in the receiver compartment (µM), M is the amount of drug in the system (nmol), V_D and V_R are the volumes of the donor and receiver compartment (mL), respectively, and t is the time that has elapsed from the start of the interval (s).[7] P_{app} was obtained from nonlinear regression of the accumulated dose in the receiver compartment over time, minimizing the sum of squared residuals in the equation.

Uptake studies with CHO cells were performed at 37°C in HBSS pH 6.0. Initially, cells were gently rinsed and left to equilibrate for 30min at 37°C. After the equilibration period, buffer was removed and replaced by compound diluted in HBSS pH 6.0 and cells were incubated for 15 min at 37°C. Uptake was terminated by buffer removal followed by two washes with ice-cold phosphate buffer saline (PBS). Cells were then lysed with acetonitrile/water (60:40, v/v) and centrifuged at 3,500 rpm and 4°C for 20min. In the case of experiments with [^{14}C]-GlySar, sodium hydroxide 1M solution was used for cell lysis. The results were expressed as pmol/mg of protein/min.

16 Data Analysis (Compound 5–9)

All experiments were performed in, at least, triplicates, and samples were subjected to liquid chromatography/mass spectrometry analysis with a ThermoFinnigan TSQ Quantum Discovery triple-quadrupole (electrospray ionization, ESI; Thermo Electron Corp. Waltham, USA) coupled to an Acquity Ultra High Performance LC system (Waters, Milford, MA). For chromatographic separation, an Acquity UPLC C18 column (1.7 µm; Waters, Milford, MA) and a flow rate of 600 µL/min were used. During the analysis 10 µL of the samples were injected with a gradient with 0.1% formic acid in acetonitrile and 0.1% formic acid in water. Electrospray ionization was used in positive mode, and the daughter ion of m/z 91.1 was used for quantification of the respective compounds. The internal standard warfarin (20 nM) was used throughout the analysis.

Radioactive samples ([^{14}C]-GlySar and [^{14}C]-Mannitol) were analyzed with a liquid scintillation counter (TopCount NXT, Perkin-Elmer Life Sciences, Boston, MA). Cellular protein content was determined using a protein assay kit with bovine serum albumin (BSA) as standard (Thermo Scientific, Rockford, IL).

17 References

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