

Supplementary Material

The Effects of Lipidation on a TAT-Containing Peptide-Based Inhibitor of PSD-95

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Experimental Procedures

Chemistry general

Amino acids, preloaded 2-chlorotrityl-Val resin, *N,N*-diisopropylethylamine (DIPEA), *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU), 1H-1, 2, 3-benzotriazol-1-yloxy-tris(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP) and linkers Fmoc-Glu-OtBu, Fmoc-GABA-OH were purchased from Iris Biotech (Marktredwitz, Germany). The PEG building blocks were obtained from Biomatrik Inc (Jiaying, China). The remaining reagents were obtained from Sigma-Aldrich (Schnelldorf, Germany). Semi-Preparative C18 reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on an Waters system equipped with a quaternary pump model Waters 2545 and a UV/vis detector model Waters 2489 using a C18 reverse phase column (Zorbax-C18, 300SB, 250 mm × 21.2mm × 7µm) with a linear binary gradient of H₂O/MeCN/trifluoroacetic acid (TFA) (A, 95/5/0.1, and B, 5/95/0.1) using a flow rate of 20 mL/min. Flash chromatography was performed on a Teledine Isco Combiflash RF equipped with a GraceResolv silica gel column using linear binary gradient elution of heptane and ethyl acetate (EtOAc). Analytical UPLC was performed on an Waters Acquity system with a C8 or a C18 BEH reverse phase column (BEH C8/18 column, 2.1 mm × 50 mm × 1.7µm), flow rate of 0.45 mL/min, and a linear binary gradient of H₂O/MeCN/TFA (A, 95/5/0.1, and B, 5/95/0.1). Mass spectra were obtained with an Agilent 6410 Triple Quadrupole Mass Spectrometer instrument using electron spray ionization (ESI) coupled to an Agilent 1200 HPLC system (ESI-LC/MS) with a C18 reverse phase column (Zorbax Eclipse XBD-C18, 4.6 mm × 50 mm), autosampler and diode array detector using a linear binary gradient of H₂O/MeCN/formic acid (A, 95/5/0.1, and B, 5/95/0.086) with a flow rate of 0.75 mL/min. During ESI-LC/MS analysis, evaporative light scattering (ELS) traces were obtained with a Sedere Sedex 85 Light Scattering Detector.

Synthesis of lipidated peptides

Peptides **1** and **2** were synthesized as previously described.^[1] Briefly, the peptides IETDV or IETAV were prepared on solid-phase using Fmoc chemistry on a preloaded 2-chlorotrityl-Val resin and dimerized using 0.1 eq. of the *N*PEG(4) diacid [10-((2-nitrophenyl)sulfonyl)-4,7,13,16-tetraoxa-10-azanonadecane-1,19-dioic acid] preactivated with (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP)(0.2 eq.) and *N,N*-diisopropylethylamine (DIPEA)(0.4 eq.), added to the resin in dimethylformamide (DMF) followed by 30 min of shaking and a DMF flow-wash. Coupling was repeated 7 times to obtain approximately 90 % dimerization efficiency as judged by LC-MS analysis. The nosyl group was removed by two successive treatments with 2-mercaptoethanol (2-ME, 4eq.), 1,8-diazabicycloundec-7-ene (DBU, 4eq.) and a DMF flow wash. The linkers (Fmoc-Glu-OtBu or Fmoc-GABA-OH, 4eq.) were coupled to the nitrogen of the *N*PEG(4) moiety using PyBOP (4 eq, 0.5M) and DIPEA (4 eq) in DMF for 40 min and repeated six times. The Fmoc group was removed and stearic acid (4 eq.) coupled to the free amine using PyBOP (4 eq, 0.5M) and DIPEA (4 eq, 0.5M) in DCM/DMF 1:1 and repeated 4 times. After cleavage and deprotection with TFA/ triisopropylsilane (TIPS)/H₂O (90/5/5), TFA was evaporated and peptides were extracted with heptane/water (20mL 1:1), the aqueous phase was freeze-dried and the crude purified by HPLC to >95% purity. **1**: linear gradient of 35-100% of acetonitrile in water + 0.1% TFA in 30 minutes, 20 mL/min, rt 24 min. **2**: linear gradient of 35-100% of acetonitrile in water + 0.1% TFA in 30 minutes, 20 mL/min, rt 25 min.

Peptides **3**. First, the dimeric peptide UCCB01-144 was prepared on solid-phase as previously described.^[2] Then, the stearic acid was coupled twice (4eq, 45 min) to the *N*-terminus of UCCB01-144 using HATU (4 eq, 0.5 M) and collidine (4 eq) in DCM/DMF 1:1. After cleavage and deprotection with TFA/TIPS/H₂O (90/5/5) for 4 h, TFA was evaporated and the peptide was extracted with DCM/water (20mL, 1:1), the aqueous phase was freeze-dried and purified by HPLC to >95% purity. **3**: linear gradient of 35-50% of acetonitrile in water + 0.1% TFA in 30 minutes, 20 mL/min, rt 20 min.

Peptide **4**. First the dimeric peptide UCCB01-144 was prepared on solid-phase as previously described.^[2] Then, the linker Fmoc-GABA-OH was coupled to the *N*-terminus of UCCB01-144 using HATU (4 eq, 0.5M) and collidine (4 eq) in DMF for 45 min, Fmoc deprotected, and coupled to stearic acid twice (4eq, 45 min), using HATU (4 eq, 0.5 M) and collidine (4 eq) in DCM/DMF 1:1. After cleavage and deprotection with TFA/TIPS/H₂O (90/5/5) for 4 h, TFA was evaporated and the peptide was extracted with DCM/water (20mL, 1:1), the aqueous phase was freeze-dried and purified by HPLC to >95%. **4**: linear gradient of 35-50% of acetonitrile in water + 0.1% TFA in 30 minutes, 20 mL/min, rt 22 min.

Peptide **5**. The 11-mer sequence YGRKKRRQRRR was prepared by an automated peptide synthesizer (Liberty Blue, CEM) on solid-phase using Fmoc chemistry on a rink amide resin. Amino acids were double coupled using *N,N'*-diisopropylcarbodiimide (DIC) in DMF (0.5 M) and ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) in DMF (1.0 M), and after the sequence was complete, the tyrosine *N*-terminal Fmoc group was removed and double coupled manually with stearic acid using HATU (4 eq, 0.5 M) and collidine (4 eq) in DCM/DMF 1:1. After cleavage and deprotection with TFA/TIPS/H₂O (90/5/5) for 4 h, TFA was evaporated and the peptide was extracted with DCM/water (20mL, 1:1), the aqueous phase was freeze-dried and purified by HPLC to >95%. **5**: linear gradient of 10-60% of acetonitrile in water + 0.1% TFA in 50 minutes, 20 mL/min, rt 42 min.

Fluorescence polarization assay

Saturation binding experiment was performed using a probe described previously.^[3,4] A fixed concentration of Cy5-Cys-N-PEG probe (0.5 nM, prepared as previously) was incubated with an increasing concentration of PSD-95 PDZ12 (0.01-30 nM final concentration) in PBS buffer (pH 7.4) in a black flat bottom 384-well plate (Corning Life Sciences, NY) at 25°C.^[5] The fluorescence was measured in Safirell (Tecan) equipment using an excitation/emission wavelengths of 649/670 nm. Fluorescence polarization values were plotted versus PSD-95 PDZ12 concentration and fitted to the equation $Y = (B_{max} \times X)/(K_d + X)$ using GraphPad Prism. v. 7.0. B_{max} is the maximum polarization value, X is PSD-95 PDZ12 concentration and K_d is the dissociation constant. The affinity of non-labeled peptides to PSD-95 PDZ12 was evaluated by a heterologous competition binding assay in which tested peptides were added in increasing concentrations (0.4-960 nM, final concentration) to a fixed amount of PSD-95 PDZ12 (4 nM) and probe (0.5 nM) using the same conditions as for the saturation binding experiment. Fluorescence polarization values were plotted against the logarithm of non-labeled peptide concentrations and fitted to a four-parameter logistic equation $Y = Bottom + (Top - Bottom)/(1 + 10^{-(LogIC50 - X) \times HillSlope})$ where Bottom is the lower limit, Top is upper limit, LogIC50 is the logarithm of the IC50, HillSlope is the relative slope around LogIC50 and IC50, IC50 is the non-labeled peptide concentration that produces a fluorescence signal half-way between the upper and lower limit. IC50 values were converted to K_i values as described previously.^[6]

In vitro plasma and microsomal stability

Peptides were dissolved in 2% DMSO and incubated at 37°C with human plasma (3H Biomedical, Uppsala, Sweden, 400 µL) to a final concentration of 50 µM. Aliquots (45 µL) were sampled at 0, 1, 2, 4, 6 and 24 h and extracted with 25 mg guanidinium chloride and 90 µL of 10% trichloroacetic acid in acetone (w/v) for 24 h at 5°C. Samples were then centrifuged at 14000g at 5°C and the acetone phase was analyzed by UPLC. After normalization to T0, data were fitted to a one-exponential decay equation $Y = (Y_0 - P) \times \exp(-K \times X) + P$ where K is the rate constant, and P is Y value at infinite time.

For microsomal peptides (final concentration 25 µM in 1% DMSO) were incubated with a mixture of mouse liver microsomes (Life Technologies, Denmark, strain C57BL/6J, final conc. 0.5 mg/mL, diluted with PBS 100mM pH 7.4) and MgCl₂ (final conc. 3 mM) for 5 min at 37°C in a thermomixer (500rpm). The reaction was initiated by adding NADPH solution (final conc. 1 mM, prepared immediately before incubation), 100 µL aliquots were taken at 0, 5, 15, 45 and 60 min and extracted with 150 µL of a mixture of 6 M urea in water : 20% TCA in acetone 1:2. Samples were centrifuged at 14000g at 5°C and the supernatant was analyzed by UPLC. Intrinsic clearance (CL_{int}) values were obtained using the in vitro $t_{1/2}$ method.^[7] The slope of the linear regression of \log_e of integrated peak areas versus time for each replicate results in the time constant $-k$ and the in vitro half-life equals $t_{1/2} = -0.693/k$. CL_{int} is then calculated using the following equation $CL_{int} = 0.693/t_{1/2} \times V/M$, where V is the volume of incubation (700µL) and M the total protein content in the incubation volume (0.35 mg)

In vivo plasma and brain pharmacokinetics

The in vivo pharmacokinetic study was done by Wuxi APS in an AAALAC-accredited animal facility. All compounds administered were diluted in DMSO/PEG400/H₂O 6:2:2 (v/v/v) in doses of 5 mg/kg for i.v administrations or 10 mg/kg for s.c administrations in fasted male Wistar rats. Blood samples were taken periodically, transferred to plastic microtubes containing EDTA and centrifuged to obtain plasma. Each animal was subject to just one blood draw and each time-point was measured in triplicate. The analytes were quantified with calibration curves using control biological matrixes (plasma or brain homogenate) spiked with eight concentrations, processed similarly and analyzed using LC-MS/MS (AB Sciex, API 4000) instrument with an ACE 3 AQ (2.1×100 mm, 3 µm, 50°C) column and detected in positive or negative ionization modes using SRM tandem mass spectrometry. Statistical differences among groups were calculated with Graph-Prism v. 7.0 using one-way analysis of variance with Bonferroni's post-hoc test and reported when significant (adjusted $p < 0.05$)

Sample preparation for plasma and brain pharmacokinetic analysis

Plasma samples from peptides **1** and **2** were prepared according to Method A, **3** according to Method B and **4** according to Method C. For brain pharmacokinetics, brain tissues were dissected and homogenized with deionized water (1:4 w/v). Brain samples from peptides **1** and **2** were prepared according to Method D, **3** according to Method E and **4** according to Method F.

Method A: An aliquot of 60 µL of plasma was mixed with 60 µL 8% phosphoric acid in water, 60 µL 1M ammonium formate buffer (pH=2.14) and extracted with 480 µL of an ethyl acetate/methyl tert-butyl ether solution, containing the internal standard (tolbutamide 200 ng/mL). The mixture was vortexed and centrifuged at 13000 rpm for 15 min at room temperature. 400 µL of the supernatant was transferred to a 96-well plate and evaporated to dryness under nitrogen flow at room temperature. The sample was then reconstituted with 120 µL water/ACN (v:v, 30:70), mixed and 10 µL was injected in a LC-MS/MS in negative ion mode (**1**: m/z 878.9/355.3, **2**: m/z 900.9/184.1;). The following linear gradient was used. A= 0.3% formic acid & 10 mM NH₄OAc in water/ACN (v:v, 95:5), B = 0.3% formic acid & 10 mM NH₄OAc in ACN/water (v:v, 95:5). 0'-10% B, 1.8'- 85%B, 2.2'-95%B, 2.7'-95%B, 2.71'-10%B, 3.5'-10%B. Flow rate= 0.45ml/min. 2.45(1), rt= 2.46 (2), 1.91 (IS)

Method B: An aliquot of 20 μ L of plasma was mixed with 20 μ L of 1N HCl in water, and extracted with 80 μ L acetonitrile with 0.5% of formic acid containing the internal standard (Diclofenac 200 ng/mL). The mixture was vortexed, centrifuged at 13000 rpm for 15 min at room temperature and 10 μ L of the supernatant was injected in a LC-MS/MS in positive ion mode (m/z 816.3/787.0). The following linear gradient was used. A= 1% formic acid in water, B = 1% formic acid in ACN. 0'-35% B, 2'-55%B, 2.5'-90%B, 3'-90%B, 3.01'-35%B, 4'-35%B. Flow rate= 0.45ml/min. rt= 1.12 (3), 2.85 (IS)

Method C: An aliquot of 20 μ L of plasma was mixed with 20 μ L of 8% phosphoric acid in water, and extracted with 80 μ L acetonitrile with 0.5% of formic acid containing the internal standard (Diclofenac 200 ng/mL). The mixture was vortexed, centrifuged at 13000 rpm for 15 min at room temperature and 8 μ L of the supernatant was injected in a LC-MS/MS in positive ion mode (m/z 837.3/807.9). The following linear gradient was used. A= 1% formic acid in water, B = 1% formic acid in ACN. 0'-35% B, 2'-60%B, 2.5'-95%B, 2.51'-35%B, 3.0'-35%B. Flow rate= 0.45ml/min. rt= 1.51 (4), 2.75 (IS)

Method D: An aliquot of 60 μ L of homogenate was mixed with 60 μ L, 60 μ L 1M ammonium formate buffer (pH=2.98) and extracted with 480 μ L of a methanol solution, containing the internal standard (tolbutamide 100 ng/mL). The mixture was vortexed and centrifuged at 13000 rpm for 20 min at 4°C. 5 μ L of the supernatant was injected in a LC-MS/MS using the same conditions as in plasma analysis.

Method E: An aliquot of 60 μ L of plasma was mixed with 60 μ L of 1N HCl in water and extracted with 240 μ L acetonitrile with 0.5% of formic acid containing the internal standard (Diclofenac 200 ng/mL). The mixture was vortexed, centrifuged at 13000 rpm for 15 min at room temperature and 10 μ L of the supernatant was injected in a LC-MS/MS in positive ion mode (m/z 816.3/787.0). The following linear gradient was used. A= 1% formic acid in water, B = 1% formic acid in ACN. 0'-35% B, 2'-65%B, 2.5'-90%B, 2.8'-90%B, 2.81'-35%B, 3.8'-35%B. Flow rate= 0.45ml/min. rt= 1.31 (3), 2.55 (IS)

Method F: An aliquot of 100 μ L of plasma was mixed with 100 μ L of 1N HCl in water and extracted with 400 μ L acetonitrile with 0.5% of formic acid containing the internal standard (Tolbutamine 200 ng/mL). The mixture was vortexed, centrifuged at 13000 rpm for 15 min at room temperature and 10 μ L of the supernatant was injected in a LC-MS/MS in positive ion mode (m/z 837.3/807.9). The following linear gradient was used. A= 1% formic acid in water, B = 1% formic acid in ACN. 0'-35% B, 2'-60%B, 2.5'-95%B, 2.51'-35%B, 3.0'-35%B. Flow rate= 0.45ml/min. rt= 1.51 (4), 1.93 (IS)

In vitro hemolytic assay

Human red blood cells were collected by finger puncture and ~ 2-4 drops of blood were washed 5 times with PBS buffer (100mM, pH 7.4). Cells were collected by centrifugation (1000 rpm, 1 min) and suspended to a final concentration of 0.25% in PBS. Peptides were prepared in PBS buffer (100mM, pH 8) in a stock concentration of 300 μ M, diluted serially and 20 μ L of each concentration was added to a round bottom 96-well plate (Sarstedt, Germany). 100 μ L of 0.25% cell suspension was added to each well and incubated at 37°C for 1h at 100rpm. After incubation, plates were centrifuged for 5 min at 150g and the supernatant was transferred to a flat bottom transparent 96-well plate (Sarstedt, Germany). Absorbance values were measured at 415 nm and the percentage of hemolysis was calculated by using the formula: % hemolysis = $\frac{\text{Abs}_{415}(\text{Sample}) - \text{Abs}_{415}(\text{PBS})}{\text{Abs}_{415}(\text{Triton } 1\%) - \text{Abs}_{415}(\text{PBS})} * 100$, where a 1% Triton solution was used to define 100% of hemolysis and a PBS solution defined 0% of hemolysis.

Supplementary Results

Peptide characterization

Sequence ^[a]	Compound number	Purity (%)	Chemical formula	Calculated m/z	Observed m/z
C ₁₈ -GABA-MPEG4-(IETDV) ₂	1	>95%	C ₈₄ H ₁₄₆ N ₁₂ O ₃₀	902.8, [M+2H] ²⁺	902.8
C ₁₈ -γGlu-MPEG4-(IETAV) ₂	2	>95%	C ₈₃ H ₁₄₆ N ₁₂ O ₂₈	880.8, [M+2H] ²	880.8
C ₁₈ -YGRKKRRQRRR -MPEG4-(IETDV) ₂	3	>95%	C ₁₄₄ H ₂₅₅ N ₄₃ O ₄₂	1087.7, [M+3H] ³⁺	1087.8
C ₁₈ -GABA-YGRKKRRQRRR -MPEG4-(IETDV) ₂	4	>95%	C ₁₄₈ H ₂₆₂ N ₄₄ O ₄₃	1116.2, [M+3H] ³⁺	1116.2
C ₁₈ -YGRKKRRQRRR	5	>95%	C ₈₂ H ₁₅₃ N ₃₃ O ₁₄	913.4, [M+2H] ²⁺	913.4

^[a] Capital letters indicate L-amino acids. MPEG indicates the PEG linker between the two pentameric sequences. GABA indicates gamma-aminobutyric acid, and γGlu indicates gamma-glutamic acid. C₁₈ indicates stearic acid.

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