Supporting Information

Mirror image pairs of cyclic hexapeptides have different oral bioavailabilities and metabolic stabilities

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Figure S1: $^1$H NMR spectrum of 1 in DMSO-d6.
Figure S2: $^1$H NMR spectrum of 2 in DMSO-d6.
Figure S3: $^1$H NMR spectrum of 3 in DMSO-d6.
Figure S4: $^1$H NMR spectrum of 4 in DMSO-d6.
Figure S5: $^1$H NMR spectrum of 5 in DMSO-d6.
NMR Spectroscopy

The samples for the NMR analyses of peptides 1-6 were prepared by dissolving the peptide (about 1 mg) in 600 μL of DMSO-d_6. 1D $^1$H-NMR spectra were recorded at 298 K on a Bruker Avance DRX-600 spectrometer equipped with CryoProbe. DMSO-d_6 was used as the reference peak.
Figure S7: HPLC trace for compound 1.
Figure S8: HPLC trace for compound 2.
Figure S9: HPLC trace for compound 3.
Figure S10: HPLC trace for compound 4.
Figure S11: HPLC trace for compound 5.
Figure S12: HPLC trace for compound 6.
**Mass spectrometry:** MS traces was measured on a Shimadzu 2020 mass spectrometer by direct injection of compounds in acetonitrile.

Figure S13: Mass spectrum of compound 1.

Figure S14: Mass spectrum of compound 2.

Figure S15: Mass spectrum of compound 3.

Figure S16: Mass spectrum of compound 4.
**High-resolution mass spectrometry:** HRMS was performed on a Electrospray ionisation high-resolution mass spectra (ESI-HRMS) measurements were obtained on a Bruker micrOTOF mass spectrometer equipped with an Agilent 1100 Series LC/MSD mass detector in either positive ion mode by direct infusion in water/acetonitril at 100 μL/h using sodium formate clusters as an internal calibrant.

HRMS-TOF for **1.** [M+H]+: calcd for C\textsubscript{36}H\textsubscript{67}N\textsubscript{6}O\textsubscript{6}+, 679.5117; found 679.5119
HRMS-TOF for **2.** [M+H]+: calcd for C\textsubscript{36}H\textsubscript{67}N\textsubscript{6}O\textsubscript{6}+, 679.5117; found 679.5120
HRMS-TOF for **3.** [M+H]+: calcd for C\textsubscript{41}H\textsubscript{67}N\textsubscript{6}O\textsubscript{6}+, 755.5066; found 755.5020
HRMS-TOF for **4.** [M+H]+: calcd for C\textsubscript{41}H\textsubscript{67}N\textsubscript{6}O\textsubscript{6}+, 755.5066; found 755.5100
HRMS-TOF for **5.** [M+H]+: calcd for C\textsubscript{39}H\textsubscript{61}N\textsubscript{6}O\textsubscript{6}+, 725.4596; found 725.4580
HRMS-TOF for **6.** [M+H]+: calcd for C\textsubscript{39}H\textsubscript{61}N\textsubscript{6}O\textsubscript{6}+, 725.4596; found 725.4610
PAMPA

A 96-well donor and accepter plate system pre-loaded with artificial lipid membrane (Gentest™ Pre-coated PAMPA Plate) was used. 300 mL of 50 μM peptide (5% (v/v) DMSO in PBS ph7.4) was added to the donor wells and 200 mL of buffer (5% (v/v) DMSO in PBS) was added in the acceptor wells. The acceptor plate was lowered onto the donor plate so that the artificial membrane was in contact with the peptide solution below. A cover plate was placed on the acceptor plate and the assay plate covered with foil. Incubation of peptides was carried out at room temperature for 5 h. Acceptor and donor well peptide concentrations were determined using LCMS. Percentage permeability was calculated relative to the equilibrium concentration. The permeability coefficient (Papp-pampa) values were calculated according to the equation:

$$P_{\text{app-pampa}} = \frac{-\ln\left[1 - \frac{C_A}{C_{\text{equilibrium}}}\right]}{A \times \left(\frac{1}{V_D} + \frac{1}{V_A}\right) \times t}$$

Where $C_A$ is the sampled concentration in the acceptor compartment, $V_A$ is the volume in the acceptor compartment, $V_D$ is the volume of the donor compartment, $t$ is the incubation time, $A$ is the area of the filter and:

$$C_{\text{equilibrium}} = \frac{[C_D \times V_D + C_A \times V_A]}{(V_D + V_A)}$$
Animals. Male Wistar rats (aged 8-9 weeks, 250±50 g) were bred at, and obtained from, the Australian Animal Resource Centre (Canning Vale, WA). Following Australian standard animal air transport, animals were housed at The University of Queensland Biological Resources (UQBR) Animal facility at The Australian Institute for Bioengineering and Nanotechnology at The University of Queensland, Australia. Wistar rats were housed at appropriate temperature/pressure environments in a 12 h light/dark cycle according to the standard of holding facility with food and water provided. All experiments were approved by the animal ethics committee of The University of Queensland. At least 48 h habituation in the UQBR facility was provided prior to any experimental intervention. After experimentation, rats were humanely euthanised by CO₂ inhalation as stipulated by the ethical agreements.

PK Measurements

Male Wistar rats (200-250 g) were surgically implanted with a jugular vein catheter and fasted overnight, as previously described. Blood samples (200 µL) were collected from the indwelling catheter of an unanaesthetised, unrestrained rat, 5 min prior to peptide administration (10 mg/kg p.o. in olive oil, or 1 mg/kg in neat DMSO). Oral dosing was performed by gavage (75 mm soft-tipped polyethylene 18G), i.v. dosing was directly via the indwelling catheter using a 250 µL glass Hamilton syringe. Further blood samples were collected 5 min, 15 min, 30 min and at given time points from 1 to 8 h, post administration. Volumes collected were replaced with
sterile heparinised saline (20 U/mL). Blood samples were centrifuged (8000 rpm, 5 min), plasma extracted and snap frozen on dry ice. Rats were fed after the 4-hour sample was collected.

Plasma compound concentration \( (C_p) \) was determined by extraction using simple acetonitrile (ACN)-based techniques. Briefly, thawed plasma was diluted 1:3 in neat ACN, vortexed, sonicated and then centrifuged (13K rpm, 5min RT). Spiked samples of known concentrations of peptide (i.e. 1 µg - 1 ng/mL) were prepared in both ACN and clean plasma using the same technique, for standard curve preparation. The supernatant of all centrifuged samples was used directly for LCMS analysis (ABSCIEX 4000 QTRAP Triple Quadrupole, Linear Ion-Trap LCMS mass spectrometer). Chromatography was carried out on a C18 column (Phenomenex, 5 µm, 2.1 × 50 mm) using a linear gradient (2-80 % Buffer B in 12 minutes, flow rate 0.3 mL/min). Buffer A was 0.1 % formic acid (aq) and Buffer B was 90/10 acetonitrile/0.1 % formic acid (aq). LCMS data was analyzed for area under the curve (AUC) of peaks corresponding to both protonated \( (H^+; \text{i.e. peptide MW+1}) \) and sodium \( (Na^+; \text{i.e. peptide MW+23}) \) moieties. These peaks were summed to give total AUC, which corresponded to the total concentration of compound in the original plasma sample. These AUC values were compared to those obtained from the plasma standard samples for conversion to concentration. The peptide fraction absorbed (i.e. oral bioavailability; \( F\% \)) was determined using standard arithmetic (eq. 1). The elimination half-life was calculated using the formula \( t_{1/2} = 0.693 \times (Vd/CL) \), based on the data obtained from the i.v. data sets. MS Excel was used for the analysis.
CD Spectroscopy

CD measurements were performed using a Jasco model J-710 spectropolarimeter, which was routinely calibrated with \((1S)-(+)\)-10-camphorsulfonic acid. A stock solution of 1-2 mg of peptide was dissolved in 1 mL of water. Separate 500 \(\mu\)L solutions of 100 \(\mu\)M were then prepared using an appropriate amount of stock solution and making up the difference with 10 mM Phosphate Buffer (pH 7.4) and TFE. Spectra were recorded at room temperature (298K), with a 0.1 cm Jasco quartz cell over the wavelength range 260-185 nm at 50 nm/min, with a bandwidth of 1.0 nm, response time of 1 s, resolution step width of 1 nm and sensitivity of 20-50 Mdeg. Each spectrum represents the average of 5 scans. Spectra were analysed using the spectral analysis software and smoothed using ‘adaptive smoothing’ function.

Concentrations were determined using the PULCON method (Dreier, L. and G. Wider, Concentration measurements by PULCON using X-filtered or 2D NMR spectra. Magn Reson Chem, 2006. 44 Spec No: p. S206-12). NMR solutions were prepared with 540 \(\mu\)L of stock solution and 60 \(\mu\)L of D\(_2\)O. 90\(^\circ\) pulses were accurately determined and then 1D Spectra were acquired using the standard Watergate sequence with \(n_s = 32-64\), \(d_1 = 25-35\) s. Spectra were also acquired for a 4.76 mM solution of L-histidine as the reference standard. The fully resolved, most downfield, amide resonance was integrated and used to calculate the concentration from the equation:

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F\% = 100 \times \frac{AUC\text{ p.o.} \times Dose\text{ i.v.}}{AUC\text{ i.v.} \times Dose\text{ p.o.}}
\]
where \( c \) is the concentration, \( S \) is the integral (in absolute units)/number of protons, \( T \) is the temperature in Kelvin, \( \theta_{360} \) is the 360\(^\circ\) rf pulse, \( n \) is the number of scans, and \( rg \) is the receiver gain used for measuring the reference (R) and unknown (U) samples.

**Analytical Methods**

HPLC analysis was performed by measuring light absorption at wavelength 200-600 nm on a Shimadzu UHPLC system (LC-30AD, SIL-30AC, CBM-20A, SPD-M20A, CTO-20A) using solvent mixtures of H\(_2\)O/0.1 \% trifluoroacetic acid (buffer A) and MeCN/H\(_2\)O (9/1) (aq)/0.1 \% trifluoroacetic acid (buffer B) with a flow rate of 0.5 mL/min on a Eclipse Plus C18 column (2.1 \( \mu \)m x 100 mm).

**References**
