

Supporting Information

Anti-inflammatory effect of a cell penetrating peptide target-ing the Nrf2/Keap1 interaction.

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1. Peptide sequences

TAT-10:

YGRKKRRQRRRLDEETGEFLP

TAT-14:

YGRKKRRQRRRLQLDEETGEFLPIQ

TAT-14Sc:

YGRKKRRQRRREFGTDIQLIEPQLE

14mer:

LQLDEETGEFLPIQ

TAT-16:

YGRKKRRQRRRAFFAQLQLDEETGEFL

2. Peptide Synthesis

All peptides were synthesised using solid phase Fmoc chemistry on a Multisynthetech Syro I automated peptide synthesiser. Couplings were performed using O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and N,N-diisopropylethylamine (DIPEA) in N,N-dimethylformamide (DMF) and with double coupling. Removal of Fmoc protecting groups was achieved using piperidine (40% in DMF).

TAT-10 peptide was synthesised on 2-chlorotrityl chloride resin (Merck, loading: 1.01 mmol/g). Attachment of the first amino acid was carried out manually on a shaker. The resin was first swollen in dry DCM, followed by addition of the first amino acid (4 equivalents) and DIPEA (5 equivalents). The reaction proceeded for 2 hours after which the resin was capped using a mixture of DCM:MeOH:DIPEA 80:15:5 (10 mL) for 5 minutes, twice.

TAT-14 and TAT-16 peptides were synthesised on Wang resin preloaded with the appropriate amino acid (Merck, loading: 0.59 and 0.64 mmol/g respectively).

TAT-14Sc and 14mer peptides were synthesised on Wang resin (AGTC, loading: 3.8 mmol/g). Attachment of the first amino acid was carried out manually on a shaker. A symmetrical anhydride of the first amino acid was formed under an inert atmosphere at 0°C using the appropriate amino acid (10 equivalents) in DCM, followed by addition of DIC (5 equivalents) dropwise. The reaction was stirred for 30 min before removal of solvent under reduced pressure. The anhydride was then taken up in the minimum amount of DMF and added to the pre-swollen resin along with 0.1 equivalents DMAP. The reaction proceeded for 3 hours before capping the resin using acetic anhydride (2 equivalents) and pyridine (2 equivalents) in a minimum amount of DMF for 30 min.

Chain elongation was performed using 4 equivalents each of amino acid, HBTU and DIPEA. Amino acids were dissolved in NMP at 0.69 M with the exception of arginine which was dissolved in DMF. DIPEA was prepared at 2M in NMP and HBTU at 0.45 M in DMF. Couplings proceeded for 25 min followed by washing with DMF and repetition of coupling. Removal of Fmoc protecting groups was achieved using piperidine (40% in DMF) for 10 min followed by 20% in DMF for 5 min, twice.

Cleavage from the resin and global deprotection was performed using TFA:H₂O:TIPS 95:2.5:2.5 (10 mL) for 3 hours with constant agitation, followed by rinsing with TFA and removal of solvent under reduced pressure. Peptide was precipitated from the residue using cold diethyl ether and recovered by filtration. The white precipitate was then dissolved in acetic acid (30% in water) and lyophilised.

3. Peptide Purification

Analytical RP-HPLC was used to assess the purity of the product after extraction and between purification steps. Analytical HPLC was performed on an Agilent Technologies 1200 series chromatograph with an Agilent Technologies ZORBAX Eclipse XDB-C18 (5 µm, 4.6x150 mm) column and a gradient of 95:5 water:methanol with 0.05% TFA additive to 5:95 water:methanol over 15 min returning to 95:5 water:methanol over 5 min at a flow rate of 1 mL/min.

Peptides were isolated by reverse-phase chromatography using a Biotage Isolera Four providing gross purification using a 12g C18 cartridge with a gradient of 95:5 water:methanol with a 0.05% TFA additive going to 5:95 water:methanol over 40 min with a flow rate of 20 mL/min, collecting at 214 and 254 nm. Fractions were further purified by semi-preparative HPLC on an Agilent Technologies 1200 series chromatograph using an Agilent Technologies ZORBAX Eclipse XDB-C18 (5 µm, 9.4x250 mm) column.

TAT-10 was pure after Isolera purification.

14mer was further purified by semi-prep RP-HPLC using a gradient of 95:5 water:methanol to 5:95 water:methanol with 0.05% TFA additive over 30 min returning to 95:5 water:methanol over 5 min at a flow rate of 4 mL/min collecting at 214 nm.

TAT-14 and TAT-14Sc were further purified by semi-prep RP-HPLC using a gradient of 95:5 water:methanol to 5:95 water:methanol with 0.05% TFA additive over 45 min returning to 95:5 water:methanol over 5 min at a flow rate of 4 mL/min collecting at 214 nm.

TAT-16 was further purified by semi-prep RP-HPLC using a gradient of 95:5 water:methanol to 5:95 water:methanol with 0.05% TFA additive over 60 min returning to 95:5 water:methanol over 5 min at a flow rate of 4 mL/min collecting at 214 nm.

Peptides were made up at a concentration of 10mM in PBS

Unless otherwise noted all reagents for cell experiments were purchased from Fisher Scientific and used according to manufacturer's instructions.

4. Cell Culture

THP-1 cells were cultured in RPMI1640 medium with 10% Foetal Calf Serum (FCS) (PAA), 2mM L-glutamine and penicillin/streptomycin (P/S) (Sigma). Cells were grown at 37 °C in an atmosphere of 5% CO₂.

5. Stimulation

Cells were seeded at a concentration of 5×10^5 /mL and allowed to return to an unstressed state before stimulation. Cells were stimulated at a concentration of 1×10^6 /mL with 10 μ L peptide from a 10 mM stock to give a final concentration of 100 μ M. Cells were then incubated at 37 °C in an atmosphere of 5% CO₂ for the indicated time interval.

6. RNA extraction and real-time PCR

The cells were spun down and the supernatant removed. The pellet was resuspended in 1ml TRI Reagent (Ambion) and RNA was extracted according to the manufacturer's instructions.

RNA concentration was quantified using a Nanodrop ND-1000 spectrophotometer and concentrations adjusted as necessary to 200-300 ng μ L⁻¹. RNA reverse transcription was achieved using a TaqMan kit (Applied Biosystems) according to the manufacturer's instructions and carried out on a PTC-100 Peltier thermal cycler using the following profile: 21 °C for 10 min, 42 °C for 15 min, 99 °C for 5 min and 4 °C for 5 min.

HO-1, TNF α and GAPDH primers were acquired from Invitrogen with the following sequences, HO-1 forward: 5'-ATGGCCTCCCTGTACCACATC-3', reverse: 5'-TGTTGCGCTCAATCTCCTCCT-3', TNF α forward: 5'-GCCCAGGCAGTCAGATCATC-

3' reverse: 5'-CGGTTTCAGCCACTGGAGCT-3' GAPDH forward: 5'-AACAGCCTCAAGATCATCAGCA-3', reverse: 5'-TGCTAAGCAGTTGGTGGTGC-3'. mRNA expression was measured by real-time PCR using a QIAGEN Rotor-Gene Q and SYBR Green technology (Sigma). DNA was replicated for 40 cycles of 95°C for 15 s and 60°C for 1 min. Each mRNA expression was normalised against GAPDH mRNA expression using the comparative cycle threshold method.

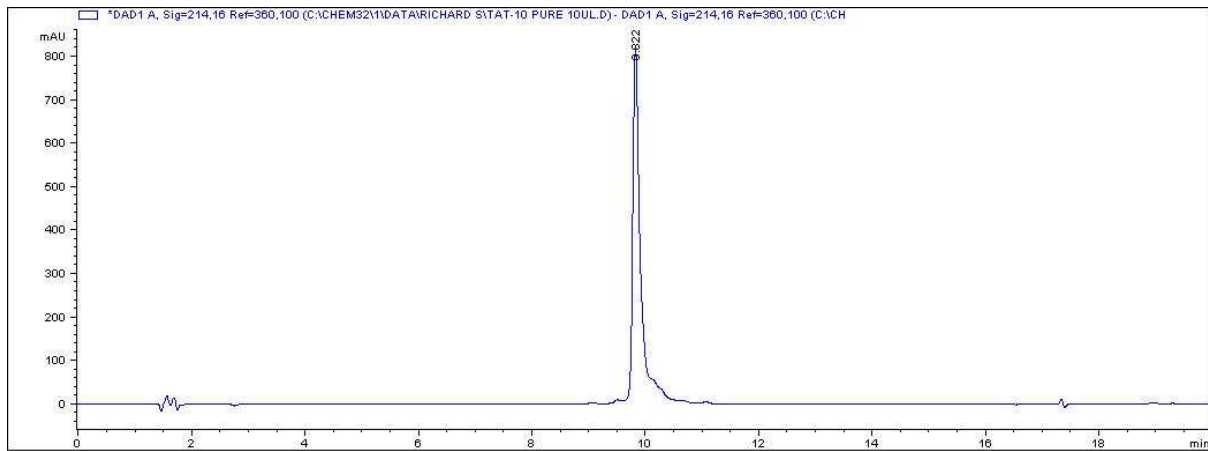
7. Western Blotting

Cells were stimulated as previously and following incubation, cells were lysed by centrifugation and the supernatant removed. Cell lysates were resuspended in 100µl SDS sample buffer (Invitrogen) before boiling for 5 min. Separation was performed using premade Bis-Tris PAGE gels (Invitrogen) before being transferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk (Marvel) and incubated with primary antibodies for 1 hour at room temperature. Immunoblotting was carried out using the following antibodies. Rabbit anti-human Nrf2 AB62352 (abcam) 1:1000; goat anti-human HO-1 AF3776 (R&D Systems), 1:2000; mouse anti-human β-actin A1978 (Sigma), 1:100,000. Membranes were blocked and washed in TBST before incubating with secondary antibodies for 30 minutes at room temperature. Secondary antibodies goat anti-rabbit, donkey anti-goat (Santa Cruz Biotechnology), and goat anti-mouse (Dako) were used at 1:1000.

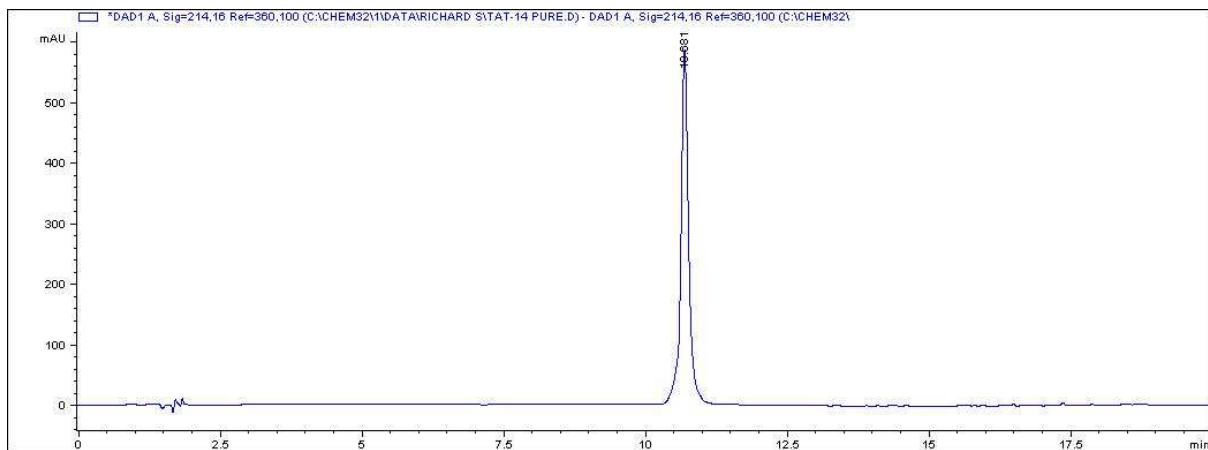
Membranes were blocked and washed in TBST before they were visualised using Amersham ECL Prime (GE Healthcare).

8. HPLC Chromatograms

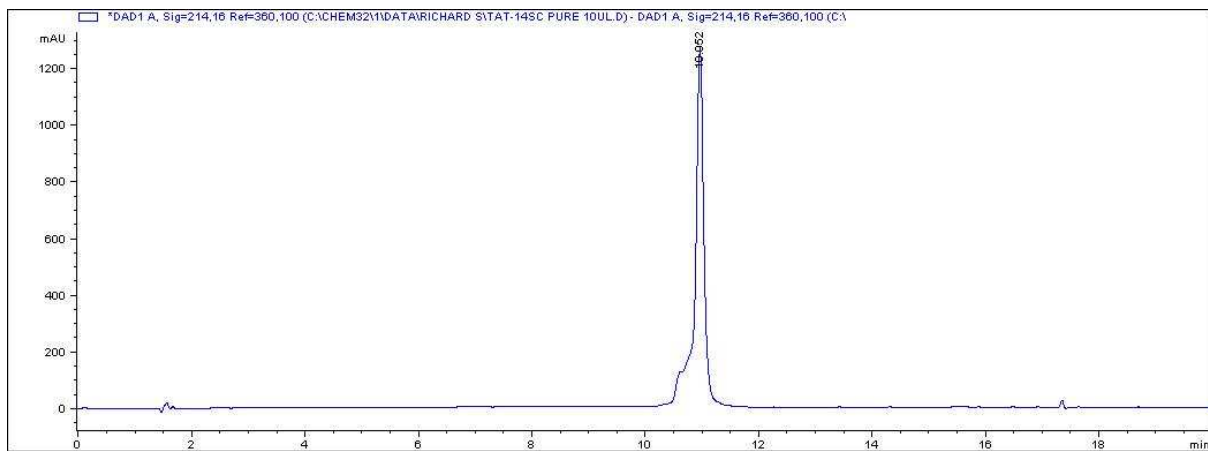
TAT-10



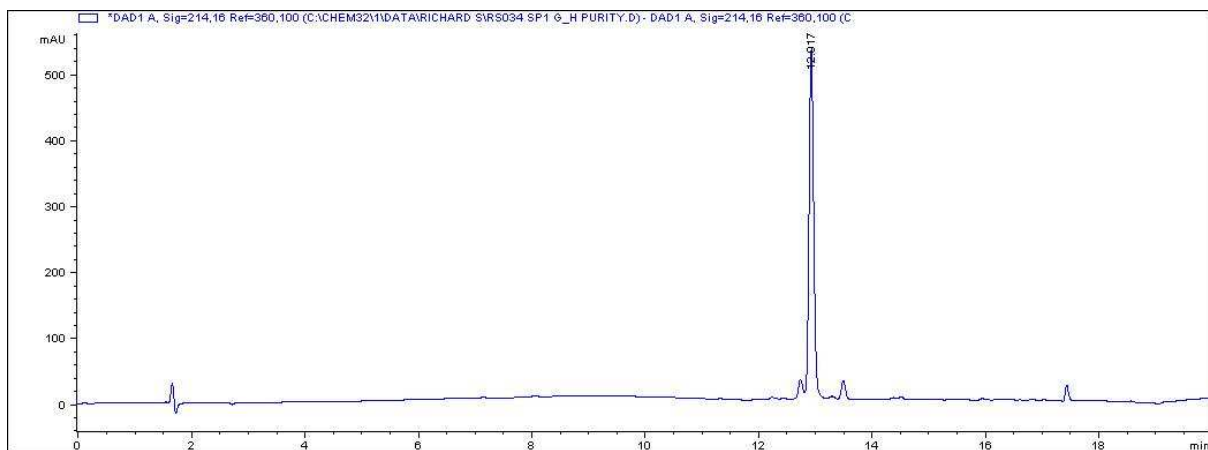
TAT-14



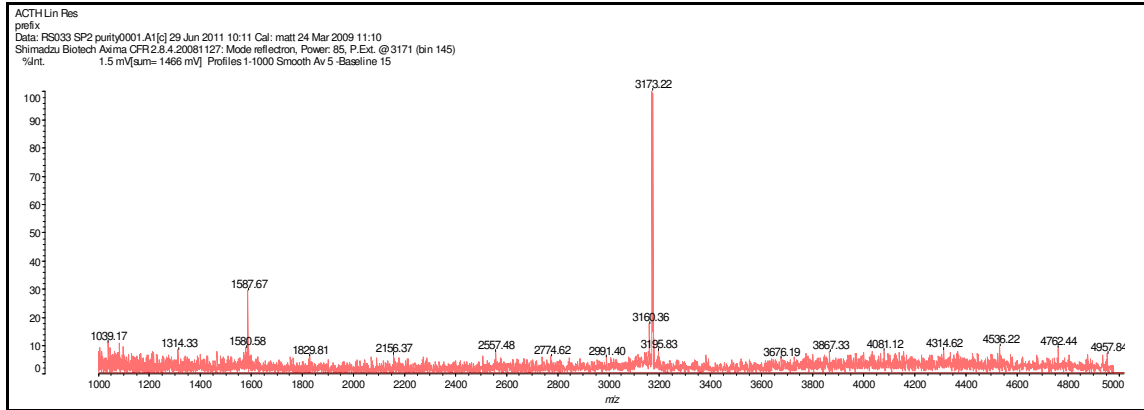
TAT-14Sc



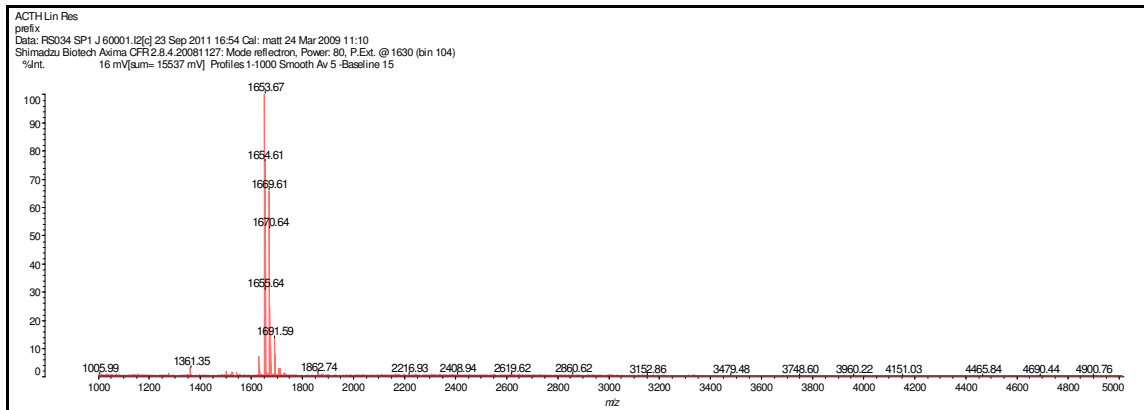
14mer



TAT-16



14mer: Expected: 1630.81 Found: 1653.67 (M+Na)



TAT-16: Expected: 3399.82 Found: 3399.00

