# A succinyl lysine-based photo-cross-linking peptide probe for Sirtuin 5

Karunakaran A. Kalesh<sup>1</sup> and Edward W. Tate<sup>1\*</sup>

<sup>1</sup>Department of Chemistry, Imperial College London, South Kensington Campus, Exhibition Road, London SW7 2AZ, UK, E-Mail: <u>e.tate@imperial.ac.uk</u>; Fax: +44 (0)20 7594 1139.

## 1. General

All chemical were purchase from commercial suppliers and used without further purification. Recombinant Sirt5 protein was purchased from Merck Millipore (catalogue # 03-230). The following abbreviations were used: Fmoc (9-fluorenylmethylcarbamate), tBu (tertiary butyl), DMF (dimethylformamide), DIEA (diisopropylethyl amine), NMM (N-methylmorpholine), HBTU (N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate), NMP (1-methyl-2-pyrrolidinone), TFA (trifluoroacetic acid), TBME (*tert*-Butyl methyl ether), TIS (triisopropyl silane), DMEM (dulbecco's modified eagle's medium), FBS (fetal bovine serum), TCEP (tris(2-carboxyethyl) phosphine hydrochloride), TBTA (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine), SDS (sodium dodecyl sulfate), EDTA (ethylenediaminetetraacetic acid), and DTT (dithiothreitol), FA (formic acid).

## 2. Synthesis of Fmoc-Lys(tBu-succinyl)- OH

The compound was synthesised as reported before<sup>1</sup>. Briefly, to a solution of mono-*t*Butyl-succinate (3.5g, 20.09 mmol) in anhydrous DMF (14 mL) was added N-hydroxysuccinimide (2.2g, 19.11 mmol) with stirring at room temperature. To this was added a solution of N,N<sup>2</sup>-dicyclohexylcarbodiimide (3.94g, 19.11 mmol) in anhydrous DMF (20 mL) and the reaction was left to stir at r.t for 2h.The reaction was then quickly filtered and the filtrate was added to a solution of Fmoc-Lys-OH (4.8g, 13 mmol) in anhydrous DMF (20 mL) with 6.6 mL (37.89 mmol) of DIEA. The reaction was stirred for another 30 min. To the reaction mixture was then added approximately 70 mL water followed by 1N HCl to adjust the pH to 2-3. The mixture was then extracted five times with ethyl acetate and washed once with brine. The organic layer was dried under anhydrous sodium sulphate followed by removal of solvent under vacuum. The residue obtained was purified by Flash column chromatography on Biotage Isolera system using 50g snap silica gel column with solid loading using gradient elution of hexanes and ethyl acetate at 50 mL/min flow rate. The desired product was eluted out at 60% ethyl acetate, which upon concentration under vacuum was obtained as a white solid at about 76% yield (5.2g, 9.9 mmol).

<sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD):  $\delta$  7.80 (d, 2H, J = 8.0 Hz), 7.67 (dd, 2H, J = 4.0, 8.0 Hz), 7.40 (t, 2H, J = 7.2 Hz), 7.31 (t, 2H, J = 7.3 Hz), 4.36 (m, 2H), 4.22 (tm 1H, J = 7 Hz), 4.12 (dd, 1H, J = 4.0, 8.0 Hz), 3.17 (t, 2H, J = 6.8 Hz), 2.52 (t, 2H, J = 6.8 Hz), 2.41 (t, 2H, J = 6.8 Hz), 1.77 (m, 2H), 1.51 (m, 2H), 1.43 (s, 9H), 1.39 (m, 2H). LC-MS (ESI) calculated for C<sub>29</sub>H<sub>37</sub>N<sub>2</sub>O<sub>7</sub> [M+H<sup>+</sup>] 525.3, observed 525.1

### 3. Peptide synthesis

Solid phase peptide synthesis was performed on an automated peptide synthesiser (Intavis ResPep SL Peptide Synthesiser) using standard Fmoc chemistry on rink amide resin (TentaGel S RAM resin from RAPP Polymere GmbH). All Fmoc amino acids were activated by HBTU/NMM (4 eq. and 5 eq. each) in NMP. Fmoc removal was carried out by treatment with 20% piperidine in DMF. Peptide cleavage from the resin was performed by treatment with 95% TFA, 2.5% water and 2.5% TIS for 2h at r.t. Peptides were isolated by precipitation with cold TBME followed by centrifugation. The peptides were subsequently purified by reverse-phase HPLC and characterised by LC-MS (Fig. S1).

### Probe 1 (P1)

Amino acid sequence: NH<sub>2</sub>-B<sub>p</sub>ISGASEK<sub>succ</sub>DIVHSGLAG<sub>p</sub>GG-(CONH<sub>2</sub>)





## Probe 2 (P2)

Amino acid sequence: NH<sub>2</sub>-B<sub>p</sub>ISGASEK<sub>ac</sub>DIVHSGLAG<sub>p</sub>GG-(CONH<sub>2</sub>)





## Probe 3 (P3)

Amino acid sequence: NH<sub>2</sub>-B<sub>p</sub>ISGASEKDIVHSGLAG<sub>p</sub>GG-(CONH<sub>2</sub>)

m/z 972.68 (M+2H<sup>+</sup>), calculated m/z 972.53 (M+2H<sup>+</sup>), m/z 648.81 (M+3H<sup>+</sup>), calculated m/z 648.69 (M+3H<sup>+</sup>)



## Probe 4 (P4)

Amino acid sequence: NH2-ISGASEK<sub>succ</sub>DIVHSGLA-(CONH2)

m/z 1583.95 (M+H<sup>+</sup>), calculated m/z 1583.74 (M+2H<sup>+</sup>), m/z 792.09 (M+2H<sup>+</sup>), calculated m/z 792.37 (M+2H<sup>+</sup>)





**Fig. S1** LC-MS analysis of the peptides **P1** to **P4**. HPLC runs used gradients of 0.1% FA in water (solvent A) and 0.1% FA in methanol (solvent B).

#### 4. Cell culture and preparation of whole cell lysate

HeLa cells were grown in DMEM supplemented with 10% FBS without antibiotics in a humidified atmosphere with 10% CO<sub>2</sub> at 37°C. Upon confluence, the cells were washed with 1X PBS and trypsinised. The cell pellets were washed three times with PBS and suspended in lysis buffer (50 mM Tris hydrochloride at pH 7.5 with 150 mM NaCl and Roche Complete EDTA-free protease inhibitors) and sonicated to complete lysis (50 round of 1s on and 1s off pulse at 100% amplitude) at 4°C. The suspension after lysis was centrifuged at 16,000 g for 15 min at 4°C and the supernatant was collected, which gave the whole cell lysate. Total protein concentration of the lysate was quantified by Bradford's assay (Bio-Rad DC<sup>TM</sup> Protein Assay) and adjusted to 4 mg/mL. The lysate was stored in -80°C freezer and used for all subsequent labelling experiments.

### 5. Protein labelling

**P1** was incubated with either recombinant Sirt5 (in Tris hydrochloride pH 7.5 with 150 mM NaCl and 2 mM MgCl<sub>2</sub>) or recombinant Sirt5 spiked in HeLa whole cell lysate (the lysate stock was diluted to working concentrations in Tris hydrochloride pH 7.5 with 150 mM NaCl and 2 mM MgCl<sub>2</sub>) for 10 min at r.t. The samples were then irradiated at 365 nm (long wavelength UV) using a UVGL-58 UV-lamp (6W) for 30 min on ice. After UV cross-linking, 100  $\mu$ M trifunctional capture reagent (AzTB, structure shown below in Fig. S2)<sup>2</sup> was added followed by CuSO<sub>4</sub> (1 mM), TCEP (1 mM) and TBTA (100  $\mu$ M). The click reactions were incubated for 2h at r.t. with shaking. The reactions were quenched

by adding 10 mM EDTA and proteins were precipitated by adding 10 fold excess of ice-cold methanol and leaving the solutions at -80°C overnight. The precipitated proteins were pelleted by centrifugation at 16,000 g for 30 min at 4°C. The supernatants were removed and the protein pellet was washed twice with 10 volumes of ice-cold methanol followed by centrifugation at 16,000 g for 20 min at 4°C. The pellets were air-dried and re-dispersed by sonication in 1X PBS with 2% SDS and 10 mM EDTA to 10 mg/mL concentration. The samples were then diluted to 1 mg/mL using 1X PBS. 4X SDS loading buffer (with  $\beta$ -mercapto ethanol) was added and the samples were heated to 95°C for 10 min. After cooling, proteins were resolved by 1D SDS-PAGE (12% gel). Labelled proteins were visualised by in-gel fluorescence scanning on an Ettan<sup>TM</sup> DIGE Imager (GE Healthcare) using Cy3 channel (excitation 532 nm, emission filter 580 nm with 30 nm band-pass) while the molecular weight marker was imaged using Cy5 channel.



Fig. S2 Chemical structure of the capture reagent (AzTB)

### 6. Affinity enrichment

The precipitated and re-dispersed protein samples (1 mg/mL concentration in PBS with 0.2% SDS and 1mM DTT) were incubated with NeutrAvidin-Agarose resin (Thermo Pierce) at r.t for 1.5 h. The beads were then spun at 4000 g for 4 min at r.t. and the supernatants were removed. The beads were subsequently washed with 0.5% SDS in 1X PBS (3X), 4M Urea in 1X PBS (2X) and 50 mM ammonium bicarbonate (5X). For in-gel fluorescence and Western-blot analysis, the bound proteins were eluted from the beads by boiling at 95°C for 10 min in PBS with 4X SDS loading buffer containing  $\beta$ -mercapto ethanol.



Fig. S3 In-gel fluorescence scans (a) before and (b) after pull-down. The corresponding Coomassie blue stains of the gels are also shown.

### 7. Western blot

Proteins resolved by 1D SDS-PAGE on 12% tris-glycine gels were electrotransferred onto a PVDF membrane. Western-blotting for Sirt5 was performed using anti-Sirt5 antibody from Millipore (Cat. # ABE198) following manufacturers protocol. Briefly, the PVDF membrane after electrotransfer was blocked in freshly prepared 5% BSA with 0.05% Tween®-20 for 1 h at r.t with constant shaking. The membrane was then incubated with anti-Sirt5 antibody at 0.5  $\mu$ g/mL concentration in freshly prepared 5% BSA for 1 h at r.t. The membrane was then washed three times with TBST followed by incubation with goat anti-rabbit IgG (H+L)-HRP conjugated secondary antibody (Advansta, cat. no. R-05072-500) in freshly prepared 5% milk for 1 h with constant agitation at r.t, washed again with TBST three times and visualised with enhanced chemiluminescence Western blotting detection reagent (Merck Millipore, Luminata<sup>TM</sup> Crescendo Western HRP Substrate, cat. no. WBLUR0100). Western blotting for biotin was performed using anti-biotin-HRP antibody (Cell Signaling, cat. no. 7075). Briefly, the PVDF membrane after electrotransfer was blocked in freshly prepared 3% BSA for 1 h at r.t with constant shaking. The membrane was then incubated with anti-biotin-HRP antibody at 2000 fold dilution in freshly prepared 0.3% BSA for 1 h at r.t. The membrane was then washed four times with TBST followed by visualisation using enhanced chemiluminescence Western blotting detection reagent.



Fig. S4 (a) Anti-Sirt5 blot and anti-biotin blot, respectively, of Sirt5-spiked HeLa lysates after labelling with P1.

### 8. References

J. Du, Y. Zhou, X. Su, J. J. Yu, S. Khan, H. Jiang, J. Kim, J. Woo, J. H. Kim, B. H. Choi, B. He,
W. Chen, S. Zhang, R. A. Cerione, J. Auwerx, Q. Hao and H. Lin, *Science*, 2011, 334, 806.

2. (a) W. P. Heal, M. H. Wright, E. Thinon and E. W. Tate, *Nat. Protoc.*, 2012, **7**, 105; (b) M. H. Wright, B. Clough, M. D. Rackham, K. Rangachari, J. A. Brannigan, M. Grainger, D. K. Moss, A. R. Bottrill, W. P. Heal, M. Broncel, R. A. Serwa, D. Brady, D. J. Mann, R. J. Leatherbarrow, R. Tewari, A. J. Wilkinson, A. A. Holder and E. W. Tate, *Nat. Chem.*, 2014, **6**, 112.

3. J. Cox and M. Mann, Nat. Biotechnol., 2008, 26, 1367.