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Supporting Information

Development of a smart activity-based probe to detect subcellular activity of asparaginyl endopeptidase in living cells

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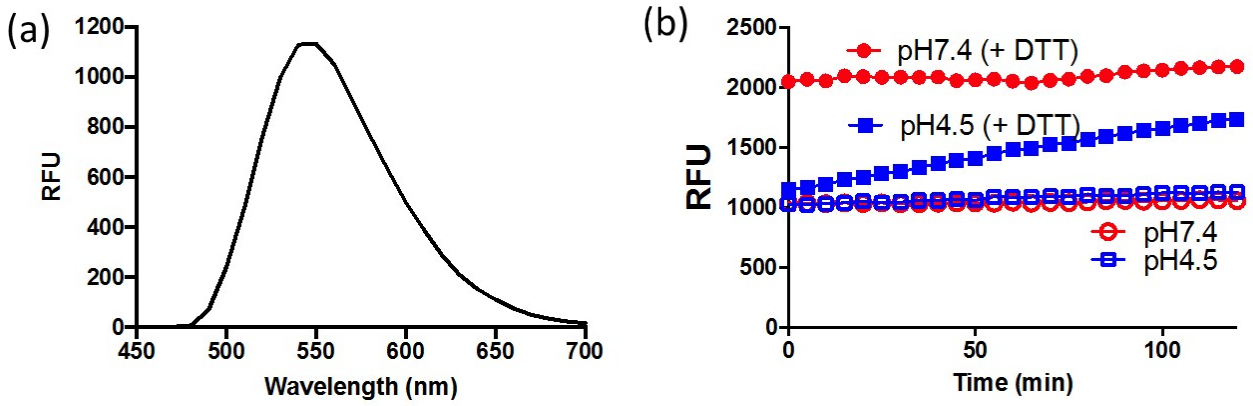


Figure S1. (a) Fluorescence emission spectra of AP-1 (ex. 450 nm) ; (b) Fluorescence emission intensity of AP-1 in buffers in the presence and absence of DTT (ex. 450 nm, em. 550 nm)

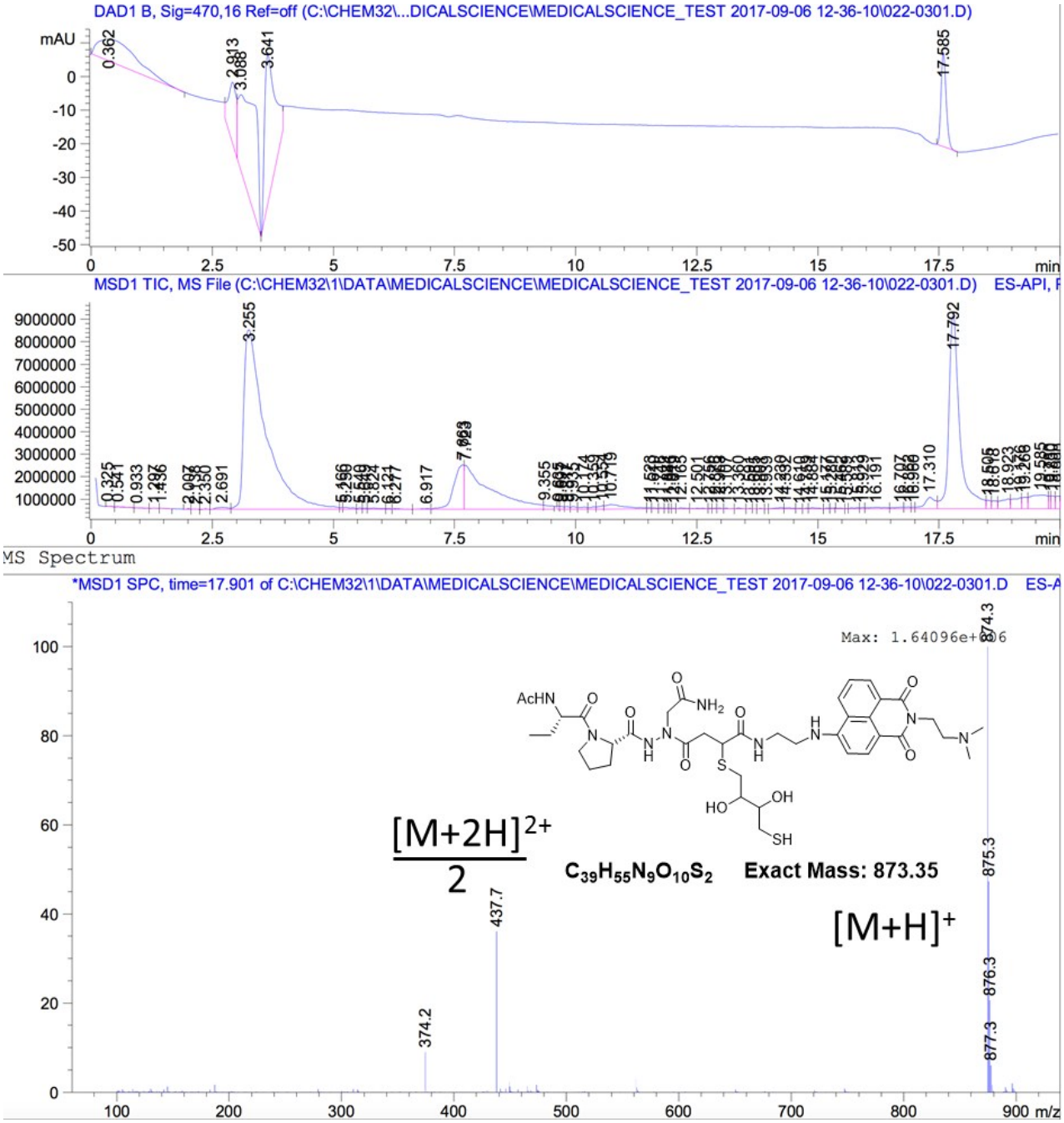


Figure S2. Mass spectrometry data of **AP-1** in the presence of excess DTT, showing a mass peak corresponds to a DTT adduct

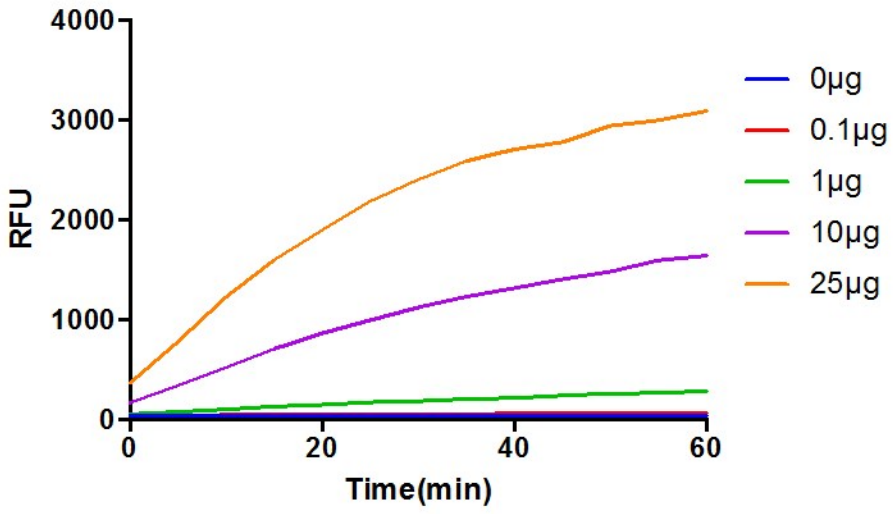


Figure S3. AEP activity in RAW 264.7 cell lysates determined by Z-AAN-AMC, a fluorogenic AEP substrate

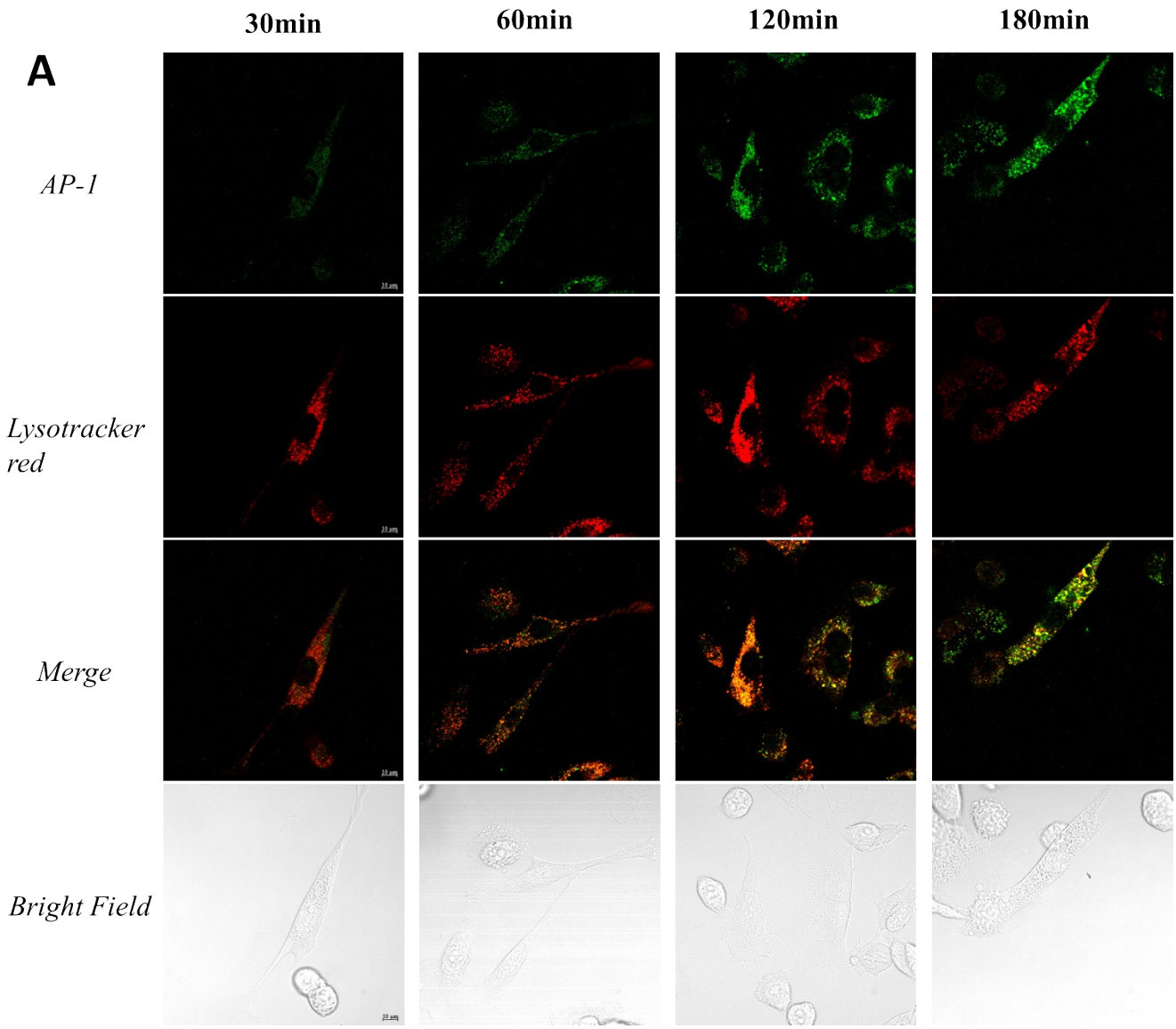


Figure S4 (a) Representative images of NIH-3T3 cells treated with AP-1 at 10 μM.

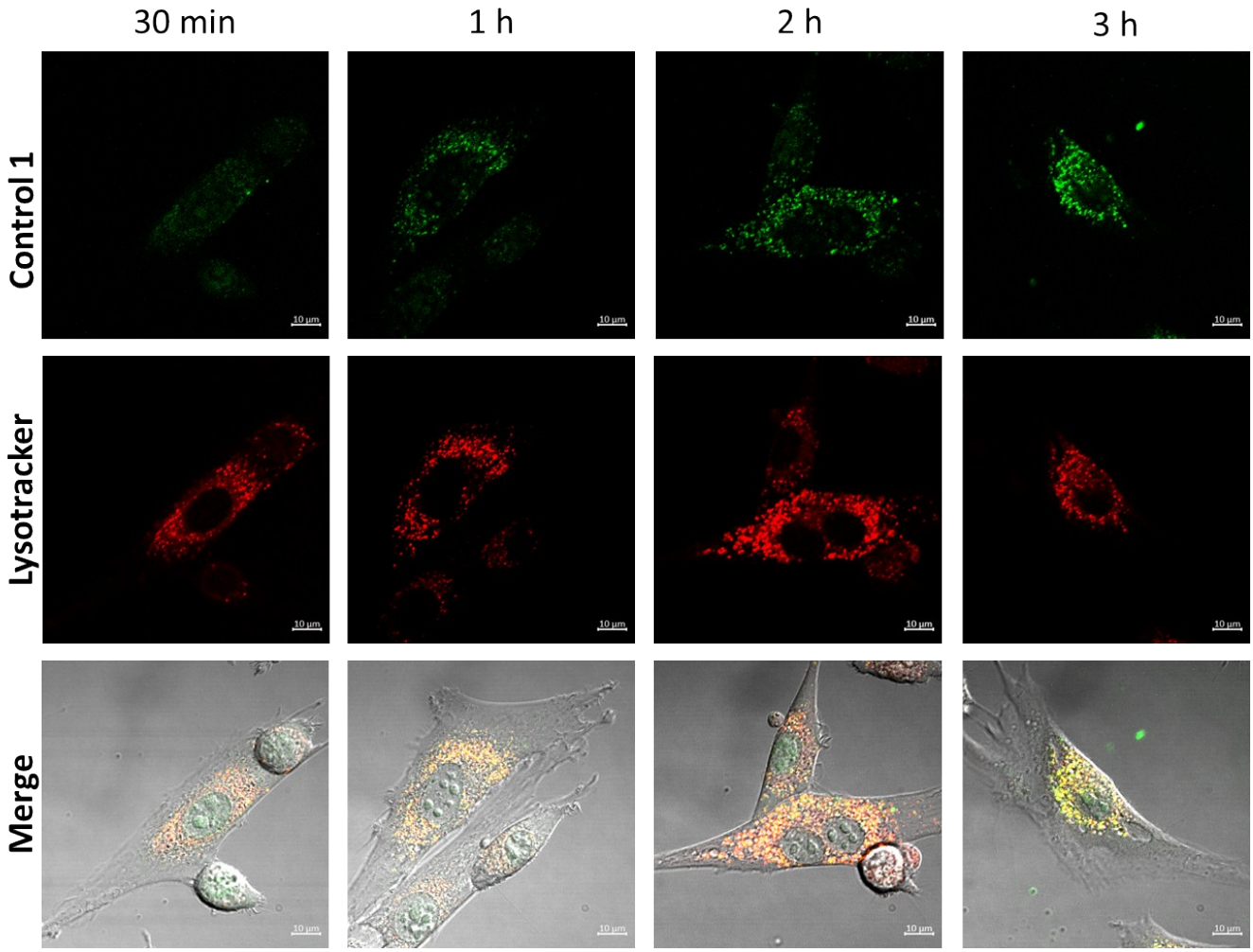


Figure S4 (b) Representative images of NIH-3T3 cells treated with Control 1 at 10 μM.

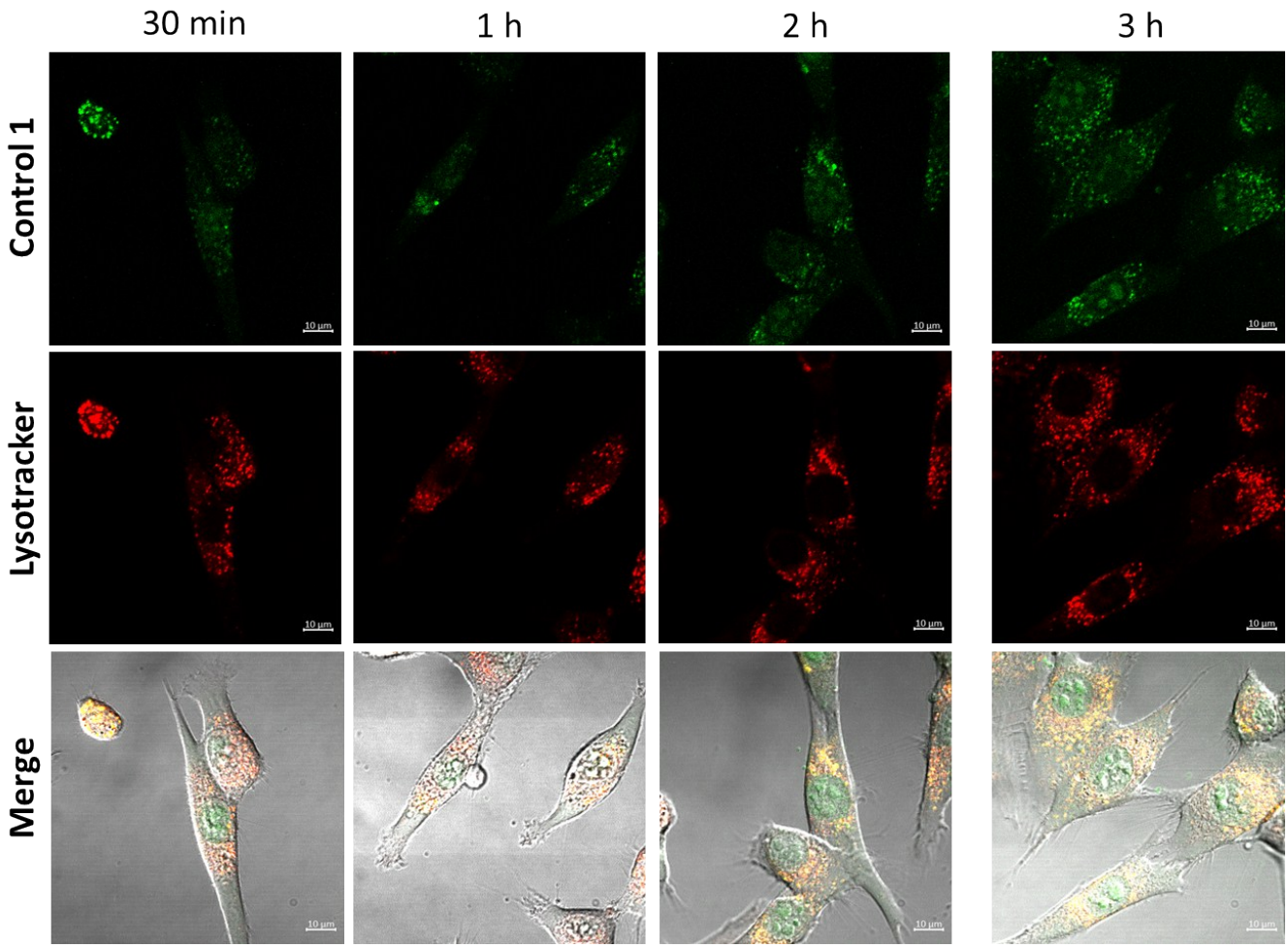


Figure S4 (c) Representative images of NIH-3T3 cells pretreated with AEP inhibitor (50 μM), and labelled with **Control 1** at 10 μM

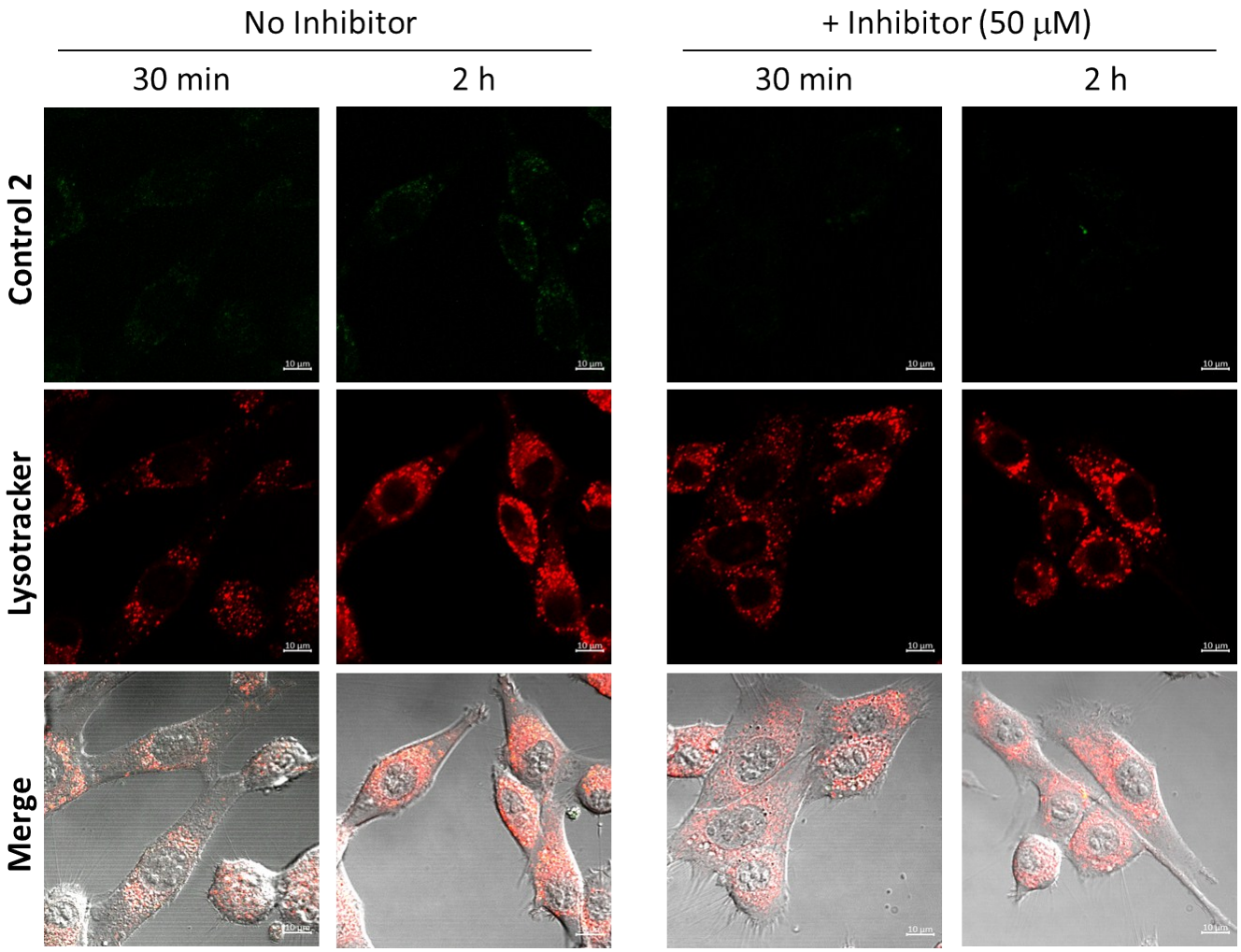


Figure S4 (d) Representative images of NIH-3T3 cells treated with **Control 2** at 10 μM

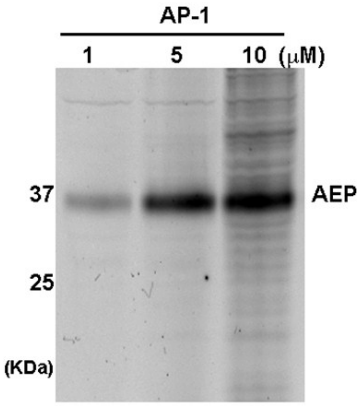
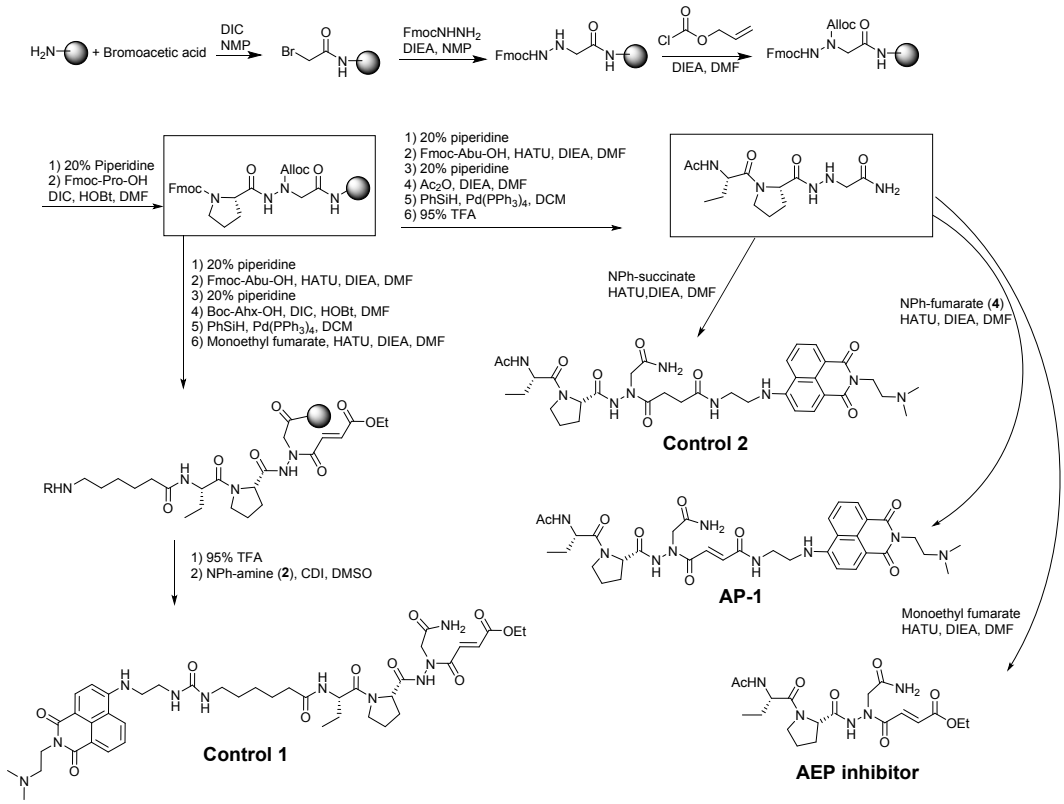


Figure S5. AEP labelling by AP-1 in intact HT-22 cells

Scheme S1. Synthesis of AP-1, the control probes and the AEP inhibitor

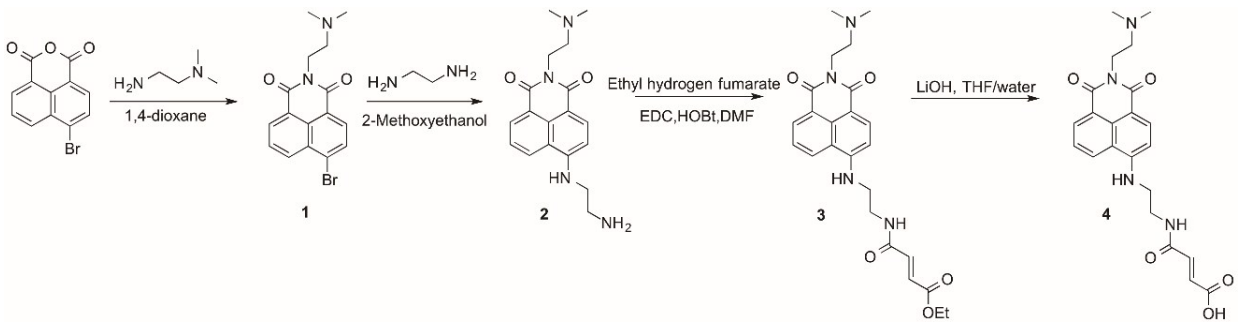


Experimental

Synthesis and characterization Unless otherwise noted, materials and solvents were purchased from commercial suppliers and used without further purification. Flash chromatography was carried out using silica gel 60 (Merck-Millipore). Nuclear magnetic resonance (NMR) spectra were obtained on a Varian VNMRS500 spectrometer (Varian). NMR chemical shifts are reported in ppm and referenced to residual protonated solvent. Reactions were analysed by liquid chromatography-mass spectrometry (LC-MS) using an Agilent 6130 single quadrupole instrument (Agilent). High-performance liquid chromatography (HPLC) purifications were carried out on an Azura preparative HPLC system (Knauer) with a SunFire C18 OBD prep column (Waters). The mobile phase consisted of 10:90:0.1 water/acetonitrile/formic acid (solvent A) and 0.1% formic acid in water (solvent B). AP-1 and the AEP selective inhibitor were

synthesized by following a previously reported procedure [1] with slight modifications, as described in **Scheme S1**. All synthesized peptides were cleaved from the resin by applying a cleavage cocktail containing 95% TFA and then purified by HPLC. The purified peptides were coupled with compound **4** (1 eq) in DMSO with HATU (1.1 eq) and DIEA (2.2eq) for 16 h and then purified by HPLC. The purity and identity of all compounds were assessed by LC-MS. Synthesis and characterization data of the AEP inhibitor was reported elsewhere.[1] Compound **4** was synthesized by following **Scheme S2**.

Scheme S2



Synthesis of 6-bromo-2-(2-(dimethylamino)ethyl)-1H-benzo [de]isoquinoline-1,3(2H)-dione (1). 4-Bromo-1,8-naphthalic anhydride (3.01 g, 10.82 mmol) was dissolved in 108.3 mL of 1,4-dioxane, and *N,N*-dimethylethylenediamine (1.42 mL, 13 mmol) was added to the solution. The reaction mixture was refluxed at 140 °C for 4 h and cooled. The resulting precipitate was filtered and washed with distilled water to afford **1** as an ivory solid. Yield: 3.564 g (95%). ESI-MS: m/z calcd for $C_{16}H_{16}BrN_2O_2$ $[M + H]^+$: 347.04; found: 347.0 ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 8.60–8.55 (m, 2H), 8.35 (d, J = 7.9 Hz, 1H), 8.23 (d, J = 7.9 Hz, 1H), 8.00 (dd, J = 33.2, 16.6 Hz, 1H), 4.15 (t, J = 6.9 Hz, 2H), 2.55–2.35 (m, 2H), 2.20 (s, 6H).

Synthesis of 6-((2-aminoethyl)amino)-2-(2-(dimethylamino)ethyl)-1H-benzo[de]isoquinoline-1,3 (2H)-dione (2). Compound **1** (345.80 mg, 1 mmol) and ethylenediamine (133.56 μL, 2 mmol) were

dissolved in methoxyethanol (8 mL). The reaction mixture was refluxed at 130 °C for 7 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH/ammonium hydroxide (10:1:0.1) as the eluent to afford compound **2** as an orange solid. Yield: 280.50 mg (86%). ESI-MS: m/z calcd $C_{18}H_{22}N_4O_2$ $[M + H]^+$: 327.17; found: 327.1 ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.61 – 8.53 (m, 1H), 8.44 (dd, *J* = 8.1, 5.5 Hz, 1H), 8.17 (d, *J* = 5.2 Hz, 1H), 7.61 (dd, *J* = 12.8, 8.0 Hz, 1H), 6.69 (dd, *J* = 8.3, 5.1 Hz, 1H), 6.20 (s, 1H), 4.32 (d, *J* = 6.6 Hz, 2H), 3.41 (dd, *J* = 15.9, 11.2 Hz, 2H), 3.18 (d, *J* = 4.9 Hz, 2H), 2.66 (d, *J* = 6.7 Hz, 2H), 2.37 (d, *J* = 5.0 Hz, 6H).

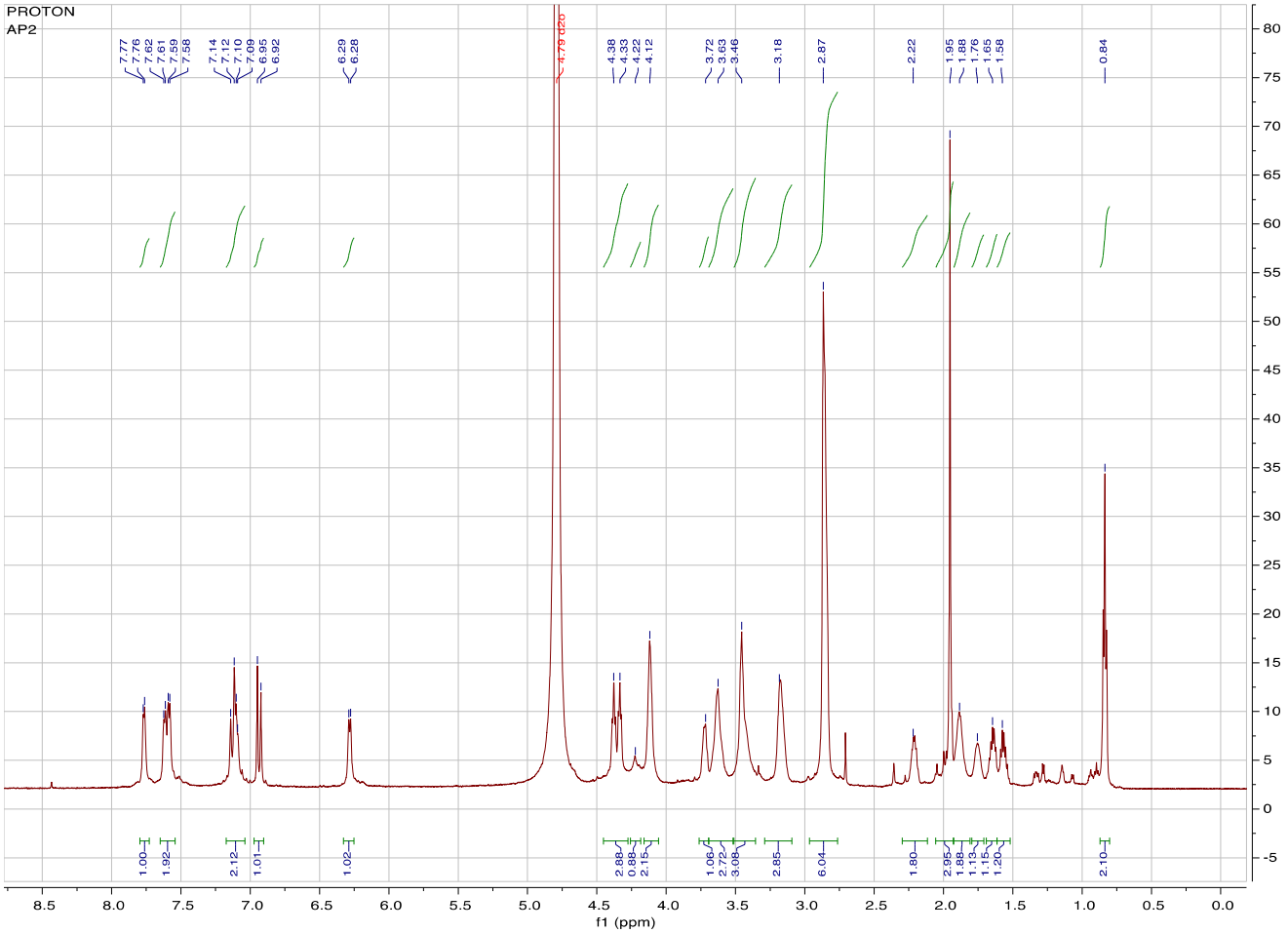
Synthesis of ethyl (E)-4-((2-((2-(2-(dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]iso-quinolin-6-yl)amino)ethyl)amino)-4-oxobut-2-enoate (3). Ethyl hydrogen fumarate (72.06 mg, 0.5 mmol), EDC•HCl (115.02 mg, 0.6 mmol) and HOBt (81.06 mg, 0.6 mmol) were dissolved in DMF (0.7 mL) and stirred for 1 h at room temperature. Ethyl hydrogen fumarate solution was added dropwise to **2** (163.0 mg, 0.5 mmol) dissolved in DMF (0.7 mL) at 0 °C, and the solution was then stirred for 1 h at room temperature. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (10:1) as the eluent to afford **3** as a yellow solid. Yield: 105.6 mg (46.67%). ESI-MS: m/z calcd $C_{24}H_{28}N_4O_5$ $[M + H]^+$: 453.21; found: 453.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70 (d, *J* = 8.0 Hz, 1H), 8.46 (d, *J* = 6.4 Hz, 1H), 8.28 (d, *J* = 8.8 Hz, 1H), 7.72 (t, *J* = 7.8 Hz, 1H), 7.00 (d, *J* = 15.6 Hz, 1H), 6.89 (d, *J* = 8.4 Hz, 1H), 6.60 (d, *J* =15.6 Hz, 1H), 4.25 - 4.12 (M, 4H), 3.52 (t, *J* = 6.0 Hz, 6H), 2.91 (s, 2H), 2.58 (s, 6H), 1.24 (t, *J* =14.4 Hz, 3H).

Synthesis of (E)-4-((2-((2-(2-(dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]iso-quinolin-6-yl)amino)ethyl)amino)-4-oxobut-2-enoic acid (4). Compound **3** (20 mg, 0.044 mmol) was dissolved in 1 mL of THF in a 25-mL round-bottomed flask and stirred at 0 °C. In a separate flask, lithium hydroxide (3.2 mg, 0.132 mmol) was dissolved in 1 mL of distilled water. The lithium hydroxide solution

was added dropwise to the solution of compound **3** at 0 °C. The mixture was stirred at room temperature for 2 h. After 2 h, the mixture was acidified with 1 M HCl (to pH 2) and extracted with ethyl acetate. The combined organic layers were concentrated under reduced pressure. The residue was purified by preparative HPLC to yield compound **4** as a light yellow solid. Yield: 12.6 mg (67.47%). ESI-MS: m/z calcd $C_{22}H_{24}N_4O_5$ $[M + H]^+$: 424.17; found: 425.2 ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.60 (t, J = 6.6 Hz, 2H), 8.41 (d, J = 7.3 Hz, 1H), 8.23 (d, J = 8.4Hz, 1H), 7.85 (t, J = 5.3 Hz, 1H), 7.69 – 7.64 (m, 1H), 6.85 (d, J = 8.7 Hz, 1H), 6.66 (d, J = 15.5 Hz, 1H), 6.38 (d, J = 15.5 Hz, 1H), 4.13 (t, J = 6.8 Hz, 2H), 3.49 (dd, J = 17.8, 8.0 Hz, 4H), 2.55 (t, J = 6.8 Hz, 2H), 2.24 (s, 6H).

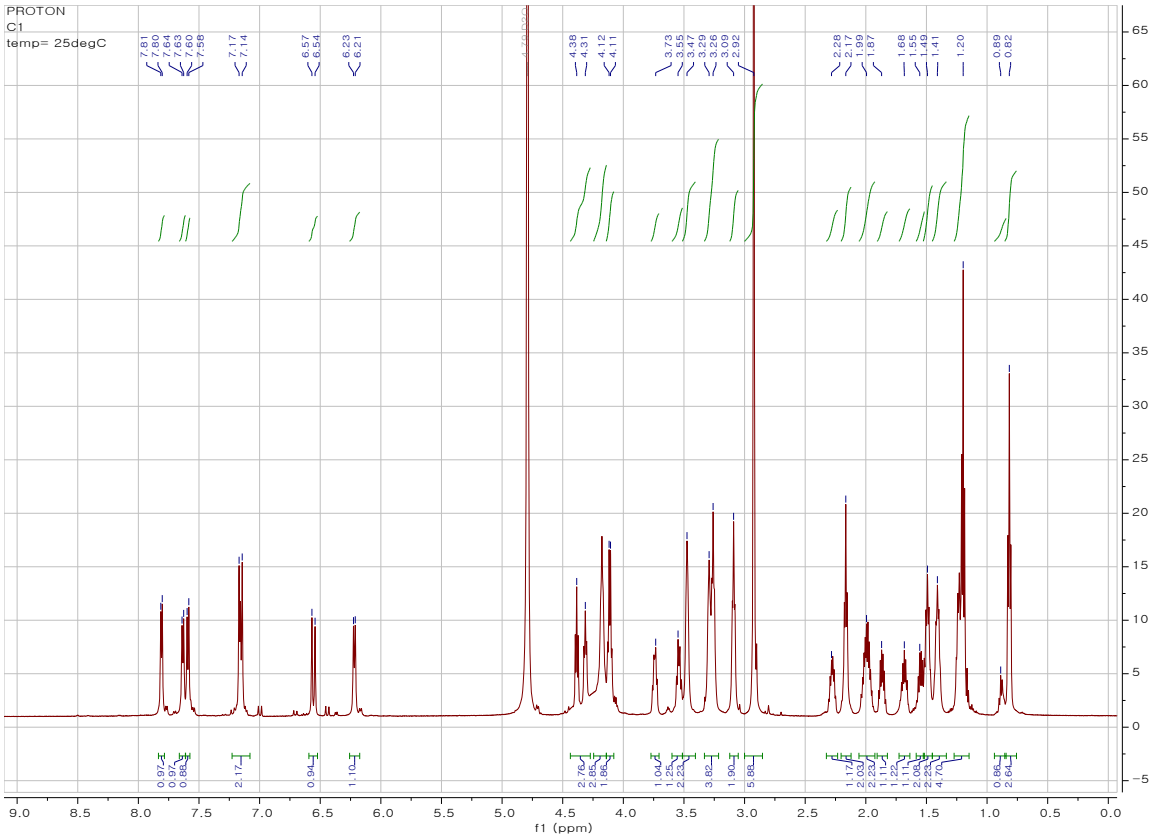
^{1}H NMR data

AP-1



^{1}H NMR (600 MHz, Deuterium Oxide) δ 7.80 – 7.73 (m, 1H), 7.60 (dd, J = 19.3, 7.0 Hz, 2H), 7.17 – 7.04 (m, 2H), 6.94 (d, J = 15.4 Hz, 1H), 6.28 (d, J = 7.4 Hz, 1H), 4.36 (d, J = 26.3 Hz, 3H), 4.22 (s, 1H), 4.12 (s, 2H), 3.72 (s, 1H), 3.63 (s, 3H), 3.46 (s, 3H), 3.18 (s, 3H), 2.87 (s, 6H), 2.22 (s, 2H), 1.95 (s, 3H), 1.88 (s, 2H), 1.76 (s, 1H), 1.65 (s, 1H), 1.58 (s, 1H), 0.84 (s, 2H).

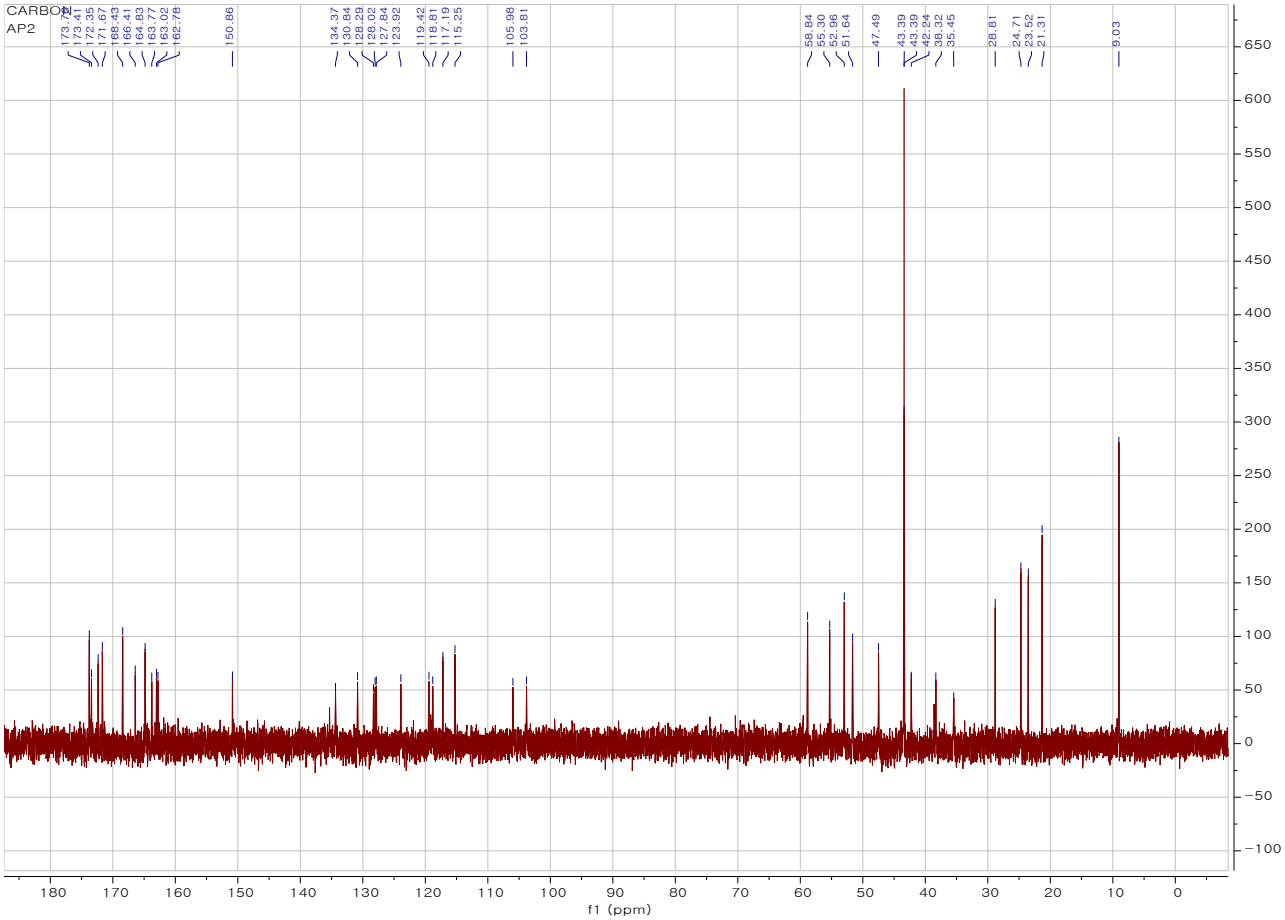
Control 1



^{1}H NMR (600 MHz, Deuterium Oxide) δ 7.81 (d, *J* = 7.1 Hz, 1H), 7.63 (d, *J* = 8.1 Hz, 1H), 7.59 (d, *J* = 8.4 Hz, 1H), 7.16 (d, *J* = 15.4 Hz, 2H), 6.56 (d, *J* = 15.5 Hz, 1H), 6.22 (d, *J* = 8.5 Hz, 1H), 4.35 (d, *J* = 42.6 Hz, 3H), 4.18 (s, 3H), 4.11 (d, *J* = 7.0 Hz, 2H), 3.73 (s, 1H), 3.55 (s, 1H), 3.47 (s, 2H), 3.28 (d, *J* = 19.7 Hz, 4H), 3.09 (s, 2H), 2.92 (s, 6H), 2.28 (s, 1H), 2.17 (s, 2H), 1.99 (s, 2H), 1.87 (s, 1H), 1.68 (s, 1H), 1.55 (s, 1H), 1.49 (s, 2H), 1.41 (s, 2H), 1.20 (s, 5H), 0.89 (s, 1H), 0.82 (s, 3H).

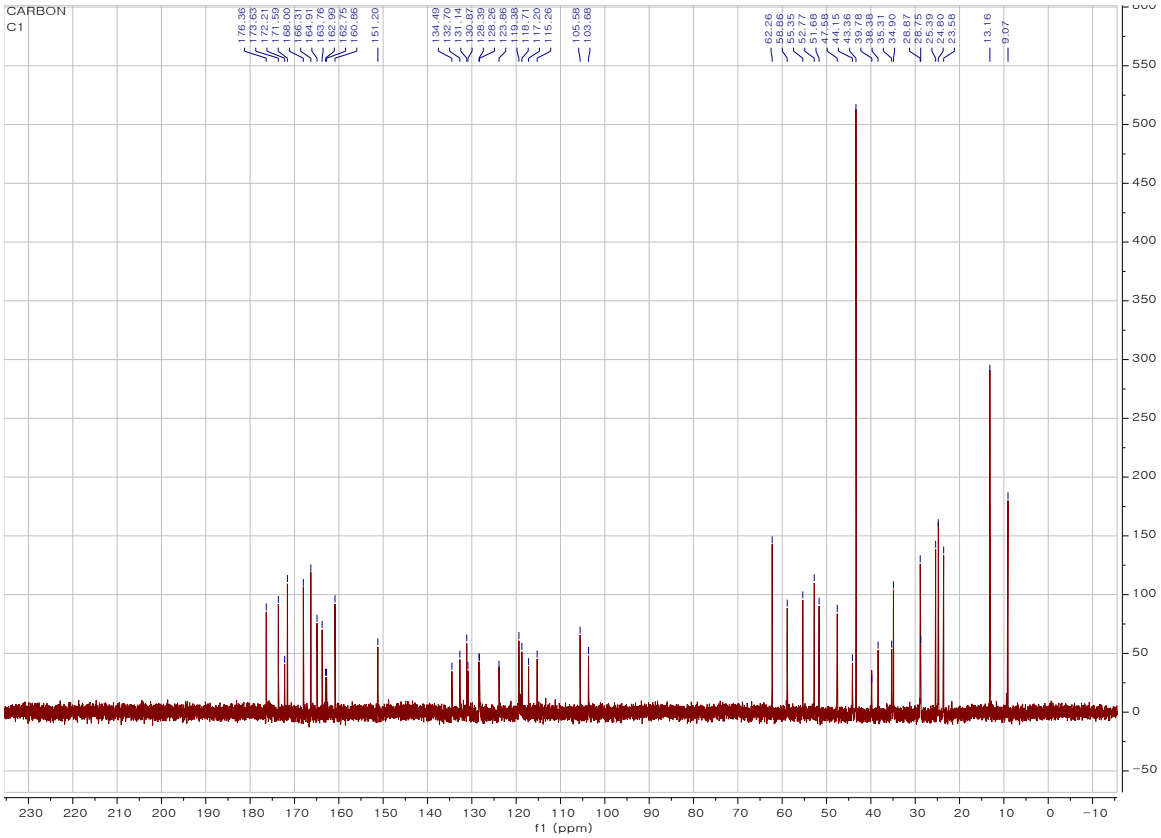
^{13}C NMR

AP-1



^{13}C NMR (151 MHz, Deuterium Oxide) δ 173.76, 173.41, 172.35, 171.67, 168.43, 166.41, 164.83, 163.77, 163.02, 162.78, 150.86, 134.37, 130.84, 128.29, 128.02, 127.84, 123.92, 119.42, 118.81, 117.19, 115.25, 105.98, 103.81, 58.84, 55.30, 52.96, 51.64, 47.49, 43.39, 42.24, 38.32, 35.45, 28.81, 24.71, 23.52, 21.31, 9.03.

Control 1



^{13}C NMR (151 MHz, Deuterium Oxide) δ 176.36, 173.63, 172.21, 171.59, 168.00, 166.31, 164.91, 163.76, 162.99, 162.75, 160.86, 151.20, 134.49, 132.70, 131.14, 130.87, 128.39, 128.26, 123.86, 119.38, 118.71, 117.20, 115.26, 105.58, 103.68, 62.26, 58.86, 55.35, 52.77, 51.68, 47.58, 44.15, 43.36, 39.78, 38.38, 35.31, 34.90, 28.87, 28.75, 25.39, 24.80, 23.58, 13.16, 9.07.

High-resolution mass spectrometry data

AP-1 $[M+H]^+$ calcd. for $C_{35}H_{47}N_9O_8^+$ 720.3464; found 720.3480

Control 1 $[M+H]^+$ calcd. for $C_{42}H_{59}N_{10}O_{10}^+$ 863.4410; found 863.4458

Control 2 $[M+H]^+$ calcd. for $C_{35}H_{48}N_9O_8^+$ 722.3620; found 722.3640

Spectroscopic measurements of AP-1 in RAW 264.7 cellular extracts. The absorbance and emission profiles of AP-1 were measured in a citrate-phosphate buffer (pH 4.5) or a phosphate-buffered saline (PBS) solution (pH 7.4) containing RAW 264.7 lysates at the indicated amount. RAW cell lysates were diluted and incubated for 1 h at 37 °C prior to each measurement using a citrate-phosphate buffer or a PBS solution. Fluorescence emission signals were recorded at 540 nm (ex. 450 nm). The AEP activity of the cell lysates was measured using the fluorogenic substrate Cbz-Ala-Ala-Asn-AMC (AnaSpec). The fluorescence intensity was monitored on a Spectramax M5 fluorescence plate reader (Molecular Devices).

Direct labelling of endogenous AEP in intact cells and cell lysates. RAW 264.7 cells (350,000 cells/well) and NIH-3T3 cells (350,000 cells/well) were seeded on a 24-well plate 24-30 h prior to labelling. The cells were pretreated with an AEP inhibitor (at 50 μM) for 1.5 h and labelled by the addition of AP-1 or the control probes for 1 h; the final DMSO concentration was maintained at less than 0.2%. The cells were washed with PBS and lysed by the addition of sample buffer. The crude lysates were collected and separated by 12.5% SDS-PAGE. The labelled proteins were analysed by scanning the gel with a ChemiDoc MP imaging system (ex. 466-490 nm/em. 580 nm for green; ex. 520-545 nm/em. 602 nm for red). For lysate labelling, cytosolic lysates of RAW 264.7 cells were prepared as previously described [2]. The lysates were diluted to 1 mg/ml in 50 mM citrate phosphate buffer (pH 4.5) containing 5 mM DTT and subjected to direct labelling. Twenty-five microlitres of the lysate samples were pretreated with inhibitors for 90 min and labeled with probes for another 60 min. The labeled samples were separated by 12.5% SDS-PAGE and analysed by scanning the gel with a ChemiDoc scanner.

Microscopy. Cells were seeded at a density of 100,000 (NIH-3T3 and HT-22) or 200,000 (HCT-116) cells in an eight-well coverslip chamber (Lab-Tek). Twenty-four hours later, the cells were treated with AP-1 or Control 1 (10 μM) for 30 min/1 h/2 h/3 h (NIH-3T3) or 2 h (HCT-116 and HT-22). For the

competition experiment, the cells were pretreated with an AEP inhibitor (50 μM) for 1.5 h before the addition of each probe. After treating cells with 100 nM of LysoTracker red for 5 min, the cells were then washed with PBS three times, and live cell imaging solution (Molecular Probes) was added. The cells were imaged live with an inverted Zeiss LSM 510 confocal microscope with an oil immersion lens (63x) in green and red channels. The mean fluorescence intensities were determined using ZEN software (Carl Zeiss Microscopy), and Pearson's correlation coefficients (Rr) were determined using ImageJ software [3] based on the fluorescence images of individual cells in optical slices from five independent Z-stacks, each containing 10-12 fluorescently labeled cells. The background was subtracted from each image with a manually selected ROI, and the values are reported as the mean ± SD.

[1] J. Lee, M. Bogyo, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 1340.

[2] K. B. Sexton, D. Kato, A. B. Berger, M. Fonovic, S. H. Verhelst, M. Bogyo, *Cell Death Differ.* **2007**, *14*, 727.

[3] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, *Nat. Methods* **2012**, *9*, 676.