Supporting Information

Real-Time Imaging and Quantification of Peptide Uptake *In Vitro* and *In Vivo*

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MATERIALS AND METHODS

Materials and reagents
The peptides r8C (4a, GL Biochem) r4C-c (GL Biochem), TatC-c (Biomatik), PenC-c (Biomatik), HMD2-LP (Aurigene Discovery Ltd.), HMD2-SP (Aurigene Discovery Ltd.), 2-cyano-6-hydroxybenzothiazole (CBT, ENH078, Endotherm GmbH), and succinimidyl 3-(2-pyridylthio)propionate (SPDP, abcr) were purchased from commercial sources and used without further purification. r8C-c, r8C, r4C-c were desalted (VariPure IPE, Agilent) before use. All other reagents were purchased from Sigma-Aldrich and used without further purification. D-cystine was used as a HCl salt. Trifluoroacetic acid (TFA) salts of the SLPs were used for the experiments shown in Figure 3E,F because we found that desalted PenC-c has relatively low aqueous solubility.

Chemistry
RP-HPLC purification of the final peptides (E4C, r8C-c and E4C-c) was achieved using H2O (0.1% formic acid) and acetonitrile (ACN; 0.1% formic acid) as the eluents. The intermediates and the final peptide conjugates were tested for purity (> 95%) using a Waters ACQUITY UPLC Class H instrument using a BEH C18 1.7 μm 2.1×50 mm column with following gradient: 0-2.5 min 0%, 2.5-3.8 min 0% to 98%, 3.80-3.85 min 98%, 3.85-4.0 min 98% to 2% acetonitrile in water containing 0.1% formic acid. HRMS characterization of the final peptides (E4C, r8C-c and E4C-c) was achieved using a Micromass Q-TOF Ultima (Waters Corp., Milford). When suitable, intermediates were characterized with 1H-NMR, 13C-NMR (Bruker Ascend 400 MHz) and LC-MS (ESI+) (Agilent Technologies 6120 Quadrupole LC/MS and Waters Acquity TQD). Preparative HPLC was performed on a Waters HPLC system using an XTerra Prep MS C18 OBD 5 μm 19×50 mm column with a 10-min gradient of 5% to 100% acetonitrile in water containing 0.1% formic acid.

RP-HPLC purification of the final peptides (HMD2-LP-c and HMD2-SP-c) was achieved using H2O (0.1% HCl) and ACN as eluents. The final peptide conjugates were characterized and tested for purity (> 95%) using a Waters Acquity UPLC equipped with a CSH C18 column (1.7 μm, 100×2.1 mm) and a Synapt G1 QTOF MS (Waters Corp., Milford, MA). The final peptide conjugates were characterized and tested for purity (> 95%) using a Waters Acquity UPLC equipped with a CSH C18 column (1.7 μm, 100 × 2.1 mm, Column Temperature = 80°C) and a Synapt G1 QTOF MS (Waters Corp., Milford, MA). The gradient elution was from 5 to 98 % B in
9.4 min. (Eluent A = H2O + 0.05 % TFA and Eluent B = ACN + 0.04 % TFA) with a flow rate equal to 0.5 mL/min.

Chemical shifts were reported in ppm relative to TMS. CD3OD (3.31 ppm) was used as the internal standard for 1H-NMR. CD3OD (49.2 ppm) was used as the internal standard for 13C-NMR spectra.

**Synthesis**

To synthesize the r8C-c and r8C-c (5a and 5b, respectively) used in the study, D-Cys (2) was activated with 2,2-dithiopyridine (1) and then treated with the peptide of interest (4a-4b) modified with a C-terminal L-Cys residue (Schemes S-1-5). To synthetize the two MDH2-p53 PPI inhibitors, HMD2-LP-c and HMD2-SP-c (5c and 5d, respectively), the N-terminus of each peptide (4c-4d) was functionalized with SPDP to obtain the corresponding 3-(pyridin-2-ylthio)propanamidyl derivatives, which quickly reacted with tert-butoxycarbonyl (BOC)-D-Cys to yield the BOC-protected product. BOC deprotection with TFA provided the desired product.

**S-(pyridin-2-ylthio)-D-cysteine (3)**

A method similar to that used earlier was applied1. Briefly, 0.88 g (4 mmol) of 2,2-dithiopyridine was dissolved in 50 ml of dry methanol under a nitrogen atmosphere. To this mixture, a solution of D-Cys (0.12 g, 1 mmol) in 3 ml of water was added dropwise over 1 h, and the reaction was stirred under a nitrogen atmosphere at room temperature for an additional 1 h. Then, the reaction mixture was filtered to remove any precipitate. The filtrate was evaporated, and the remaining crude compound was initially washed with CH2Cl2 and further purified through RP-HPLC (Waters, XTerra Prep MS-C18 OBD™, 5 µm, 19×50 mm), yielding 0.07 g of white powder (30% yield). The values were as follows: HRMS (ESI): m/z calc. for C8H11N2O2S2 [M + H]⁺ 231.0256, found 231.0262; 1H NMR (400 MHz, CD3OD) δ: 8.54 (ddd, 1H, J= 5.1, 1.9, 1.0 Hz), 7.78 (td, 1H, J= 7.7, 1.7 Hz), 7.65-7.58 (m, 1H), 7.30 (ddd, 1H, J= 7.4, 4.9, 1.1 Hz), 3.81 (dd, 1H, J= 10.4, 3.5 Hz), 3.51 (dd, 1H, 14.7, 3.5 Hz), and 3.16 (dd, 1H, J= 14.7, 10.4 Hz); 13C NMR (100 MHz, CD3OD): 172.34, 159.60, 151.04, 139.32, 123.50, 123.20, 54.85, and 41.57.

**r8C-c (5a) (Scheme S-1)**

To a solution of r8C (4a) (20 mg, 8 µmol, TFA salt) in 2 ml of pH 4-5 H2O (pH adjusted with TFA), intermediate 3 (3.8 mg, 16 µmol) in 1.5 ml of pH 4-5 H2O solution was added over 30 min, and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was directly injected into an RP-HPLC column (Zorbax Prep SB-C8, 21.2×250 mm, 7 µm) for purification. Then, the purified compound was desalted (VariPure IPE, Agilent) and lyophilized to
obtain 7.2 mg of rC-c (5a) as a white solid (58% yield). The values were as follows: HRMS (ESI): m/z calc. for [M + 2H]^{2+} 812.4420, found 812.4426; [M + 3H]^{3+} 541.9638, found 541.9644; [M + 4H]^{4+} 406.7247, found 406.7252; and [M + 5H]^{5+} 325.5812, found 325.5817.

**E:C-c (5b) (Scheme S-1)**

To a mixture of E:C (4b) (0.025 g, 0.04 mmol) in 12 ml of a 1:1 mixture of H_{2}O and ACN, intermediate 3 (0.009 g, 0.04 mmol) was added, and the reaction mixture was stirred at room temperature for 1 h before purification through RP-HPLC (Waters, X Terra Prep MS-C_{18} OBD™, 5 \mu m, 19\times50 mm). The values were as follows: m/z calc. for C_{28}H_{43}N_{6}O_{17}S^{2+} [M + H]^{+} 799.2121, found 799.2128.

**E:C (4b) (Scheme S-2)**

The peptide intermediate (4b) was synthesized with Fmoc chemistry using H-Cys(Trt)-preloaded 2-chlorotrityl chloride resin (0.2 mmol). DIC/HOBt was used as the coupling reagent. The peptide was then cleaved from the resin together with removal of all side chains using a cleavage cocktail (TFA:TES:DTT:H_{2}O). The filtrate was evaporated, and the remaining crude compound was washed with ether before purification through RP-HPLC (Zorbax Prep SB-C_{8}, 21.2\times250 mm, 7 \mu m), which yielded 50 mg of white solid (35% yield). The values were as follows: HRMS (ESI): m/z calc. for C_{25}H_{38}N_{5}O_{15}S [M + H]^{+} 680.2080, found 680.2085.

**HMD2-LP-c (5c) (Scheme S-3)**

A solution of HMD2-LP (4c) (20 mg, 0.011 mmol) in DMF (3 ml) was added dropwise to a solution of SPDP (4.83 mg, 0.015 mmol) and DIPEA (14.46 \mu l, 0.083 mmol) in ACN (3 ml). The reaction was stirred at room temperature for 3 h. BOC-D-Cys (0.181 M in ACN) (91 \mu l, 0.017 mmol) was added to the stirring reaction mixture, which turned yellow. The reaction mixture was stirred at room temperature for 1 h and then evaporated to dryness. Next, 95% TFA (500 \mu l, 6.17 mmol) was added, and the reaction was stirred for 1 min at room temperature before purification through RP-HPLC (Waters, SunFire Prep C_{18} OBD™, 5 \mu m, 50\times150 mm). The purified compound was lyophilized to obtain 8.1 mg of HMD2-LP-c (5c) as a white solid (39% yield). The values were as follows: HRMS (ESI): m/z calc. for C_{82}H_{115}F_{3}N_{19}O_{19}S_{2} [M + H]^{+} 1790.8005, found 1790.8075.

**HMD2-SP-c (5d) (Scheme S-4)**

A solution of HMD2-SP (4d) (22 mg, 0.014 mmol) and DIPEA (22.04 \mu l, 0.126 mmol) in DMF (2 ml) was added to a solution of SPDP (7.3 mg, 0.023 mmol) in ACN (4 ml) at room temperature. After 3 h, the reaction was recharged with a solution of SPDP (2.1 mg, 6.72 \mu mol) in ACN (0.07 ml) and stirred for 2 h. A solution of BOC-D-Cys (0.2 M in ACN) (155 \mu l, 0.031 mmol) was added,
and the reaction was allowed to proceed for 10 min at room temperature before being evaporated to dryness. Next, 95% TFA (500 µl, 6.17 mmol) was added to the crude material, and the deprotected compound was stirred for 10 min before purification through RP-HPLC (Waters, SunFire Prep C18 OBD™, 5 µm, 50×150 mm). The purified compound was lyophilized to obtain 6.2 mg of HMD2-SP-c (5d) as a white solid (26% yield). The values were as follows: HRMS (ESI): m/z calc. for C_{79}H_{114}ClN_{14}O_{19}S_{2} [M + H]^+ 1661.7509, found 1661.7634.

**Cell lines and culture**

MDA-MB-231-luc breast cancer cells were acquired from PerkinElmer (the full abbreviation for this cell line is MDA-MB-231-luc-D3H2LN). The cells, which carried regular firefly luciferase enzyme (luc), were cultured in alpha MEM supplemented with 1% Glutamax. U87MG-luc cells were cultured in EMEM and carried Luc2 luciferase. BT-474-luc cells were cultured in RPMI 1640 and were manually transfected with red luciferase (the full abbreviation for this cell line is BT-474 Red Luc) see section below for more details). All the cell media contained 10% FBS and a 1% penicillin/streptomycin mixture. Cells were seeded at a density of 1×10^4 cells/well (MDA-MB-231-luc and BT-474-luc) or 1.5×10^4 cells/well (U87MG-luc2) for the uptake experiments.

**Transduction of the BT-474 cell line**

BT-474 cells in RPMI-1640 medium were transduced with RediFect Red-FLuc-Puro lentiviral particles (PerkinElmer) for 24 h. The medium was then replaced with fresh RPMI-1640 medium, and cell culture continued. Then, the transduced cells (1.5×10^6) were seeded in a 25 cm^2 cell culture flask in RPMI-1640 containing 2 µg/ml puromycin (Calbiochem). The cells were maintained under puromycin stress for a week, and the medium containing 2 µg/ml puromycin was renewed every other day.

**Cell-free enzymatic assay**

The assay was performed in Tris buffer (pH 8.5) using a 96-well flat-bottom black/clear tissue culture plate (BD Falcon). To 100 µl of solution containing the test compound (0.65 nmol), 15 µl of 5 mM GSH, 15 µl of 86.7 µM CBT (1.3 nmol) and 20 µl of luciferase mixture was added before the bioluminescence signal was read with a Tecan Infinite M-1000 plate reader (Tecan) at 37 °C. The luciferase mixture was composed of 22.5 µl of 2 mg/ml firefly luciferase (*Photinus pyralis*, Sigma), 3 µl of 100 mM ATP, 3 µl of 240 mM MgSO_4 and 273 µl of Tris buffer (pH 8.5).
LCMS characterization of D-cysteine labelled peptides

E4C-c (5b)
HMD2-LP-c (5c)

HMD2-SP-c (5d)
Supplementary figures, schemes and tables

**Supplementary Figure 1** | Structures and sequences of the peptides used in the uptake studies.
To simplify the nomenclature, one letter abbreviations of amino acids are used as follows: L- and D-amino acids are represented with uppercase and lowercase letters respectively (e.g. C = L-Cys, c = D-Cys, r = D-Arg). PenC-c and TatC-c are full Penetratin and Tat peptides linked to D-Cys.
through a C-terminal L-cysteine residue. HMD2-SP-c and HMD2-LP-c are p53-HMD2 PPI peptide inhibitors which are labeled with D-Cys through an N-terminal 3-mercaptopropionate group (3-MPA). X in the peptide sequence represents the hydrocarbon stapling linkage (see the structure).

Supplementary Figure 2  | In vitro validation of SLP uptake assay. (A) Experimental design of the cell-free luciferase assay. Glutathione (GSH), CBT, luciferase enzyme with co-factors ATP and MgSO₄ were added to a solution of r₈C-c and the resulting bioluminescent signal was monitored with a plate reader. (B) Comparison of total bioluminescent signal from r₈C-c (4.3 µM) with D-cystine (2.15 µM) or D-Cys (4.3 µM) in the cell-free luciferase assay. To a solution of each probe, GSH, CBT and a luciferase mixture were added, and bioluminescent signal was monitored with a plate reader for 120 minutes. Unpaired two-tailed Student's t-test was used for the statistical analysis. (C) Monitoring total peptide uptake in live breast cancer cells (MDA-MD-231-luc-D3H2LN) stably expressing luciferase. Total bioluminescent signal over 3 h is shown as calculated from AUC of real-time kinetic curves in Figure 2C. (D) Comparison of r₈C-c with negative control r₈C, which lacks the D-Cys modification. Cells were pre-incubated with CBT (50 µM) for 30 minutes followed by addition of the SLP (10 µM). (E) Representative images of cells 20 min after peptide addition. Error bars are represented as ± SD.
Supplementary Figure 3 | The canonical pathway for the SLP uptake assay and D-luciferin formation is shown. Extracellular and endogenous L-cysteine (L-Cys) condensation with CBT form L-luciferin, which subsequently undergoes racemization in the presence of firefly luciferase and causes background bioluminescence (red frame).

Supplementary Figure 4 | Bioluminescent signal from MDA-MB-231-luc cells treated with different concentration of r8C-c peptide. Real-time kinetic curves for 0.63–10 µM concentrations are shown. Error bars are represented as ± SD.
Supplementary Figure 5 | Comparison of imaging total SLP uptake using an IVIS spectrum (A) and a plate reader (B) under the same conditions. Cells (MDA-MB-231-luc-D3H2LN) were pre-incubated with CBT (50 µM) for 30 minutes followed by addition of the SLP (10 µM) and imaging with IVIS spectrum or plate reader.

Supplementary Figure 6 | Comparison of various cell permeable peptides for their total uptake in MDA-MB-231-luc-D3H2LN cells at different time points as calculated from AUC of real-time kinetic curves in Figure 3E. Error bars are represented as ± SD.
Supplementary Figure 7  |  Comparison of intra-cellular uptake of different cell permeable peptides octaarginine (r8C-c), TAT (TatC-c), Penetratin (PenC-c)) in various live cell lines using SLP assay. Real-time bioluminescent signal resulting from octaarginine, TAT and Penetratin SLP for their uptake in (A) U87MG and (B) BT474 luciferase-expressing cell lines, respectively. Error bars are represented as ± SD.

Supplementary Figure 8  |  Application of the SLP assay for the assessment of cellular permeability of p53-HDM2 PPI peptide inhibitors, HMD2-LP-c and HMD2-SP-c. SJSA-1-luc cell were pre-incubated with CBT (50 µM) for 30 minutes followed by addition of the therapeutic peptides at (A) (1 µM) and (B) (20 µM). The total uptake was calculated from the area under the corresponding kinetic curves (AUC) at 24 h post incubation.
Table S1: Rate constants for octaarginine, TAT peptide and penetratin.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Rate constant [ph/s²/cm²/sr]</th>
</tr>
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<tbody>
<tr>
<td>r8C-c</td>
<td>0.01589</td>
</tr>
<tr>
<td>TatC-c</td>
<td>0.01024</td>
</tr>
<tr>
<td>PenC-c</td>
<td>0.02419</td>
</tr>
</tbody>
</table>

Supplementary Scheme 1: General synthesis of SLPs (5a-5b).

Supplementary Scheme 2: Synthesis of E4C-c.
Supplementary Scheme 3: Synthesis of HMD2-LP-c
Supplementary Scheme 4: Synthesis of HMD2-SP-c