

Supplementary Information

In-tether chiral center induced helical peptide modulators target p53-MDM2/MDMX and inhibit tumor growth in stem-like cancer cell

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Methods

1. Peptide Synthesis

All peptides were synthesized by manual Fmoc-based solid-phase synthesis. The intramolecular thiol-ene reactions were conducted via the method reported in previous literature.[1-3] The resultant cyclic diastereomers were separated by HPLC. The purified peptides were detected by ESI/LC-MS and the pure fractions were combined and then lyophilized. The detailed synthesis route for PhR was attached to **Supplementary Scheme 1**, other peptides synthesis in this article is similar to this route.

2. Sphere formation assay

Cells were collected and washed to remove serum and then suspended in serum-free MEM supplemented with 20 ng/ml human recombinant epidermal growth factor (hrEGF), 10 ng/ml human recombinant basic fibroblast growth factor (hrbFGF), 5 µg/ml insulin, and 0.4% BSA. The cells were subsequently cultured in ultralow attachment 6-well plates at a density of less than 5000 cells/well for 14 days. Spheres were observed under a microscope and images were photographed under a phase contrast fluorescence microscope[4].

2. Protein production

Human MDM2 LBD residues 25-117 were cloned into pGEX-4t-1 *via* EcoRI and XhoI to generate GST-tagged constructs. Expression was carried out in *E. coli* BL21 (DE3) and was induced with

0.1 mM IPTG. Cultures were grown in LB medium at 37 °C to an OD₆₀₀ of 0.6 before being transferred to 18 °C for 24 hr. Cells were harvested by centrifugation and flash frozen. Harvested cells were lysed by sonication in lysis buffer (20 mM Tris-Cl pH 7.9, 500 mM NaCl). Cell debris was removed by centrifugation and the supernatant was purified on a 5mL GST affinity column (GE healthcare) and eluted with elution buffer (10 mM GSH in 20 mM Tris-Cl pH 7.9, 500 mM NaCl). The protein was further purified with a Superdex 200 column equilibrated in 20 mM Tris-Cl pH 7.9, 500 mM NaCl, 1 mM DTT.

Primer sequences:

MDM2-EcoRI-25: CCGGAATTCGAGACCCTGGTTAGACCAAA

MDM2-XhoI-117 : GTAGGCACTCGAGTCAGTCCGATGATTCCT

3. Flow Cytometry.

1) Transfection efficiency. For the flow cytometry of transfection efficiency experiments, cells were treated with fluoresceinated peptides (5 μ M) for up to 4 hours at 37°C. After washing with media, the cells were digested with trypsin (0.25%; Gibco) digestion (2 min, 37°C), washed with PBS, and resuspended in PBS. Cellular fluorescence was analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson) and CellQuest Pro (or CFlow plus). 2) Cell cycle. For the flow cytometry of cell cycle arrest experiments, cells were treated with peptides and Nutlin-3a for 48 hours. Subsequently, cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization. The cells were fixed with cold 70% ethanol for 4 hours and then underwent centrifugation at 2000 rpm for 5 min to remove the ethanol. Then the fixed cells were dispersed in PBS with 1% Triton-100, 1 mg/mL RNase and 5 mg/mL PI, stained at 37°C for 30 min (double fixation was not needed for this experiment). The samples were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Mississauga, CA). The percentages of cells in G1, S, and G2/M phases were determined by FlowJo software. 3) Apoptosis assay. The apoptosis assay was conducted using the FITC Annexin-V/PI Apoptosis Detection Kit I (BD Pharmingen™) according to the manufacturer's instructions. Approximately 1×10^6 cells were seeded in a six-well plate. Then, the cells were treated with the peptides and nutlin-3a and incubated for 48 hours. The cells were then collected and washed twice with cold PBS and suspended in binding buffer. The induction of the apoptosis process enables the FITC-labeled Annexin-V to bind with phosphatidylserine (PS) since it appears on the outer surface of the cell membrane at the onset of apoptosis, and is confined to the inner boundary of the cell membrane in healthy cells. The cell nuclei were stained with propidium iodide (PI). The stained cells were analyzed by flow cytometry to distinguish the apoptotic cells. The cells with positive fluorescent intensity signals for both FITC and PI were representative of the apoptotic cell count. The extent of apoptosis was measured through the detection of caspase-3 activity by exposing the cells to a caspase-3-specific substrate (Oncogene). Fluorescence as a result of substrate cleavage was measured in a Spectramax M5 microplate reader (Molecular Devices).

4. Preparation of paraffin section histological analysis (IHC)

For histological experiments, organ tissues were collected on the final day treatment and fixed in 4(wt/vol) % buffered formalin-saline at room temperature for 24 hours. Following this, tissues were embedded in paraffin blocks and 4 mm thick paraffin sections were mounted on a glass

slide for hematoxylin and eosin (H&E) staining. The H&E staining slices were examined under a light microscopy (Olympus BX51).

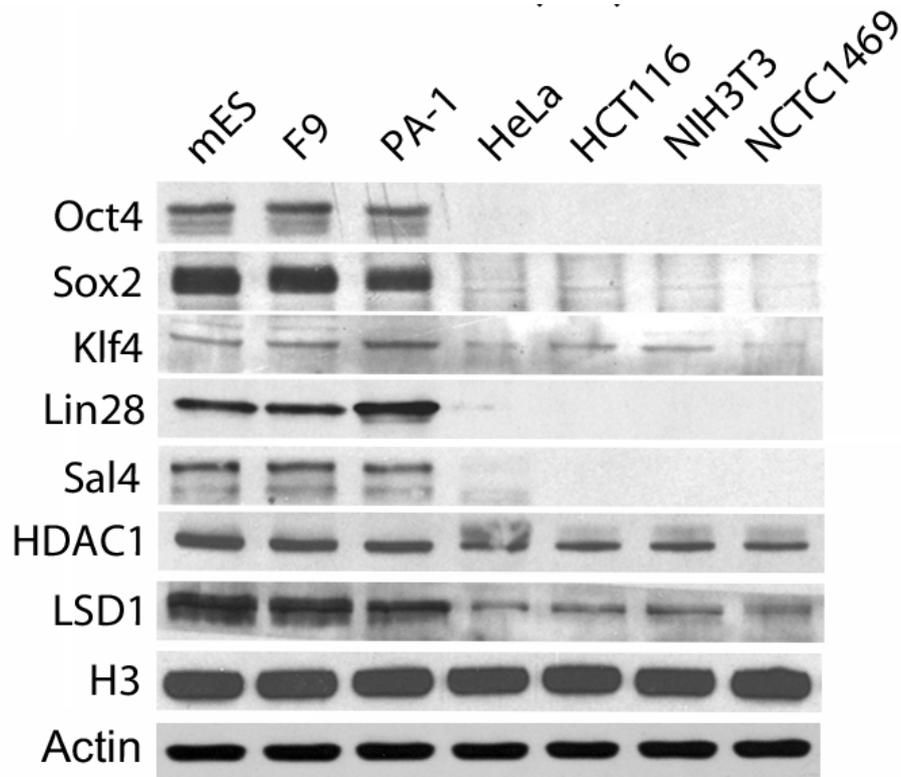
5. Mice voluntary cage-wheel exercise

BALB/c mice (female; 6 weeks old) were obtained from Vital River Laboratory Animal Technology Co. Ltd. of Beijing, People's Republic of China and allowed an acclimation period of 1 week at 22 ± 2 °C with a 12-hour light: dark cycle (lights on at 8am, lights off at 8pm). Subsequently, BALB/c mice were randomly divided into 2 groups (3-4 mice per group) and were subcutaneously injected with PBS or peptide (10 mg/kg). Voluntary running was performed by these two groups at the start of exercise following reported protocol. A voluntary running system consisting of six separated chambers (Chengdu TME Technology Co., Ltd, China) was used in the animal performance study. During the training session, mice were placed on the motorized rod (30 mm in diameter) in the chamber. The rotation speed gradually increased from 0 to 100 rpm over the course of 100s. The rotation speed was recorded when the animal fell off from the rod. Each rotarod training session consisted of 7 trials and lasted around 7 minutes. Performance was measured as the average rotation speed animals achieved during the training session. The two different groups were all trained at the same five continuous time points (day 0, day 2, day 4, day 8, day 12, day 16 and day 20). No significant differences were found between the two groups.

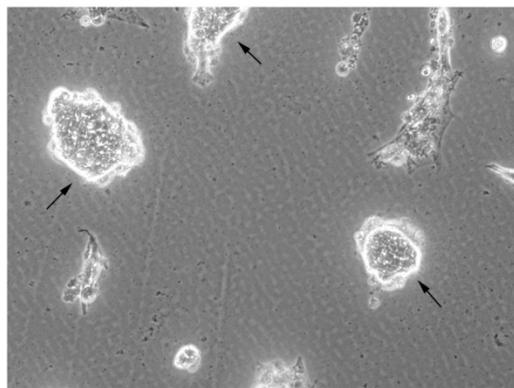
6. In vivo imaging

When the tumor reached an appropriate volume of 200–300 mm³, the mice were injected with 100 µL of Cy3-labeled PhR peptide by intratumoral injection. After injection, mice were anesthetized with isoflurane. The induction concentration was 5% isoflurane/1L O₂, and the maintenance concentration was 2–3% isoflurane/1L O₂. Once the mice were properly anesthetized, they were imaged at indicated time points to monitor the metabolization of PhR^{Cy3} peptide in tumors using the IVIS LuminaII small animal in vivo optical imaging system (Caliper). In this study, a scanning wavelength ranging between 500 and 950 nm was used for in vivo imaging.

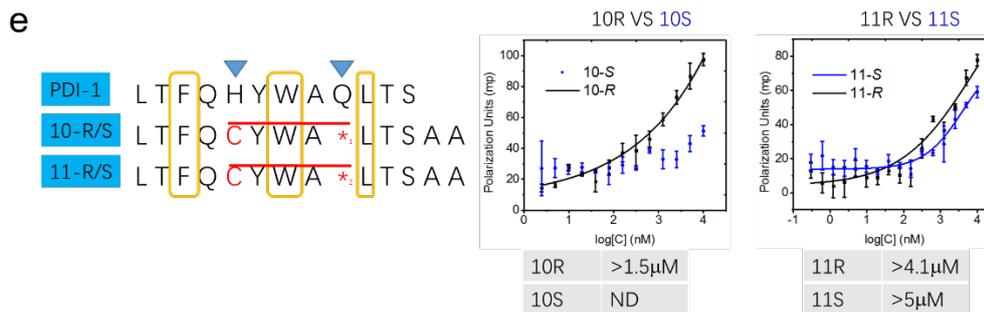
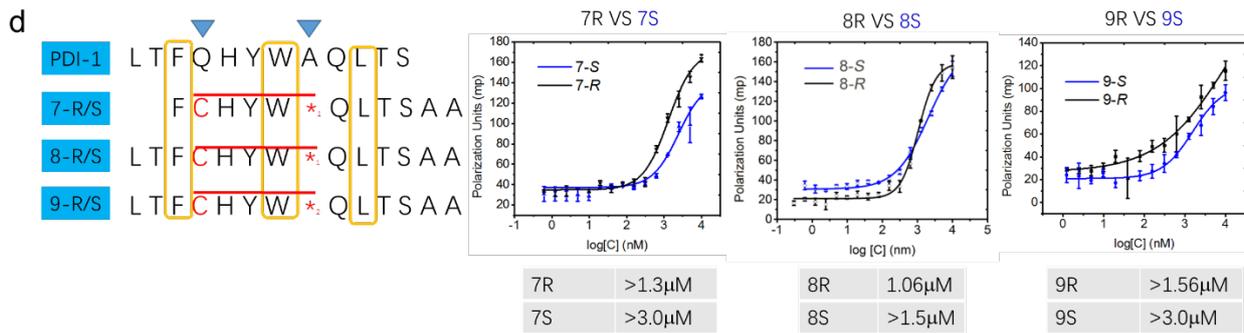
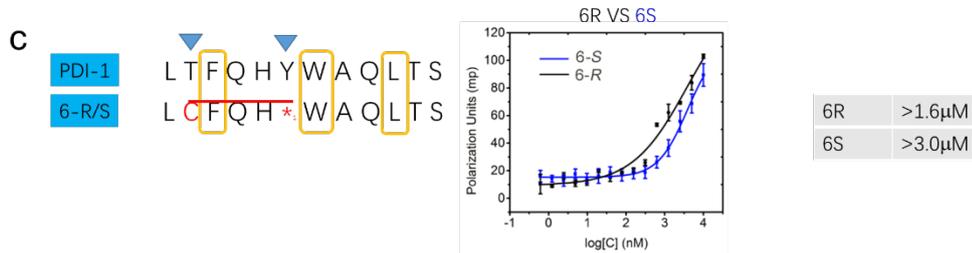
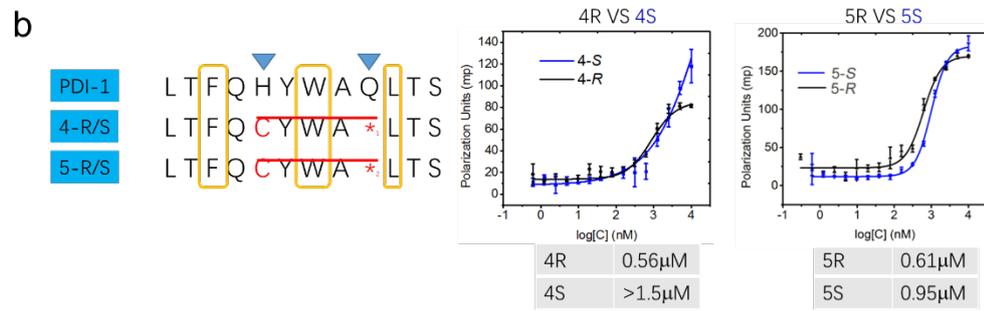
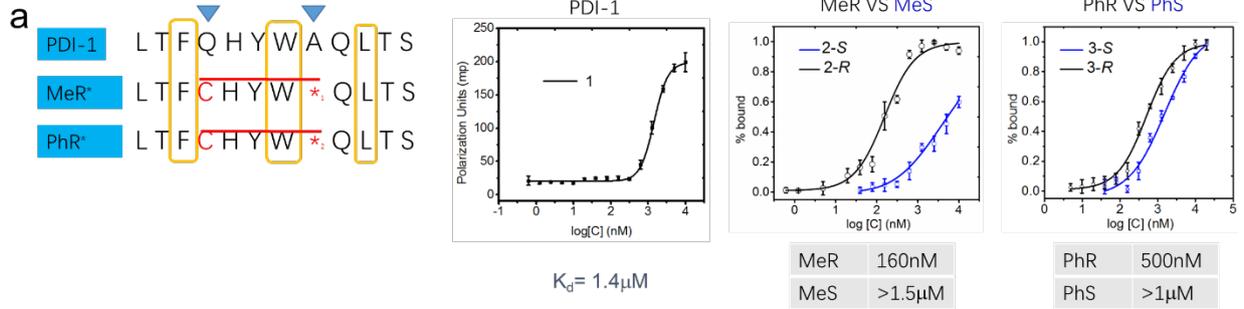
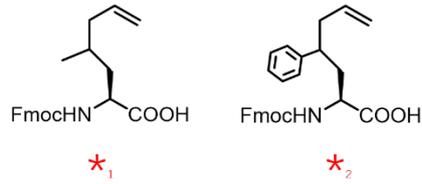
Figures and Figure legends

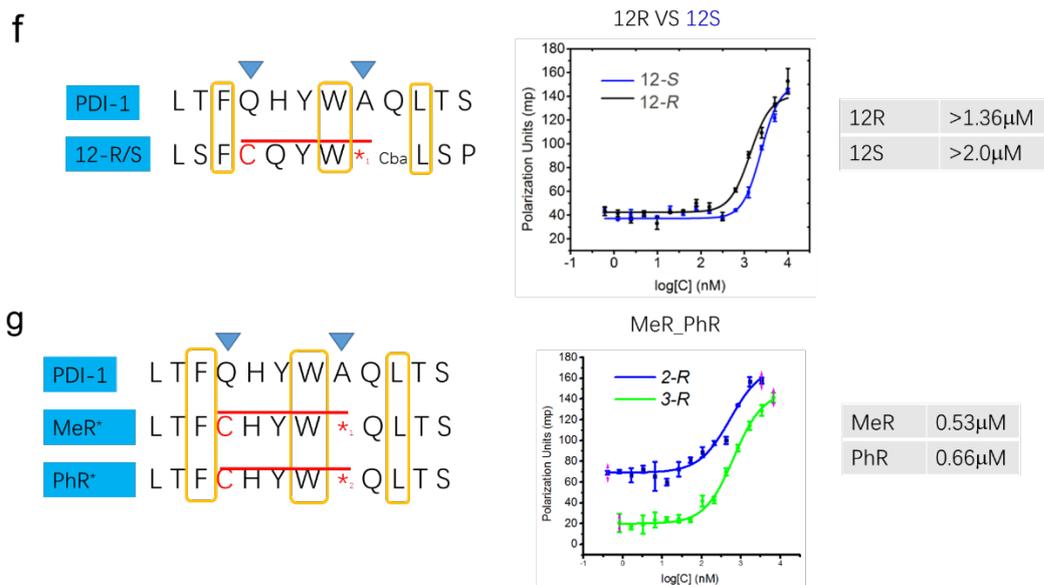


Supplementary Figure 1. The levels of the HDAC1, HDAC2, HDAC3, and HDAC6, CoREST, and LSD1 proteins were compared between the various cell lines by western blotting, as indicated. Histone H3 and Actin were used as the protein loading control.

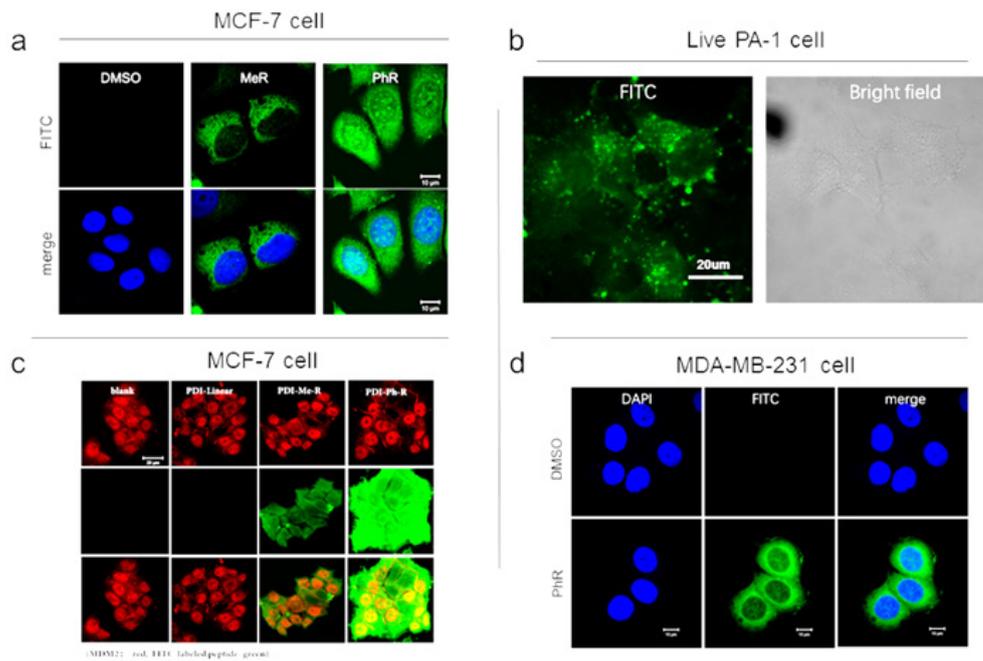


Supplementary Figure 2. The sphere images of PA1 cell colonies. The sphere images were taken on day 14 by a phase contrast microscope.

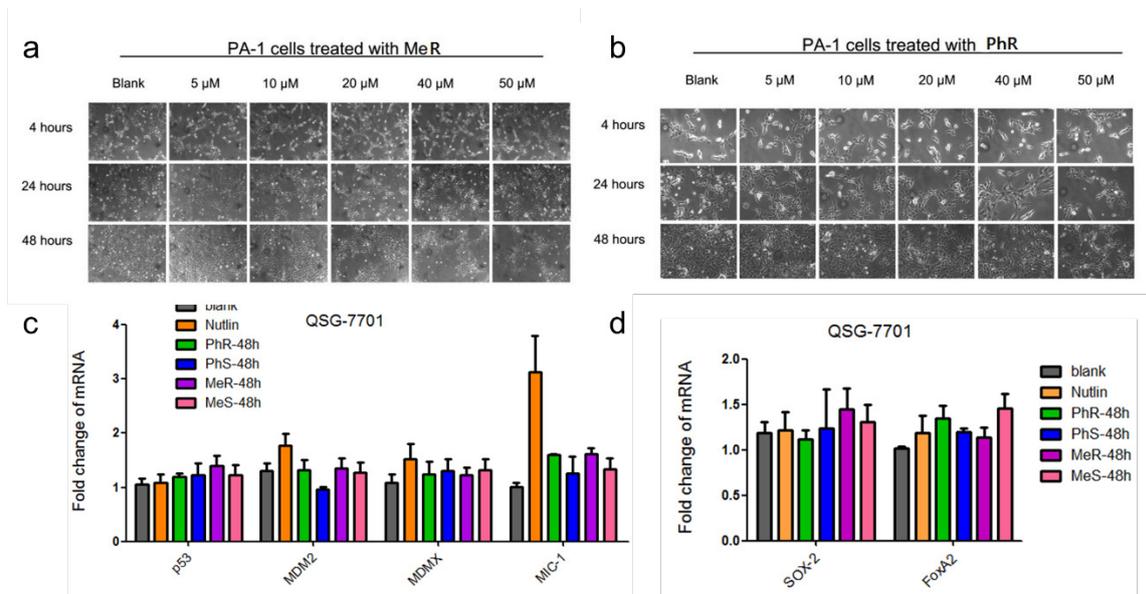




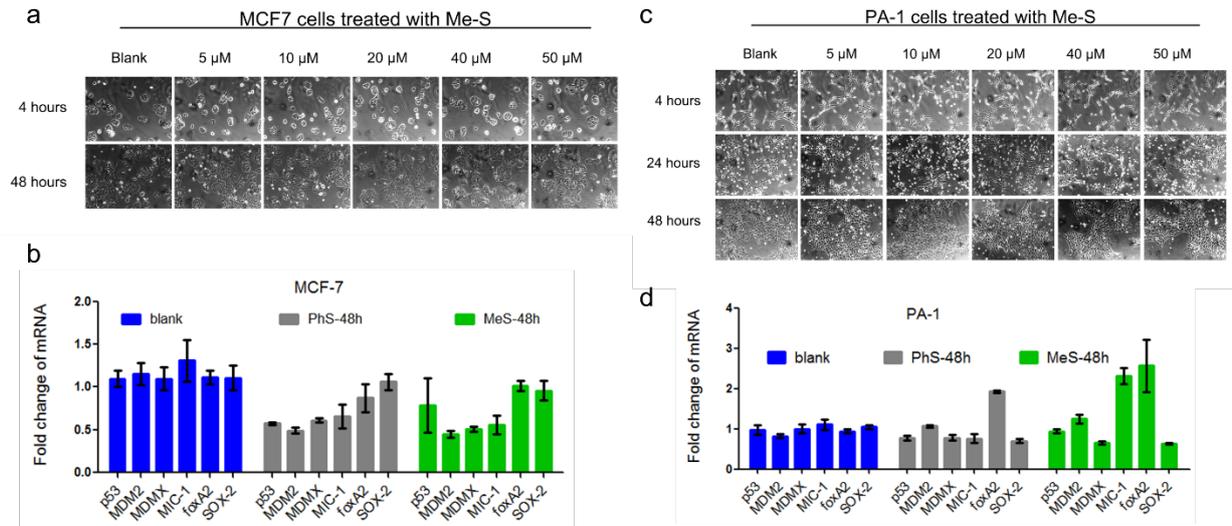
Supplementary Figure 3. Fluorescence polarization assay to determine the K_d of fluorescein-labeled peptides 1-12 bind to protein MDM2/X. Data were demonstrated the S diastereomers have higher K_d value compared to R diastereomers of each peptide. MeR(2R) and PhR(3R) were screened to show the best affinity to MDM2/X. ‘a’ represents the S diastereomer peptides, ‘b’ represents the R diastereomers, respectively. (a-f) The binding curve of peptide 1-12 binds to MDM2. The blue triangle indicates the positions for cyclization. From the fluorescence polarization data, we found the original peptide length is optimized for binding, either adding (8-11 compared to 2-5) or minus (7 compared to 8) amino acid(s) would decrease the binding ability. The best position to deposit the unnatural amino acid S₅(Me or Ph) is position of Ala in PDI. (g) The K_d values of MeR/PhR binding to MDMX were measured. K_d tendency fit well with previous reports and K_d variations were caused by different protein expression systems. Notes: The orange column indicated the key residues (also called ‘hot spots’) for PDI peptides binding to MDM2/X. The red letters indicate the residues used for cyclization. The inverted triangle indicates the positions for cyclization.



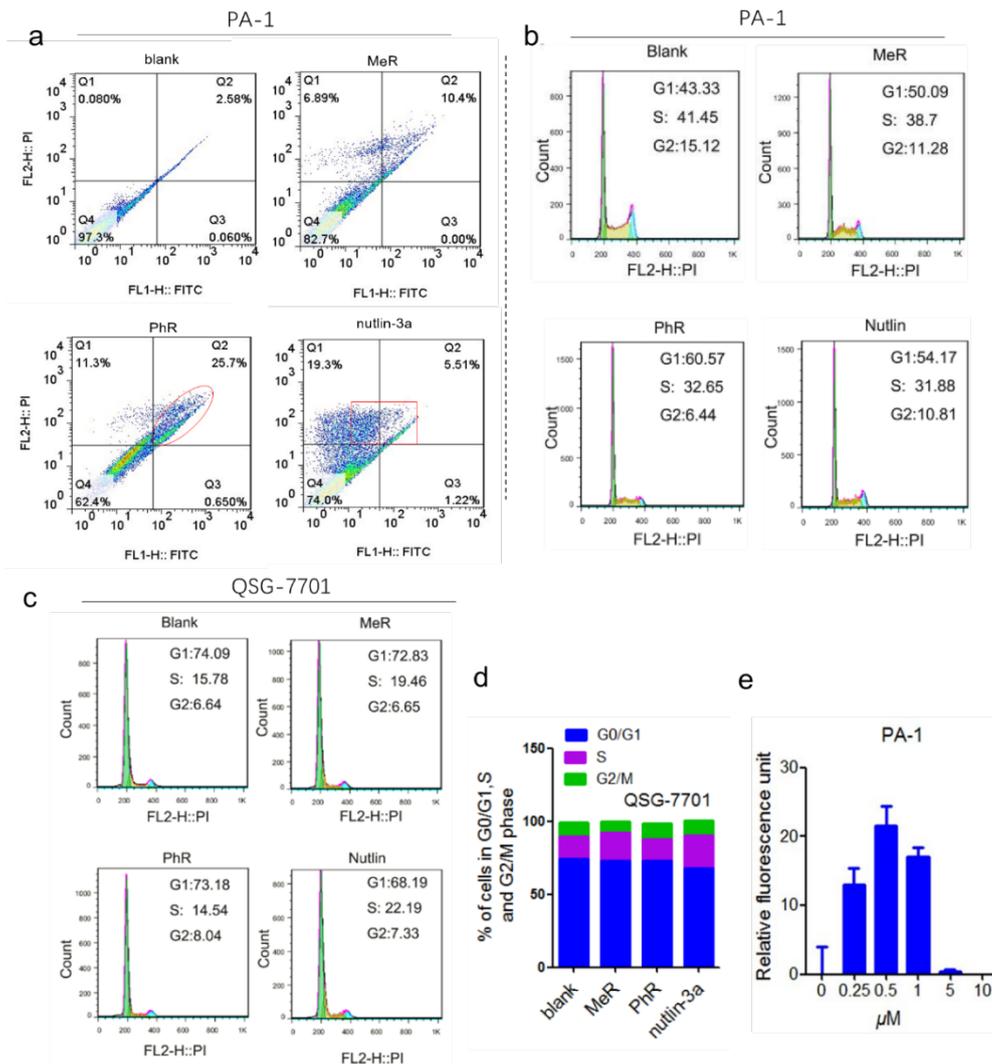
Supplementary Figure 4. (a) Confocal microscopy images of FITC-labeled peptide incubated with MCF-7 cells for 2hours at 37°C. DAPI(blue), FITC(green). (b) The live cell confocal microscopy images of PhR in PA-1. 5µM of PhR was incubated with PA-1 cells for 2 hours at 37°C, then washed with PBS and observed in Zeiss image system. (c) Immunofluorescence imaging demonstrated FITC-labeled peptides' uptake by MCF-7 cells and that they co-localization to the MDM2 protein in cell nucleus. MDM2 was stained with Alexa Fluor 647-labeled antibody(red). (d) Confocal microscopy images of FITC-labeled peptide incubated with MDA-MB-231 cells for 2hours at 37°C. DAPI(blue), FITC(green).



Supplementary Figure 5. (a, b) Bright field images of PA-1 cells treated with MeR/PhR showing a time and dose-responsive effect to cell death. Apparent cell death was observed in PA-1 cells when incubated with 50 μ M MeR or PhR for 48 h. (c, d) The mRNA expression level of QSG-7701 cells after treated with peptide modulators. Exponentially growing QSG-7701 cell line was incubated with 40 μ M peptides or 1 μ M nutlin-3a for 48 h, and mRNA level of p53 and p53 targets MDM2, MDMX, and MIC or stemness-related genes were analyzed by quantitative PCR and expressed as fold increase. No obvious changes were observed in these genes except MIC-1 in the nutlin-3a group.

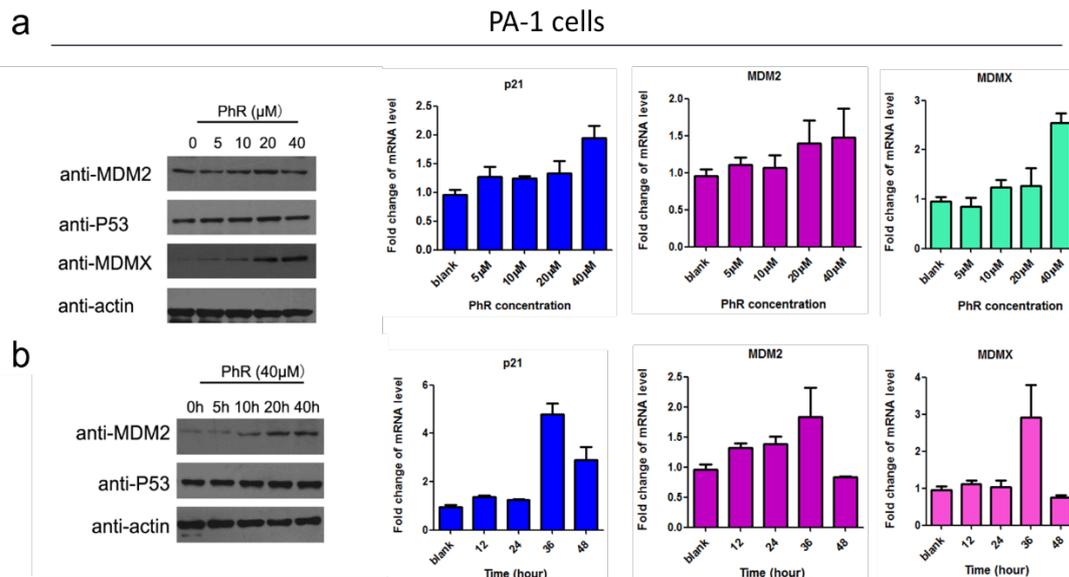


Supplementary Figure 6. Cell viability and mRNA expression levels of MCF-7 and PA-1 cells treated with PhS or MeS peptides. (a, c) The bright field images of MCF-7 or PA-1 cells were treated with MeS with a time and dose ladder. The proliferation of cells shows no obvious changes with increasing concentration. This is consistent to the cell viabilities in Figure. 2a. (b, d). Exponentially growing p53wt cancer cell lines were incubated with peptides MeS/PhS(40 μ M) for 48 hour, the p53 and p53-related genes expression levels were analyzed by quantitative PCR and expressed as fold increase. Error bars represent SEMs for triplicates of the data.

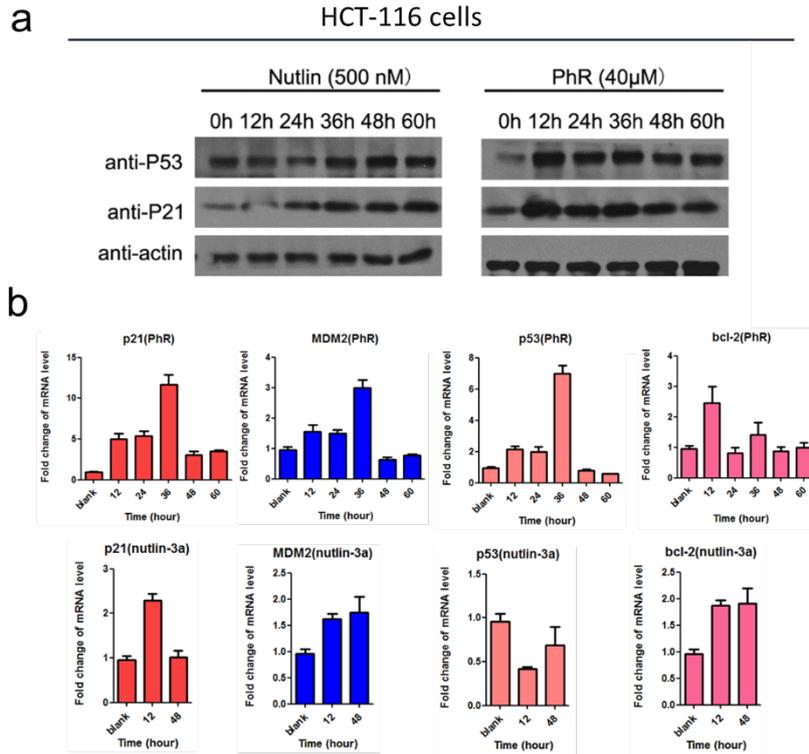


Supplementary Figure 7. (a) PA-1 cancer cell lines were treated with MeR, PhR (40 μ M) and nutlin-3a (5 μ M) for 48h. The percentage of apoptotic cells was determined through the PI/Annexin V double stain assay, the red cycles showed PhR induced higher apoptosis in both cells than MeR or nutlin-3a. The red rectangle showed higher percent of cell death when treated with nutlin-3a. (b) The cell cycle distribution was determined through PI staining in PA-1 cells after treated with MeR, PhR (40 μ M) or nutlin-3a (5 μ M) for 48hours. The flow cytometry data was analyzed with Flowjo software. The green zone represents cells in G1 phase, yellow in S phase and cyan in G2 phase. The numbers mean percent (%) in all cells. (c) The cell cycle data of QSG-7701 cells which treated with different compounds analyzed with flow cytometry. The ratio of each cell cycle phase was labeled. QSG-7701 Cells were incubated with peptides for 48hours and stained with PI. The flow cytometry data was analyzed with Flowjo software. The green zone represents cells in G1 phase, yellow in S phase and cyan in G2 phase. The numbers mean percent (%) in all cells. (d) Column graph to show the distribution of cell-cycle phase for QSG-

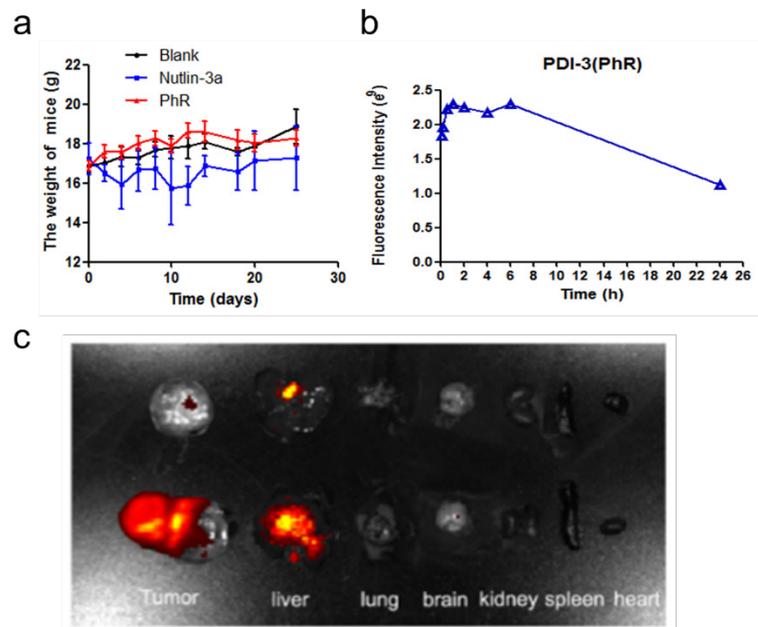
7701 after treated with MeS or PhS. These results showed MeS and PhS (40 μ M) had little effect to cell cycle arrest. (e) Caspase-3 activity in PA-1 cells treated with nutlin-3a.



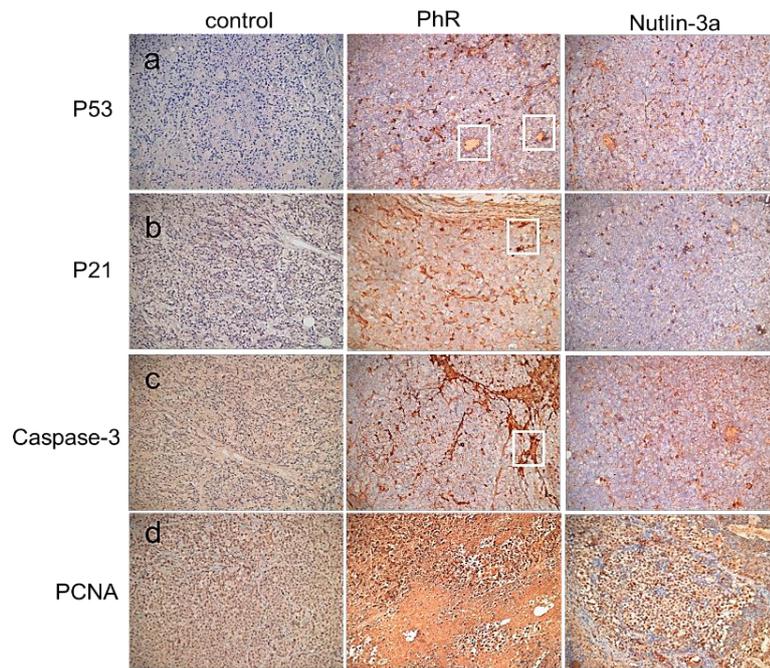
Supplementary Figure 8. PhR reactivates the p53 pathway in a time- and dose-dependent manner. (a) PhR elevates p53 and p53 targets MDM2/X protein level and shows induction of the p53 target genes p21 and MDM2 in a dose-dependent manner in PA-1 cells. Log-phase PA-1 cells were exposed to 5, 10, 20, 40 μM PhR for 48 hours and cell lysate were analyzed by western blotting. Exponentially growing p53wt cancer cell line PA-1 was incubated with 5, 10, 20, 40 μM PhR for 48 hours for quantitative PCR. (b) PhR elevates p53 protein and p21 protein levels in a time-dependent manner. The mRNA levels of p21 and MDM2 changed as the incubation time changed. For western blotting, log-phase PA-1 cells were incubated with 40 μM PhR for 5h, 10h, 20h and 40h and cell lysate were analyzed by western blotting. For quantitative PCR, PA-1 cells were incubated with PhR for 12, 24, 36 and 48 h.



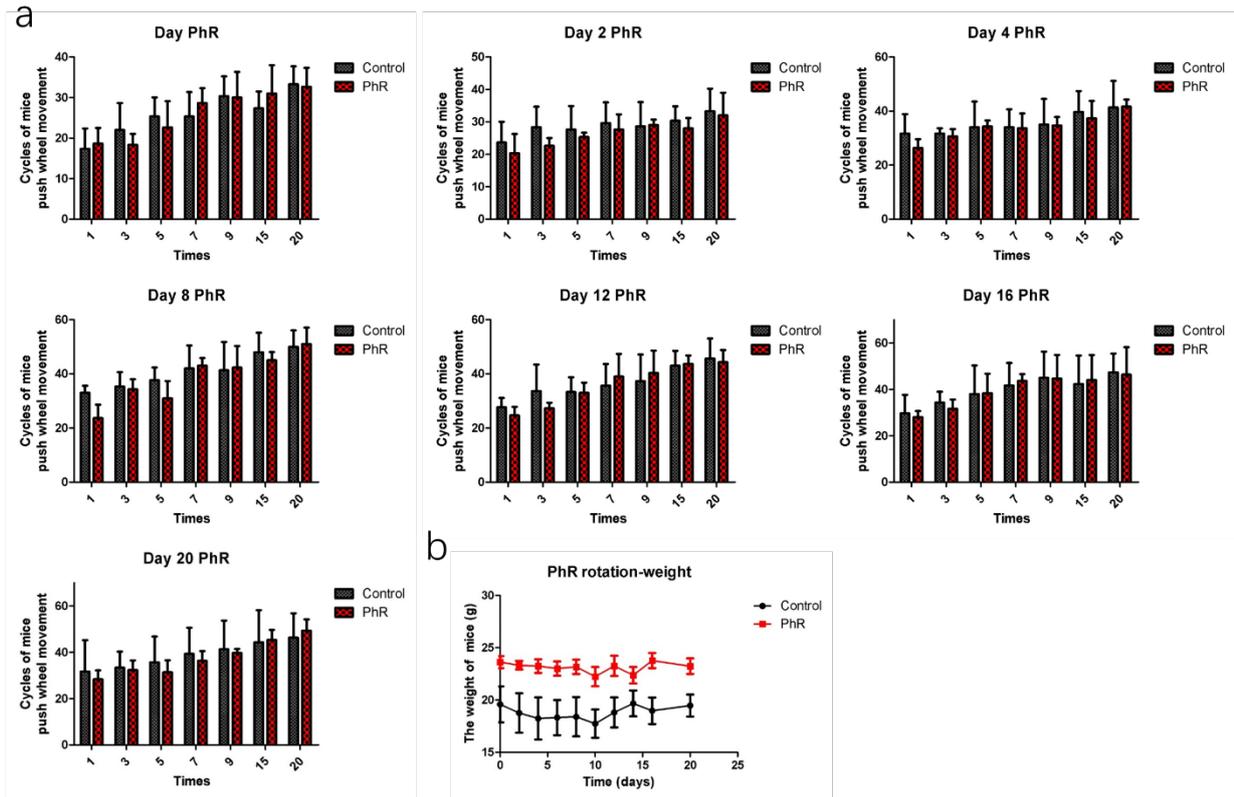
Supplementary Figure 9. (a) Transfected HCT-116 cells treated with nutlin-3a(0.5 μ M) and PhR(40 μ M), changes in the protein level expression in a time-dependent manner were detected. (b) The P53 and p53-related genes expression level in HCT-116 (p53-VKI) cells at different time points when treated with PhR(40 μ M) or nutlin-3a(5 μ M). The HCT-116 Venus knock-in cell line (p53-VKI) was a kind gift from the Lahav laboratory.



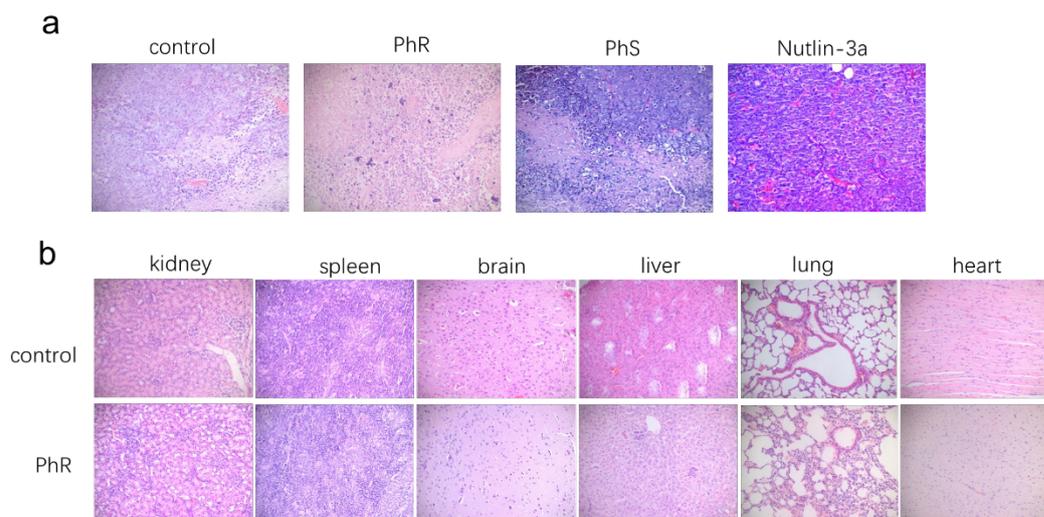
Supplementary Figure 10. (a) The weight of the mice was measured during the treated time schedule. (b) The fluorescence signal intensity quantified from Figure 5E at different time points. Half percent of intensity retained after 24 h injection. (c) The major organs' distribution of PhR-Cy3 after intratumoral injection for 24hour.



Supplementary Figure 11. Immunohistochemistry analysis of p53 and p53-related protein level in tumor tissue slides. Teratocacinoma tissues collected from different groups of mice after 3weeks treatment. (a) p53 (b) p21 (c) caspase-3 (d) PCNA were apparently elevated in the PhR treated mice. The white rectangle in the slides indicates respective proteins. MicroSpot Focusing Objective, 20X.



Supplementary Figure 12. PhR shows high biocompatibility and low toxicity *in vivo*. (a) The cage-wheel exercise assay was used to study the motor learning ability of mice. BALB/c mice were randomly divided into two groups and were subcutaneously injected with PBS or PhR (10 mg/kg). The cycle of mice push wheel in day 20 was no obvious different between control group and PhR group, which indicated no obvious effect of motor learning ability of mice treated with PhR. (b) The mice weight kept steady in 20days after injection. The error bars represent the standard derivations (3 mice per group).



Supplementary Figure 13. H&E stained tumor sections and organs collected from different groups of mice 3 weeks after treatment. (a) H&E stained tumor sections collected from different groups of mice 3 weeks after treatment. MicroSpot Focusing Objective, 20X. (b) Organs collected from two groups of mice (PBS or PhR) 3 weeks post treatment. No obvious organ hurts were observed. MicroSpot Focusing Objective, 20X.

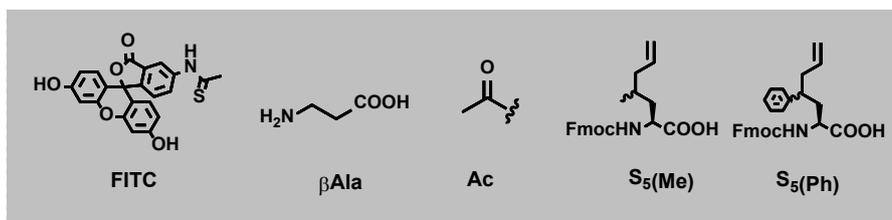
Supplementary Tables

Supplementary Table 1. Entry 2 to 11 were designed with different tether positions, tether type, and mutations at indicated positions. K_d was measured with fluorescence polarization assays using FITC-labeled peptide R epimers with MDM2 and MDMX. * means the sequences was reported in our previous paper. The relatively low binding affinity may be caused by different MDM2/MDMX expression system, as both the CIH peptide MeR and PhR showed a greater binding affinity than the linear peptide PDI-1, which was reported as 10nM in reference 39 but measured as 1 μ M with proteins expressed in our lab.

Entry													$K_d(\mu M)$			
													MDM ₂	MDM _x		
1	L	T	F	Q	H	Y	W	A	Q	L	T	S	NH ₂	1.40	ND	
2*(MeR)	L	T	F	C	H	Y	W	S ₅ (Me)	Q	L	T	S	NH ₂	0.16	0.53	
3*(PhR)	L	T	F	C	H	Y	W	S ₅ (Ph)	Q	L	T	S	NH ₂	0.50	0.66	
4R	L	T	F	Q	C	Y	W	A	S ₅ (Me)	L	T	S	NH ₂	0.56	ND	
5R	L	T	F	Q	C	Y	W	A	S ₅ (Ph)	L	T	S	NH ₂	0.61		
6R	L	C	F	Q	H	S ₅ (Me)	W	A	Q	L	T	S	NH ₂	>1.60		
7R			F	C	H	Y	W	S ₅ (Me)	Q	L	T	S	A A	NH ₂		>1.31
8R	L	T	F	C	H	Y	W	S ₅ (Me)	Q	L	T	S	A A	NH ₂		1.06
9R	L	T	F	C	H	Y	W	S ₅ (Ph)	Q	L	T	S	A A	NH ₂		>1.56
10R	L	T	F	Q	C	Y	W	A	S ₅ (Me)	L	T	S	A A	NH ₂		>1.50
11R	L	T	F	Q	C	Y	W	A	S ₅ (Ph)	L	T	S	A A	NH ₂		>4.14
12R	L	S	F	C	Q	Y	W	S ₅ (Me)	Cba	L	S	P	NH ₂	>1.36		

Supplementary Table 2. MS characterization of peptide 1-12. FITC labeled peptides were used for FP and cellular uptake, Cy3 labeled peptides were used for in vivo imaging, acetyl peptides were used for all other experiments. The observed MS was presented as (calcu. MS+1)/2, as these peptides are characterized as 2 charges. Notes: The FITC means fluorescein isothiocyanate; the β A means beta Alanine; the Ac means acetyl group; the S₅ means unnatural amino acids, and Me means methyl group, Ph means phenyl group. All the chemical structure of these abbreviation was showed below.

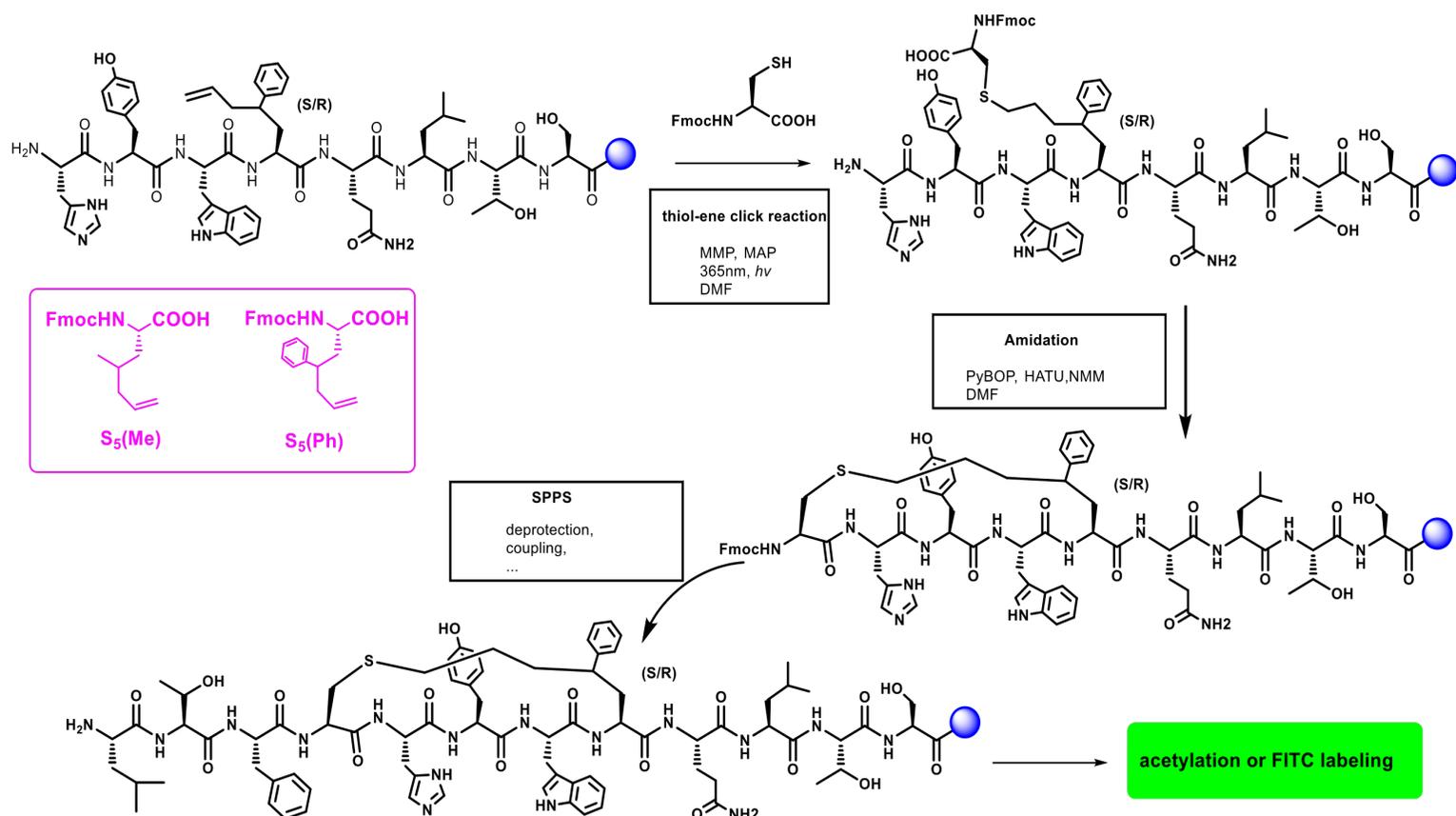
entry		sequence			calcu. MS	obsev. MS
1	FITC	βA L T F Q H Y	W A	Q L T S	NH ₂	1956.17 979.09
2	FITC	βA L T F C H Y	W S ₅ (Me)	Q L T S	NH ₂	1999.29 1000.03
3	FITC	βA L T F C H Y	W S ₅ (Ph)	Q L T S	NH ₂	2061.36 1030.2
4	FITC	βA L T F Q C Y	W A	S ₅ (Me) L T S	NH ₂	1933.23 967.63
5	FITC	βA L T F Q C Y	W A	S ₅ (Ph) L T S	NH ₂	1995.3 999.02
6	FITC	βA L C F Q H S ₅ (Me)	W A	Q L T S	NH ₂	1934.22 967.96
7	FITC	βA F C H Y	W S ₅ (Me)	Q L T S A A	NH ₂	1927.19 964.71
8	FITC	βA L T F C H Y	W S ₅ (Me)	Q L T S A A	NH ₂	2141.45 1071.75
9	FITC	βA L T F C H Y	W S ₅ (Ph)	Q L T S A A	NH ₂	2303.52 1152.5
10	FITC	βA L T F Q C Y	W A	S ₅ (Me) L T S A A	NH ₂	2075.39 1038.99
11	FITC	βA L T F Q C Y	W A	S ₅ (Ph) L T S A A	NH ₂	2137.46 1070.13
12	FITC	βA L S F C Q Y	W S ₅ (Me)	Cba L S P	NH ₂	1970.29 986.35
MeR		Ac L T F C H Y	W S ₅ (R-Me)	Q L T S	NH ₂	789.8 790.1
PhR		Ac L T F C H Y	W S ₅ (R-Ph)	Q L T S	NH ₂	821.3 821.4
PhR-Cy3	Cy3	βA L T F C H Y	W S ₅ (R-Ph)	Q L T S	NH ₂	1056.1 1056.2



Supplementary Table 3. Primer sequence of genes for RT-PCR analysis.

Primer name	Sequence (5'-3')
Foward-P53	GGAGCACTAAGCGAGCACTG
Reverse-P53	TATGGCGGGAGGTAGACTGA
Foward-MDM2	GGGCTTTGATGTTCCCTGATT
Reverse-MDM2	CTTTGTCTTGGGTTTCTTCC
Foward-MDMX	CATTTCGGCTCCTGTCGTTA
Reverse-MDMX	GTTCCCGTCTCGTGGTCTTT
Foward-MIC1	AGTTGCGGAAACGCTACGAG
Reverse-MIC1	GGAACAGAGCCCGGTGAAGG
Foward-Sox2	GTGAGCGCCCTGCAGTACAA
Reverse-Sox2	GCGAGTAGGACATGCTGTAGGTG
Foward-FoxA2	CCCCAACAAGATGCTGACGC
Reverse-FoxA2	GCGAGTGGCGGATGGAGTT
Foward-beta Actin	TCCAGCCTTCCTTCTTGGGTATG
Reverse-beta Actin	GAAGGTGGACAGTGAGGCCAGGAT

Supplementary Scheme

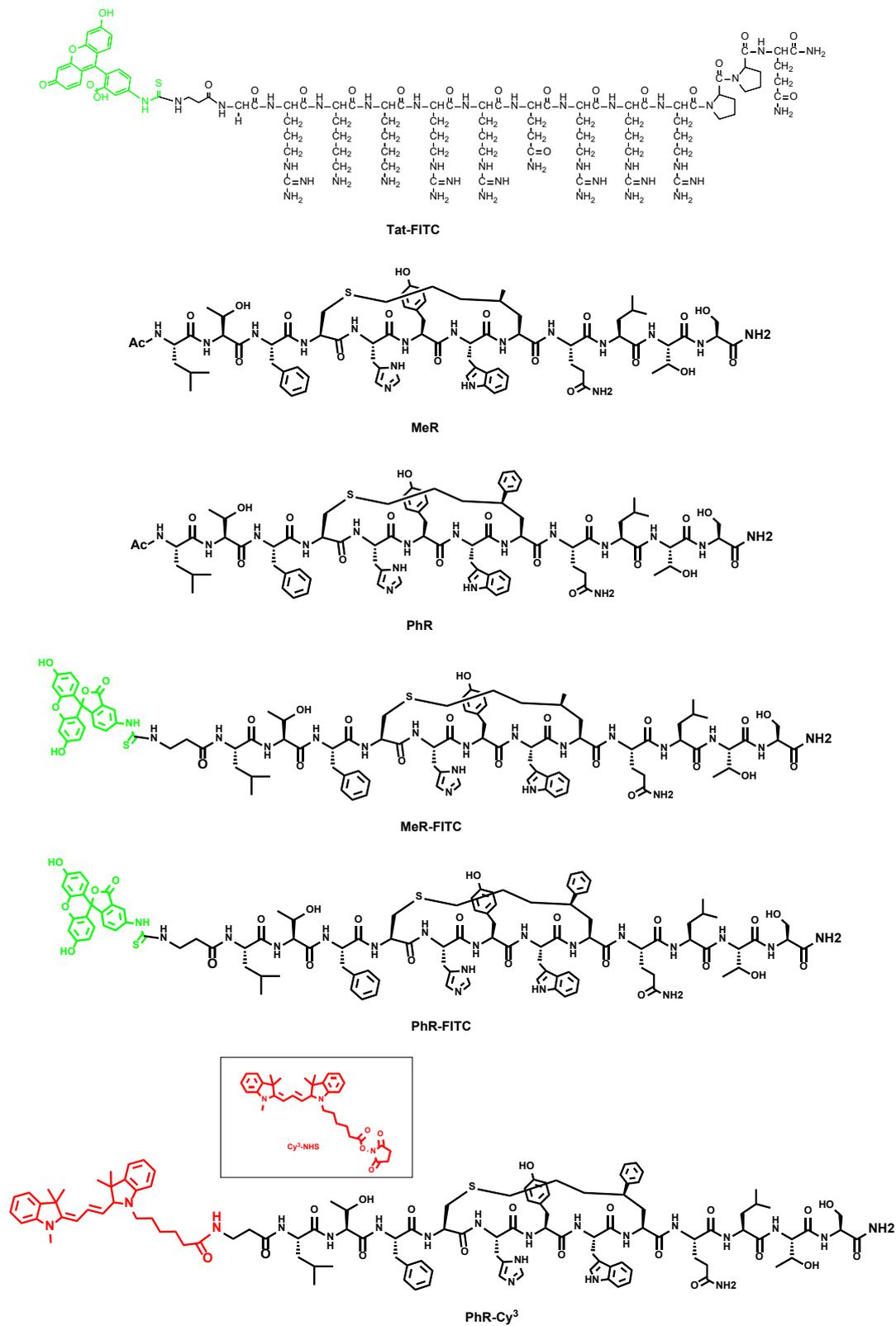


Supplementary Scheme 1. Schematic presentation of the synthesis process of peptides. Here take PhR as an example, the thiol-ene click reaction was performed in a photo-reactor under a wavelength of 365nm violet light. The photoreaction was repeated for three times, each time last for 1hour. The yield of the photoreaction step reached to 90%. The unnatural amino acids were epimer and were synthesized followed the previous reported procedures. For each peptide sequences, we got two diastereomers which we named as S or R. (Abbreviation: MMP: 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone; MAP: 4-methoxyacetophenone; PyBOP: benzotriazol-1-yl-oxytripyridinophosphonium hexafluorophosphate; NMM: N-Methylmorpholine; HATU: N-[(Dimethylamino)-1H-1,2,3-triazolo-[4,5-

b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide; FITC: fluorescein isothiocyanate; DMF: dimethylformamide).

Appendix

The chemical structure of PhR/MeR and fluorescein-labeled Tat/PhR/MeR.



Full gel image of western blot

Figure 4a

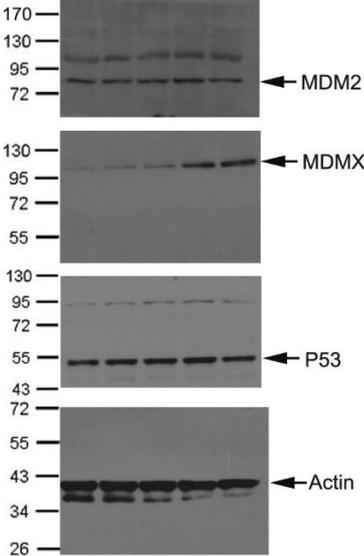


Figure 4b

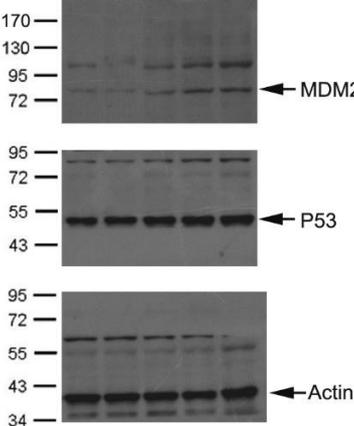
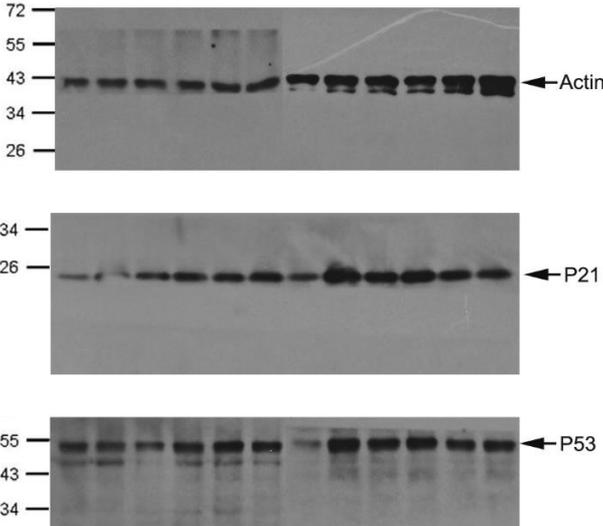
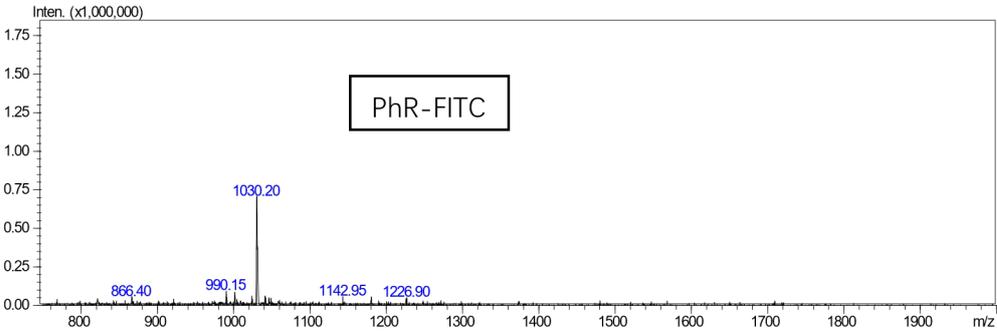
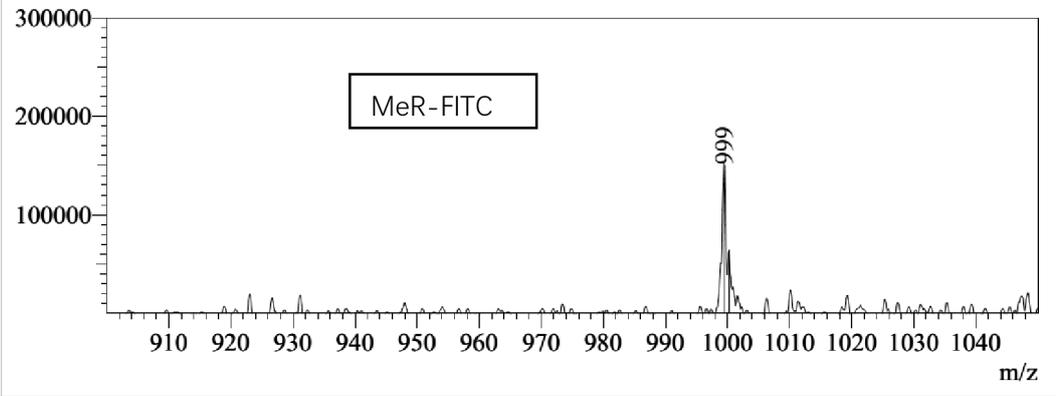
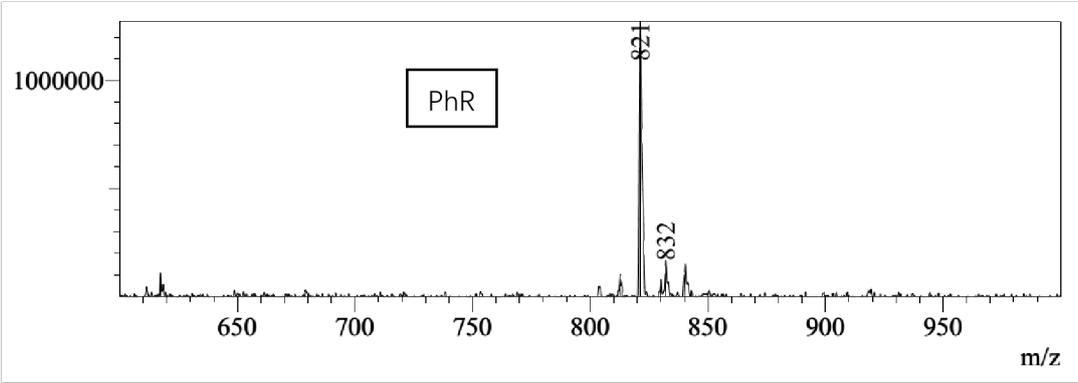
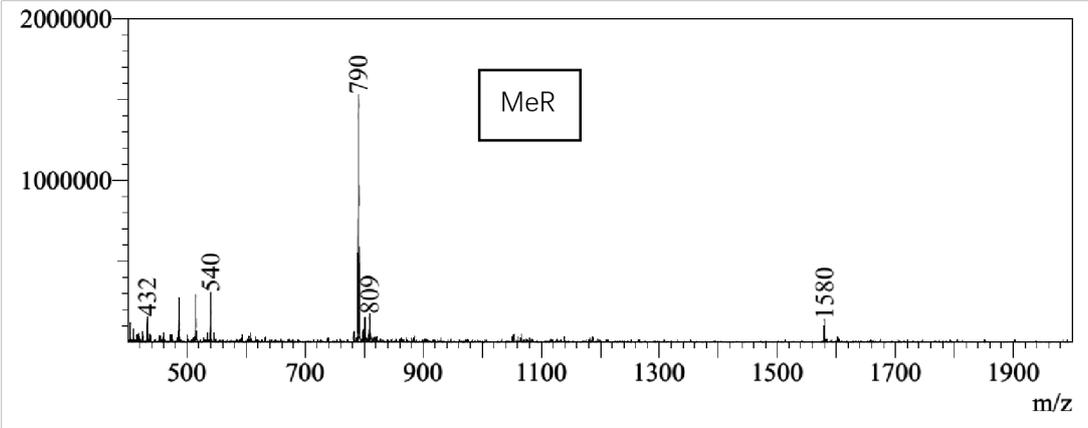


Figure 4c



MS characterization of PhR peptides.



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