Supplementary Materials

Reactivation of the p53 Tumor Suppressor Pathway by a Stapled p53 Peptide

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A. General Information

Fmoc-protected α-amino acids (other than the olefinic amino acids Fmoc-S_5-OH and Fmoc-R_8-OH), 2-(6-chloro-1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and Rink Amide MBHA resin were purchased from Novabiochem (San Diego, CA). Dimethylformamide (DMF), *N*-methyl-2-pyrrolidinone (NMP), *N*,*N*-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), 1,2-dichloroethane (DCE), fluorescein isothiocyanate (FITC), and piperidine were purchased from Sigma-Aldrich and used as supplied. The synthesis of the olefinic amino acids has been described elsewhere.[1,2]

B. Peptide Synthesis

The peptides were synthesized manually using Fmoc solid phase peptide chemistry on Rink amide MBHA resin with loading levels of 0.4-0.6 mmol/g resin. The following protocol was used:

1. The Fmoc protective group was removed with 20% piperidine in NMP for 30 min.
2. The resin was washed with NMP five times.
3. The subsequent Fmoc-protected amino acid was coupled for 30 min (60 min for a cross-linker) using Fmoc-AA (10 equiv., 4 equiv. for a cross-linker), HCTU (9.9 equiv., 3.9 equiv. for a cross-linker), and DIEA (20 equiv., 7.8 equiv. for a cross-linker).
4. The resin was washed with NMP five times. Repeat from step 1.

All peptides were capped with a β-alanine residue at the *N*-terminus. CD experiments make use of peptides that have been acetylated at the *N*-terminus. The acetylation reaction consisted of deprotection of the Fmoc group as outlined above, followed by reaction with acetic anhydride and DIEA. All other experiments shown make use of fluoresceinated peptides at the *N*-terminus. To this end, the peptides with the deprotected *N*-terminus were exposed to fluorescein isothiocyanate in DMF overnight in the presence of DIEA.

C. Olefin Metathesis, Cleavage from Resin, and Purification

The ring-closing metathesis reaction was performed on the *N*-terminal capped peptide while still on the solid support in a disposable fritted reaction vessel. The resin was exposed to a 10 m*M* solution of bis(tricyclohexylphosphine)benzylidine ruthenium (IV) dichloride (Grubbs First Generation Catalyst) in 1,2-dichloroethane for 2 hours. The catalyst addition and 2 hour metathesis reaction was repeated once. The resin-bound peptide was washed with CH_2Cl_2 three times and dried under a stream of nitrogen.

The peptide was cleaved from the resin and deprotected by exposure to Reagent K (82.5% TFA, 5% thioanisole, 5% phenol, 5% water, 2.5% 1,2-ethanedithiol) and precipitated with methyl-*tert*-butyl ether at 4°C and lyophilized.

The lyophilized peptides were purified by reverse phase HPLC using a C_{18} column (Agilent). The peptides were characterized by LC-MS and amino acid analysis. Mass spectra were obtained either by electrospray in positive ion mode or by MALDI-TOF. A representative LC trace and mass spectrum are shown below (Figure S1) and the mass spectral data for all the compounds are likewise shown below (Table 1).

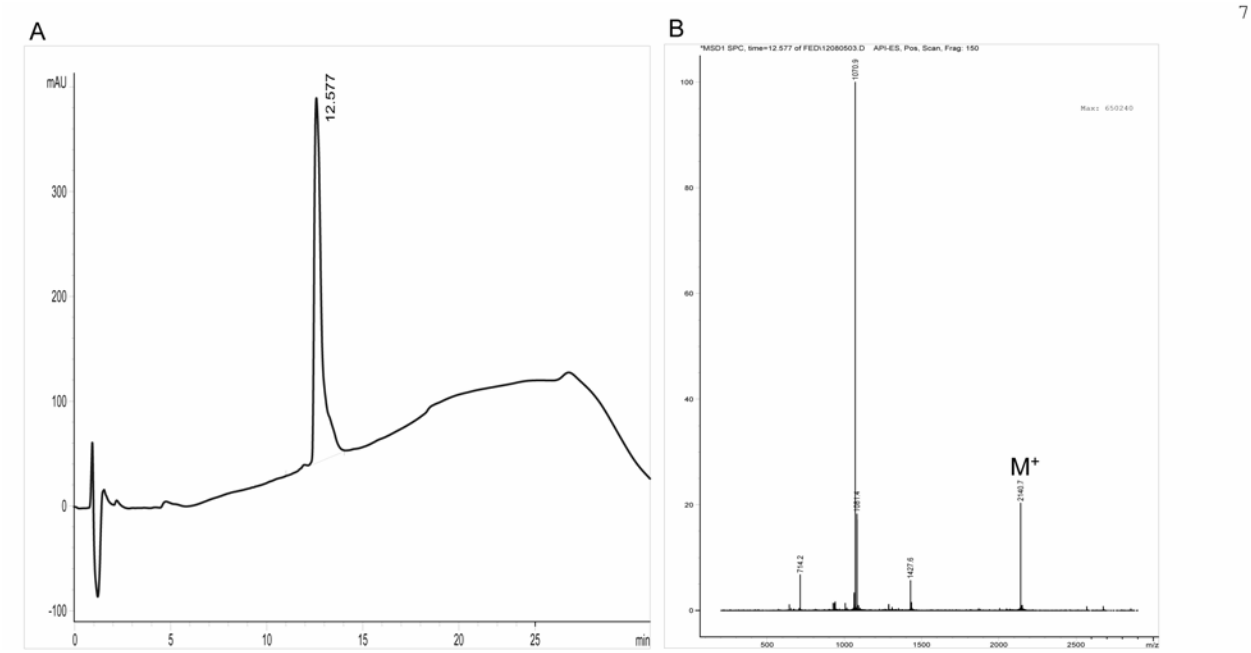


Figure S1. LC-MS traces from purified peptide **SAH-p53-4** as a representative example. All LC-MS samples were run on an Agilent 1100 instrument using a Zorbax C18 column. (A) LC chromatogram of peptide **SAH-p53-4**. (B) Electrospray mass spectrum (positive ion mode) of peptide **SAH-p53-4**.

Table 1. Mass spectrometry data for the compounds described in this study.

Compound	Calculated Mass	Found Mass	Method
WT $p53_{14-29}$	2033.26	2033.12 [M + H]	MALDI-TOF
SAH-p53-1	2097.41	2097.14 [M + H]	MALDI-TOF
SAH-p53-2	2132.40	2132.84 [M + Na]	MALDI-TOF
SAH-p53-3	2089.37	2089.18 [M + Na]	MALDI-TOF
SAH-p53-4	2140.48	2140.70 [M + H]	MALDI-TOF
SAH-p53-5	2138.5	2139.0 [M + H]	ESI
SAH-p53-6	2165.5	1083.2 [M/2 + H]	ESI
SAH-p53-7	2152.4	1077.2 [M/2 + H]	ESI
SAH-p53-8	2180.5	1112.9 [M/2 + Na]	ESI
SAH-p53-8_{F19A}	2104.4	1052.9 [M + H]	ESI
unstapled SAH-p53-8	2208.5	2209.1 [M + H]	ESI
FITC-WT $p53_{14-29}$	2401.59	2402.94 [M + Na]	MALDI-TOF
FITC-SAH-p53-1	2466.74	2467.29 [M + Na]	MALDI-TOF
FITC-SAH-p53-2	2479.74	2479.27 [M + Na]	MALDI-TOF
FITC-SAH-p53-3	2437.72	2437.31 [M + Na]	MALDI-TOF
FITC-SAH-p53-4	2509.81	2509.10 [M + Na]	MALDI-TOF
FITC-SAH-p53-5	2401.59	2402.94 [M + Na]	MALDI-TOF
FITC-SAH-p53-6	2512.8	1257.2 [M/2 + H]	ESI

FITC-SAH-p53-7	2499.8	1250.6 [M/2 + H]	ESI
FITC-SAH-p53-8	2527.8	1286.3 [M/2 + Na]	ESI
FITC-SAH-p53-8$_{F19A}$	2451.7	1248.5 [M/2 + Na]	ESI
unstapled FITC-SAH-p53-8	2555.9	1278.5 [M/2 + Na]	ESI

D. Circular Dichroism (CD) Spectroscopy

Compounds were dissolved in H_2O to concentrations ranging from 10-50 μM. The spectra were obtained on a Jasco J-715 spectropolarimeter at 20°C. The spectra were collected using a 0.1 cm pathlength quartz cuvette with the following measurement parameters: wavelength, 185-255 nm; step resolution 0.1 nm; speed, 20 nm min^{-1}; accumulations, 6; bandwidth, 1 nm. The helical content of each peptide was calculated as reported previously.[3]

E. *Ex vivo* Protease Stability

The fluoresceinated peptides (2.5 μg) were incubated with fresh mouse serum (20 μL) at 37°C for 0-24 hours. The level of intact fluoresceinated compound was determined by flash freezing the serum specimens in liquid nitrogen, lyophilization, extraction in 1:1 CH_3CN:H_2O containing 0.1% TFA, followed by HPLC-based quantitation using fluorescence detection at excitation/emission settings of 495/530 nm.

F. Protein Production and Fluorescence Polarization

Escherichia coli BL21 (DE3) containing the plasmid encoding hDM2$_{17-125}$ with an *N*-terminal hexahistidine tag and a thrombin cleavage site were cultured in kanamycin- and chloramphenicol-containing Luria Broth and induced with 0.1 mM isopropyl β-D-thiogalactoside (IPTG). The cells were harvested after 4 hours by centrifugation for 20 min at 3200 rpm, resuspended in buffer A (20 m*M* Tris pH 7.4, 0.5 M NaCl) and lysed by sonication. Cellular debris was pelleted by centrifugation for 30 minutes at 15,000 rpm, and the supernatant was incubated with Ni-NTA agarose (QIAGEN) for 2 h. The resin was washed with buffer A and eluted with a gradient of imidazole ranging from 5 m*M* to 500 m*M*. The fractions containing the eluted protein were concentrated and diluted 1:1 with thrombin cleavage buffer (5 mM $CaCl_2$, 20 m*M* Tris pH 7.4, 1 μL mL^{-1} β-mercaptoethanol, and 0.8 U mL^{-1} thrombin). The cleavage reaction was incubated overnight at 4°C. The reaction was concentrated to 2 mL and purified by gel filtration using a G75 column. Purity of the protein was assessed by SDS-PAGE, FPLC and MALDI-TOF and determined to be >90%. Its identity was further confirmed by digestion followed by mass spectrometry of the resulting peptide fragments.

Fluoresceinated compounds (L_T = 5-25 n*M*) were incubated with hDM2$_{17-125}$ in binding assay buffer (140 mM NaCl, 50 mM, Tris pH 8.0) at room temperature. Binding activity was measured by fluorescence polarization on a Perkin-Elmer LS50B luminescence spectrophotometer using a cuvette containing a stirbar or a Spectramax M5 Microplate Reader (Molecular Devices). K_d values were determined by nonlinear regression analysis of dose response curves using Prism software 4.0 Graphpad. In the case of

compounds where $L_T < K_d$ and under the assumption that $L_T \approx L_{free}$, binding isotherms were fitted to the equation

$$P = P_f + \left[\left(P_b - P_f \right) \times \frac{R_T}{K_D + R_T} \right] \quad (1)$$

where P is the measured polarization value, P_f is the polarization of the free fluorescent ligand, P_b is the polarization of the bound ligand, and R_T is the receptor/protein concentration.

With compounds where $L_T > K_d$, the assumption that $L_T \approx L_{free}$ does not hold due to ligand depletion. As such, binding isotherms were fitted to the more explicit equation

$$P = P_f + (P_b - P_f) \left[\frac{(L_T + K_D + R_T) - \sqrt{(L_T + K_D + R_T)^2 - 4L_T R_T}}{2L_T} \right] \quad (2)$$

where P is the measured polarization value, P_f is the polarization of the free fluorescent ligand, P_b is the polarization of the bound ligand, L_T is the total concentration of fluorescent ligand and R_T is the receptor/protein concentration.[4] Each data point represents the average of an experimental condition performed in at least triplicate.

G. Flow Cytometry

Jurkat T-cell leukemia cells were grown in RPMI-1640 (Gibco) medium with 10% fetal bovine serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1}, 2 mM glutamine, 50 mM Hepes pH 7, and 50 μM β-mercaptoethanol. SJSA-1 cells were cultured in McCoy's 5A media (ATCC) supplemented with 10% fetal bovine serum and 100 U mL^{-1} penicillin. Jurkat cells (50,000 cells per well) were treated with fluoresceinated peptides (10 μM) for up to 4 hours at 37°C. After washing with media, the cells were exposed to trypsin (0.25%; Gibco) digestion (30 min, 37°C), washed with PBS, and resuspended in PBS containing 0.5 mg mL^{-1} propidium iodide (BD Biosciences). Cellular fluorescence and propidium iodide positivity were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) and FlowJo software (TreeStar). The identical experiment was performed with 30 min pre-incubation of cells at 4°C followed by 4 hour incubation with fluoresceinated peptides at 4°C to assess temperature-dependence of fluorescent labeling.

H. Confocal Microscopy

Jurkat T-cell leukemia cells were incubated with fluoresceinated compounds for 24 hours at 37°C. After washing with PBS, the cells were cytospun at 600 rpm for 5 minutes onto Superfrost plus glass slides (Fisher Scientific). The cells were fixed in 4% paraformaldehyde, washed with PBS, incubated with TOPRO-3 iodide (100 nM; Molecular Probes) to conterstain nuclei, treated with Vectashield mounting medium (Vector), and imaged by confocal microscopy (BioRad 1024 or Nikon E800).

In a similar fashion, SJSA-1 osteosarcoma cells (1 x 10^5 cells) were incubated in with fluoresceinated compounds for 24 hours at 37°C in Lab-Tek™-CC2 Chamber Slides (Nunc). After washing with PBS, the

cells were fixed in 4% paraformaldehyde, washed with PBS, and treated with DAPI-containing (nuclear counterstain) Vectashield mounting medium (Vector), coverslipped and imaged by confocal microscopy (BioRad 1024 or Nikon E800).

I. Western Blotting

SJSA-1 osteosarcoma cells (1 x 10^6) incubated at 37 °C were treated with p53 peptides (20 μ*M*) in serum-free media for 4 hours, followed by serum replacement and additional incubation for 4-26 additional hours. The cells were lysed (20 m*M* Tris-HCl pH 8.0, 0.8% SDS, 1 m*M* PMSF, 1 U mL^{-1} benzonase nuclease) and the crude lysates were clarified by brief centrifugation and total protein concentration was determined by using the Pierce BCA protein assay. Aliquots containing 5 μg of total protein were run on 4-12% Bis-Tris polyacrylamide gels (Invitrogen). Proteins were detected by chemiluminescence reagent (Perkin Elmer) using antibodies specific for p53 (DO-1 clone; Calbiochem), hDM2 (IF2 clone; EMD Biosciences), p21 (EA10 clone; Calbiochem), and β-actin (Sigma-Aldrich).

J. Cell Viability and Apoptosis High-Throughput Assays

SJSA-1 osteosarcoma cells (4 x 10^5 cells per well) were incubated in 96-well plates and treated with p53 peptides in serum-free media for 4 hours, followed by serum replacement and additional incubation for 20 hours. Cell viability was assayed by addition of CellTiter-Glo™ bioluminescence reagent (Promega) and reading luminescence in a Spectramax M5 microplate reader (Molecular Devices). 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).

K. Co-Immunoprecipitation of FITC-SAH-p53 Peptides and Endogenous hDM2

SJSA-1 osteosarcoma cells (1 x 10^6) were treated with FITC-p53 peptides (15 μM) in serum-free media for 4 hours, followed by serum replacement and additional 8 hour incubation. The cells were thoroughly washed with serum-containing media and PBS and exposed to lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton-X100, 1 mM PMSF, 1 U mL^{-1} benzonase nuclease [EMD Biosciences] and complete protease inhibitor tablet [Roche]) at room temperature. All subsequent steps were all performed at 4 °C. The extracts were centrifuged, and the supernatants were incubated with protein A/G sepharose (50 μL 50% bead slurry per 0.5 mL lysates; Santa Cruz Biotechnology). The pre-cleared supernatants (500 μL) were collected after centrifugation, incubated with 10 μL of goat-anti-FITC antibody (AbCam) for 1.5 h followed by protein A/G sepharose for an additional 1.5 hours. The immunoprecipitation reactions were pelleted and washed three times with lysis buffer. The precipitated proteins were suspended in SDS-containing loading buffer, boiled, and the supernatants were processed by SDS-PAGE on 4-12% Bis-Tris gels (Invitrogen). The proteins were blotted into Immobilon-P membranes (Millipore). After blocking, the blots were incubated with either a 1:100 dilution of mouse anti-human hDM2 antibody (IF2 clone; EMD

Biosciences) or a 1:200 dilution rabbit anti-FITC antibody (Zymed) in 3% BSA in PBS followed by anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG (Pharmingen). The hDM2 protein and FITC peptides were visualized using the Western Lightning™ chemiluminescence reagent (Perkin Elmer) and exposing to film. The gels were stained using a silver stain kit (Bio-Rad) following manufacturer's instructions.

L. References

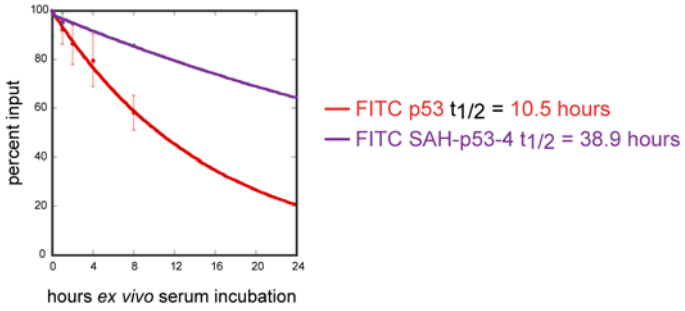
(1) Williams, R. M.; Im, M. N. *J. Am. Chem. Soc.* **1991**, *113*, 9276-9286

(2) Schafmeister, C.; Po, J.; Verdine, G. *J. Am. Chem. Soc.* **2000**, *122*, 5891-5892.

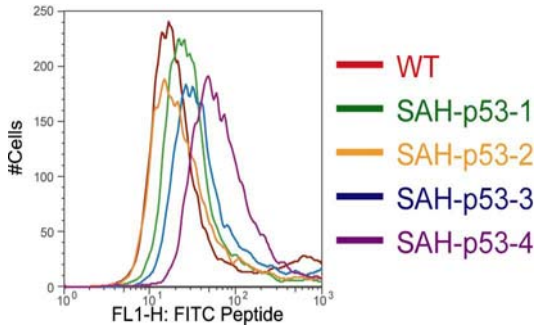
(3) Chen, Y.-H.; Yang, L.-T.; Chau, K.-H. *Biochemistry* **1974**, *13*, 3350-3359.

(4) Copeland, R. A. in *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*, 2nd. ed. (2000).

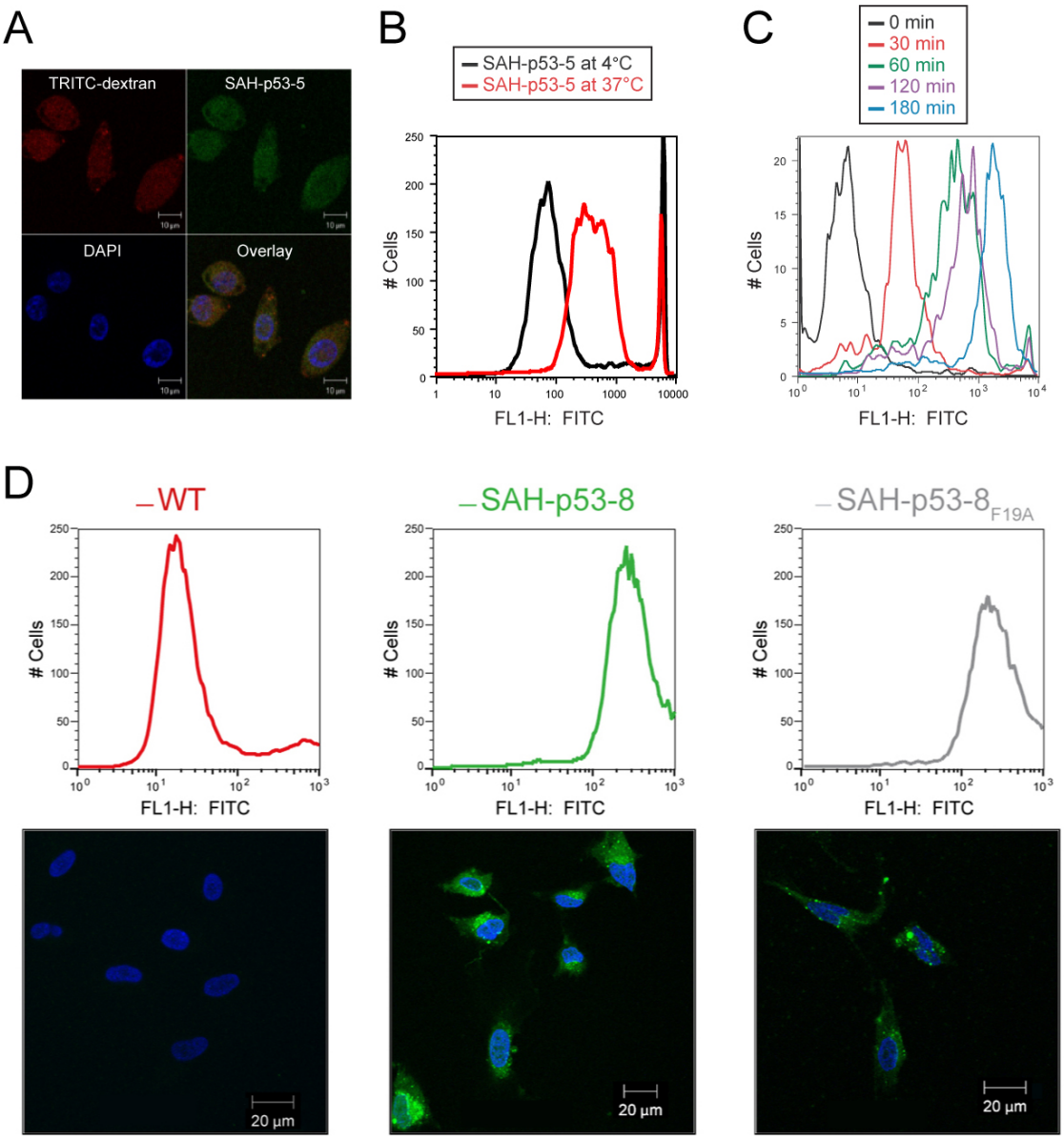
M. Supplementary Figures



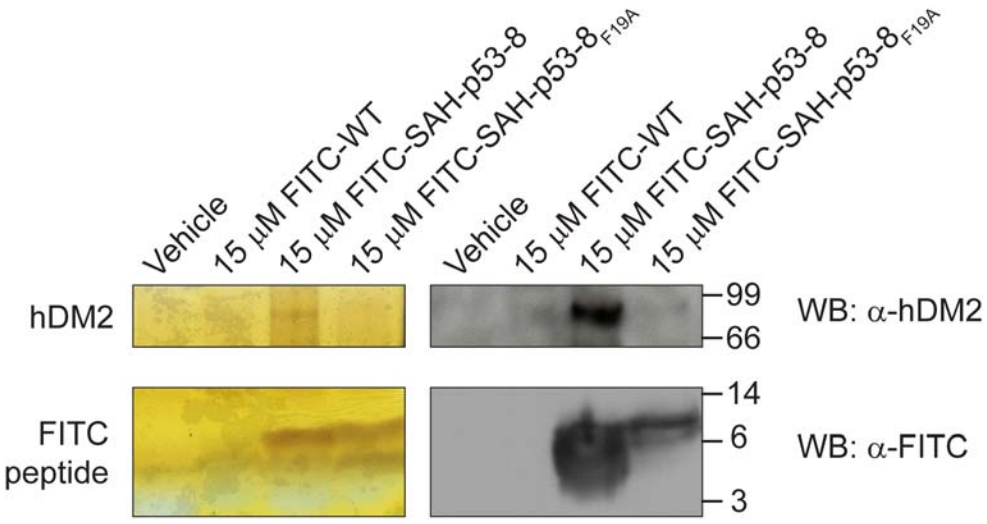
Supplementary Figure 1. To determine whether SAH-p53 peptides have increased proteolytic stability, the wild type $p53_{14-29}$ peptide and SAH-p53-**4** were exposed to serum *ex vivo*. SAH-p53-**4** displayed a serum half-life ($t_{1/2}$) almost four times longer than that of the unmodified wild type peptide.



Supplementary Figure 2. To determine if SAH-p53 peptides **1**-**4** were cell permeable, Jurkat T-cell leukemia cells were incubated with fluoresceinated p53 peptides for 4 hours followed by washing, trypsinization, and FACS analysis to evaluate cellular fluorescence. None of the peptides tested produced cellular fluorescence.



Supplementary Figure 3. (A) SJSA-1 cells were treated with FITC-SAH-p53-**5** and 4.4 kDa TRITC-dextran for 4 hours. Confocal microscopy revealed co-localization of FITC-SAH-p53-**5** peptide with TRITC-dextran in pinosomes. (B) To assess whether the permeability of FITC-SAH-p53-**5** was temperature-dependent, Jurkat T-cell leukemia cells were incubated with fluoresceinated p53 peptides for 4 hours at either 4 °C or 37 °C followed by washing, trypsinization, and FACS analysis to evaluate cellular fluorescence. (C) To determine the kinetics of cell permeability, Jurkat T-cell leukemia cells were exposed to FITC-SAH-p53-**5** peptide and cellular fluorescence was evaluated by FACS analysis at successive time points. FITC-SAH-p53-**5**-treated cells displayed a time-dependent increase in cellular fluorescence. (D) SJSA-1 cells were treated with FITC-wild type, SAH-p53-**8**, and SAH-p53-$\mathbf{8}_{\mathbf{F19A}}$ peptides for 4 hours followed by FACS and confocal microscopy analyses. Cellular fluorescence was observed after treatment with FITC-SAH-p53 peptides, but not with FITC-wild type p53 peptide.



Supplementary Figure 4. SJSA-1 cells were incubated with FITC-peptides followed by lysis and anti-FITC pull down. Native hDM2 co-immunoprecipitated with FITC-SAH-p53-**8** but not with wild-type or mutant SAH-p53-$\mathbf{8}_{\mathbf{F19A}}$ peptides. Left: silver stained gel; right: Western Blots.

N. Expanded Reference

(16h) Grasberger, B.L.; Lu, T.; Schubert, C.; Parks, D. J.; Carver, T. E.; Koblish, H. K.; Cummings, M. D.; LaFrance, L. V.l Milkiewicz, K. L.; Calvo, R. R.; Maguire, D.; Lattanze, J.; Franks, C. F.; Zhao, S.; Ramachandren, K.; Bylebyl, G. R.; Zhang, M.; Manthey, C. L.; Petrella, E. C.; Pantoliano, M. W.; Deckman, I. C.; Spurlino, J. C.; Maroney, A. C.; Tomczuk, B. E.; Molloy, C. J.; Bone, R. F. *J. Med. Chem.* **2005**, *48*, 909-912.