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Supporting Information

Virtual peptide directed binding identifies selective and dual p53-hDM2/X protein-protein interaction inhibitors.

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1. General Procedures

Reagents and Solvents

All chemicals were reagent grade and were purchased from Sigma Aldrich, Fisher Scientific and Tokyo Chemical Industry. Fmoc-amino acids and coupling reagents were purchased from Novabiochem or AGTC Bioproducts. Anhydrous solvents were bought from Sigma Aldrich and assumed to conform to specification.

Physical Characterisation and Spectroscopic Techniques

¹H- and ¹³C-NMR spectra were recorded on a Bruker spectrometer operating at 400 MHz (¹H) or 100 MHz (¹³C) using the specified deuterated solvent. The chemical shifts for both ¹H- and ¹³C-were recorded in ppm and were referenced to the residual solvent peak of CHCl₃ at 7.26 ppm (¹H) and 77.0 ppm (¹³C). Multiplicities in the NMR spectra are described as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, and combinations thereof; coupling constants are reported in Hz. Assignments, where conspicuous, have been confirmed by appropriate 2D NMR experiments. MALDI was performed on Kratos Analytical Axima MALDI-TOF. Low resolution mass spectra were recorded using a Shimadzu LCMS 2010EV operated under electrospray ionisation in positive (ES+) mode. Accurate mass spectra were recorded at the EPSRC National Mass Spectroscopy Service Centre, Swansea. Melting points were recorded using open capillary tubes on a Mel-Temp electrothermal melting point apparatus, melting points are uncorrected. Infrared spectra were recorded using a PerkinElmer Spectrum BX with ATR attachment.

Chromatographic Techniques

Analytical RP-HPLC was performed on an Agilent 1200 using an Agilent eclipse XDB-C18 column, 4.6×150 mm, 5μ M and a flow rate of 1 mL/min. Solvent A = Water + 0.05% TFA and Solvent B = MeOH + 0.05% TFA. Gradient 5% B \rightarrow 95% B over 20 min. Detection wavelength 214 nm and 254 nm.

Semi-preparative RP-HPLC was performed on an Agilent 1200 using an Agilent eclipse XDB-C18 column, 9.4 x 250mm, 5μ M and a flow rate of 4 mL/min. Solvent A = Water + 0.05% TFA and Solvent B = MeOH + 0.05% TFA. Gradient 5% B \rightarrow 95% B over 20 min. Detection wavelength 214 nm and 254 nm.

2. Protein Expression

hDM2

BL21(DE3)pLysS cells transformed with a pET14b plasmid bearing His-tagged hDM2 (17-125, kindly donated by Gary Parkinson, School of Pharmacy, University College London, UK) were cultured at 37 °C in LB medium containing 100 μg/mL ampicillin and 52 μg/mL chloramphenicol to an OD = 0.6. Protein expression was induced by 0.4 mM IPTG at 16-18 °C for 14 h. The cells were sonicated in 20 mM Tris-HCl buffer containing 300 mM NaCl and 20 mM imidazole. The lysate was spinned at 10000 rcf for 30 min at a temperature of 4 °C. The supernatant was loaded on a Ni-NTA resin (QIAGEN), washed with the same buffer and then eluted with a 20 mM Tris-HCl buffer containing 300 mM NaCl and 300 mM imidazole. Fractions containing the protein were collected and diluted with a buffer prepared with 2.323 g/L of NaH₂PO₄·2H₂O, 0.724 g/L of Na₂HPO₄ containing EDTA (1 mM) and DTT (1 mM). The protein was loaded on a HiTrap SP HP 5 mL cation exchange column (GE Healthcare). The column was washed with the previous buffer

(50 mL) and the protein was eluted with a stepwise gradient of the buffer with an increasing concentration of NaCl (0 to 1 M in ten steps, 0.1 M increase each step). Fractions containing pure protein were concentrated and the solvent was exchanged with PBS saline buffer at pH 7.4 with 10% glycerol for stocking at -80 °C. The protein construct has a mass of 14 kDa.

hDMX

The hDMX protein used in this study was purchased from DC Biosciences and is a truncated construction of the full length protein, with deletion of the RING domain, amino acid residues 393-490. The protein is fused to GST and has a C-terminal HA tag. The protein construct has a mass of 73 kDa. Plasmid was kindly provided by Professor Aart Jochemsen.^[1]

3. Synthesis

FAM-p4 peptide

The twelve amino acid p4 peptide FAM-LTFEHYWAQLTS-CONH₂ was synthesised on Rink amide MBHA resin (resin loading 0.6 mmol/g) using an automated peptide synthesiser. 100 mg of Rink Amide MBHA resin (0.06 mmol) was suspended in DMF (2 mL) and was allowed to swell for 20 min. The DMF was drained from the peptide vessel and Fmoc deprotection was carried out by addition of 2 x 2 mL of 40% piperidine in DMF, which was vortexed for 10 min. This was removed and the resin was washed with DMF (3 x 2 mL). The resin was then treated with a solution of Fmoc-Ser(OtBu)-OH (4 equiv. compared to resin loading), to which HBTU (3.9 equiv.) and HOBt (4 equiv.) and DIPEA (8 equiv.) in DMF were added. The mixture was then vortexed for 30 min. The vessel was drained and the resin washed with DMF (3 x 2 mL). The coupling reaction was then repeated followed by Fmoc deprotection (2 x 2 mL 40% piperidine in DMF, 10 min) and finally the resin was washed with DMF. Subsequent amino acids were coupled in an identical fashion. After the final amino acid coupling reaction (leucine) and Fmoc deprotection, the resin was treated with Fmoc-aminohexanoic acid (Ahx, 4 equiv.), HBTU (3.9 equiv.), HOBt (4 equiv.) and DIPEA (8 equiv.) and reacted for 45 min. Subsequent Fmoc deprotection was followed by coupling with FAM (1.5 equiv.) and HBTU (3.9 equiv.), HOBt (4 equiv.) and DIPEA (8 equiv.) in DMF overnight. The resin was washed with DCM (x 3) and 1:1 MeOH:DCM (x 3) to remove any residual DMF. The peptide was cleaved from the resin using 95:2.5:2.5 TFA:TIPS:H₂O (5 mL) and shaken for 3 h after which the cleavage cocktail was drained into a round bottom flask. The resin was washed with TFA (x 3) and the solutions combined and concentrated in vacuo. The peptide was precipitated using cold diethyl ether and filtered. The peptide was purified using automated reversed phase flash chromatography and lyophilised from water to yield a yellow solid. This was subsequently analysed using RP-HPLC (RT = 15.3 min) and MALDI (m/z 1855.5 M+H observed).

p53 peptide

The p53 peptide SQETFSDLWKLLPEN synthesis was performed as described for FAM-p53 but the peptide was acetylated at the N-terminus after the addition of the final amino acid (serine) using acetyl chloride (4 equiv.) and DIPEA (4 equiv.) in DMF and shaken for 45 min. The resin was washed with DMF (x 3), DCM (x 3) and 1:1 MeOH:DCM (x 3) to remove any residual DMF. The peptide was cleaved from the resin using 95:2.5:2.5 TFA:TIPS:H₂O (5 mL) and shaken for 3 h after which the cleavage cocktail was drained into a round bottom flask. The resin was washed with TFA (x 3) and the solutions combined and concentrated in vacuo. The peptide was precipitated using cold diethyl ether and filtered. The peptide was purified using automated reversed phase flash

chromatography and lyophilised from water to yield a white solid. This was subsequently analysed using RP-HPLC (RT = 14.3 min) and MALDI (m/z 1805.9 M+H observed).

General azide formation

Azides were either purchased directly from the supplier or prepared from the corresponding alkyl halides. Alkyl halide (e.g. 4-methoxybenzylbromide, 1 equiv.) and sodium azide (1.3 equiv.) were stirred in DMF (1 mL/mmol) at 50 °C for 4 h. After cooling to room temperature water was added and the solution was extracted with diethyl ether. The organic layer was washed with water exhaustively, dried with MgSO₄, filtered and concentrated. The obtained azide, confirmed by IR spectroscopy, was used directly in the following step without further purification.

General alkyne formation

Alkynes were either purchased directly from the supplier or prepared from the corresponding aldehyde. Aldehyde (e.g. 2-fluorobenzaldehyde, 1 equiv.), methanol (1 mL/mmol) and potassium carbonate (5 equiv.) were stirred at room temperature for 20 mins. The Bestmann-Ohira reagent^[2] (1.3 equiv.) was added dropwise and the solution allowed to stir for 4 h, at which time water was added. The solution was extracted with diethyl ether and the organic layer was washed with water and brine. The organic layer was dried with MgSO₄, filtered and concentrated. The obtained alkyne, confirmed by ¹H NMR spectroscopy, was used directly in the following step without further purification.

General Click reaction of small molecules

Azide (1 equiv.), alkyne (1 equiv.), sodium ascorbate (2.5 eq), CuSO₄.5H₂O (0.25 eq) and DMF (1 mL/0.5 mmol) were stirred together for 4 h. The mixture was diluted with distilled water and extracted using diethyl ether. The organic layers were collected, washed exhaustively with water, dried with MgSO₄, and evaporated under reduced pressure. Purification was achieved with semi-preparative RP-HPLC prior to testing.

Experimental Data

9 1-((2R,4S,5S)-4-(4-((8R,9S,13S,14S,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-17-yl)-1H-1,2,3-triazol-1-yl)-5-(hydroxymethyl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione.

M.P. 203 °C (decomp.). ¹H NMR (CD₃OD); δ 8.00 (1H, s, 5"), 7.88 (1H, s, 6), 6.98 (1H, d, ${}^{3}J$ = 8.4, 1"'), 6.53-6.46 (3H, m, 2', 2"', 4"'), 5.43 (1H, m, 4'), 4.40 (1H, m, 5'), 3.93 (1H, dd, ${}^{2}J$ = 12.4, ${}^{3}J$ = 3.2, 5'-CH_A), 3.79 (1H, dd, ${}^{2}J$ = 12.4, ${}^{3}J$ = 3.4, 5'-CH_B), 2.94 (1H, m, 6"'-H_A), 2.75 (3H, m, 6"'-H_B, 3'), 2.45 (1H, m, 10"'), 2.11 (2H, m), 2.00-1.82 (3H, m), 1.91 (3H, s, 5-Me), 1.75-1.20 (6H, m), 1.03 (3H, s, 13"'), 0.66 (1H, m, 14"'). ¹³C NMR (CD₃OD); δ 165.25, 160.02, 159.64, 154.24, 154.17, 151.03, 137.58,

137.02, 131.28, 125.80, 122.66, 116.02, 114.76, 113.19, 112.38, 110.61, 85.41, 84.93, 82.03, 78.14, 77.82, 77.49, 60.80, 59.72, 43.42, 39.62, 37.59, 37.07, 32.91, 29.28, 27.26, 26.08, 23.23, 13.54, 11.21. LRMS (APCI): $564 (100, [M+H]^+)$. HRMS (ESI): $C_{30}H_{37}N_5O_6$ calcd. $[M+H]^+ = 564.2822$, found: 564.2841.

8 (2S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(1-hydroxy-1-phenylethyl)-1H-1,2,3-triazol-1-yl)propanoic acid

Amorphous/oil. ¹H NMR (CD₃OD); δ 7.75 (2H, m, 4"",5""), 7.64 (2H, m, 1"",8""), 7.70-7.50 (5H, m, 2"'-6"'), 7.37 (2H, m, 3"",6""), 7.30 (1H, s, 5'), 7.29 (2H, m, 2"",7""), 4.46 (1H, m, 2), 4.35 (2H, m, 9""-CH₂), 4.20 (1H, m, 9""), 3.89-3.64 (2H, m, 3). ¹³C NMR (CD₃OD); δ 169.82,

157.17, 143.81, 141.16, 134.93, 128.94, 127.41, 126.80, 124.86, 123.63, 121.25, 120.37, 119.53, 118.07, 66.85, 42.86, 19.85. LRMS (APCI): 499 (100, $[M+H]^+$). HRMS (ESI): $C_{28}H_{26}N_4O_5$ calcd. $[M+H]^+$ = 499.1981, found: 499.1976.

N-((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-(4-(1-(3-fluorophenyl)-1-hydroxyethyl)-1H-1,2,3-triazol-1-yl)tetrahydro-2H-pyran-3-yl)acetamide.

M.P. 196 °C. ¹H NMR (CD₃OD); δ 7.95 (1H, s, 5"), 7.10-7.30 (19H, m, aromatic protons), 5.76 (1H, m, 2'), 4.81 (6H, m, OCH₂Ph), 4.80-4.30 (4H, m, 3'-6'), 3.75 (2H, m, 6'-CH₂), 1.87 (3H, s, 2), 1.61 (3H, s, 1"'-CMe). (2' epimers unable to be separated, most abundant epimer reported) ¹³C NMR (CD₃OD); δ 171.81, 171.73, 163.92, 161.50, 149.97, 138.28, 138.02, 137.72, 129.45, 129.36, 128.06, 128.00, 127.82, 127.79, 127.65, 127.48, 127.44, 127.38, 127.36, 121.14,

121.11, 120.95, 120.29, 113.31, 113.19, 113.10, 112.98, 112.31, 112.14, 112.08, 86.56, 86.45, 82.63, 77.67, 77.61, 74.99, 74.62, 73.07, 73.02, 70.97, 70.87, 68.18, 54.71, 54.56, 29.38, 29.09, 21.19, 21.14. LRMS (APCI): 681 (100, $[M+H]^+$). HRMS (ESI): $C_{39}H_{42}FN_4O_6$ calcd. $[M+H]^+$ = 681.3088, found: 681.3097.

 $2 \qquad (3S,4S,5R,6R)-2-(4-(1-(3-fluorophenyl)-1-hydroxyethyl)-1H-1,2,3-triazol-1-yl)-6-(hydroxymethyl) tetrahydro-2H-pyran-3,4,5-triol.$

Amorphous. 1 H NMR (CD₃OD); δ 8.00 (1H, s, 5'), 7.29 (3H, m, 4"-6"), 6.94 (1H, m, 2"), 5.56 (1H, m, 2), 3.90-3.50 (6H, m, 2-6, 6-CH₂), 1.93 (3H, s, 1"-CMe) (2' epimers unable to be separated, most abundant epimer reported). LRMS (APCI): 370 (100, [M+H]⁺). HRMS (ESI): $C_{16}H_{21}FN_{3}O_{6}$ calcd. [M+H]⁺ = 370.1414, found: 370.1422.

3 (3S,4S,5R,6R)-2-(4-(2,4-difluorophenyl)-1H-1,2,3-triazol-1-yl)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol.

M.P. 237 °C (decomp.). ¹H NMR (CD₃OD); δ 8.47 (1H, s, 5'), 8.16 (1H, m, 6"), 7.11 (2H, m, 3", 5"), 5.69 (1H, m, 2), 4.00-3.50 (6H, m, 3-6,6-CH₂) (2' epimers unable to be separated, most abundant epimer reported). LRMS (APCI): 344 (100, [M+H]⁺). HRMS (ESI): C₁₄H₁₆F₂N₃O₅ calcd. [M+H]⁺ = 344.1058, found: 344.1040.

5 4-(2,4-difluorophenyl)-1-((phenylthio)methyl)-1H-1,2,3-triazole.

M.P. 74 °C. ¹H NMR (CD₃OD); δ 8.08 (2H, m, 6′, 5), 7.40 (2H, m, 2″, 6″), 7.32 (3H, m, 3′, 5′, 4″), 7.05 (2H, m, 3″, 5″), 5.83 (2H, s, NCH₂S). ¹³C NMR (CD₃OD); δ 164.06, 163.94, 161.58, 161.45, 160.61, 160.49, 158.12, 158.01, 140.33, 132.41, 131.94, 129.07, 128.52, 128.47, 128.42, 128.35, 122.63, 122.51, 114.68, 114.64,

 $114.55, 114.51, 111.76, 111.72, 111.54, 111.50, 104.07, 103.81, 103.55, 53.51. \ LRMS \ (APCI): 304, (100, [M+H]^+). \ HRMS \ (ESI): C_{15}H_{12}F_2N_3S \ calcd. \ [M+H]^+ = 304.0720, \ found: 304.0729.$

4 1-(3-fluorophenyl)-1-(1-((phenylthio)methyl)-1H-1,2,3-triazol-4-yl)ethan-1-ol.

M.P. 84 °C. ¹H NMR (CD₃OD); δ 7.70 (1H, s, 5"), 7.20-7.30 (6H, m, 5', 6', 2"', 3"', 5"', 6"'), 7.18 (2H, m, 4', 4"'), 6.95 (1H, m, 2'), 5.72 (2H, NCH₂S). ¹³C NMR (CDCl₃); δ 163.92, 161.49, 150.21, 132.52, 131.74, 129.47, 129.39, 129.02,

128.35, 120.91, 120.88, 113.30, 113.08, 112.13, 111.90, 53.53, 29.04. LRMS (APCI): 330 (100, $[M+H]^+$). HRMS (ESI): $C_{17}H_{17}FN_3OS$ calcd. $[M+H]^+ = 330.1076$, found: 330.1080.

11 N-((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-(4-(2,4-difluorophenyl)-1H-1,2,3-triazol-1-yl)tetrahydro-2H-pyran-3-yl)acetamide.

M.P. 191 °C (decomp.). ¹H NMR (CD₃OD); δ 8.46 (1H, s, 5"), 8.13 (1H, m, 6"), 7.40-7.05 (17H, m, aromatic protons), 5.93 (1H, m, 2'), 4.80-4.45 (4H, m, 3'-6'), 3.80 (2H, m, 6'-CH₂), 1.96 (3H, s, 2) (2' epimers unable to be separated, most abundant epimer reported). LRMS (APCI): 655 (100, [M+H]⁺). HRMS (ESI): C₃₇H₃₇F₂N₄O₅ calcd. [M+H]⁺ = 655.2732, found: 655.2749.

 $\textbf{6} \hspace{0.2cm} \textit{(S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(2,4-difluorophenyl)-1H-1,2,3-triazol-1-yl)propanoic acid.}$

Amorphous solid. 1 H NMR (CD₃OD); δ 8.21 (1H, s, 5"), 8.07 (1H, m, 6""), 7.76 (2H, m, 4', 5'), 7.57 (2H, m, 3', 6'), 7.34 (3H, m, 2', 7', 5""), 7.24 (2H, m, 1', 8'), 7.03 (1H, m, 3""), 5.02 (1H, m, 2), 4.35-4.10 (4H, m, 3, 9'-CH₂). LRMS (APCI): 491 (100, [M+H] $^{+}$). HRMS (ESI): $C_{26}H_{21}F_{2}N_{4}O_{4}$ calcd.

 $[M+H]^+ = 491.1531$, found: 491.1527.

7 (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-phenyl-1H-1,2,3-triazol-1-yl)propanoic acid.

Amorphous solid. ¹H NMR (CD₃OD); δ 8.21 (1H, s, 5"), 7.80-7.20 (13H, m, aromatic protons), 4.98 (1H, m, 2), 4.50-4.10 (4H, m, 3, 9'-CH₂). LRMS (APCI): 455 (100, [M+H]⁺). HRMS (ESI): C₂₆H₂₃N₄O₄ calcd. [M+H]⁺ = 455.1719, found: 455.1721.

Table S1: Compounds tested against hDM2 and hDMX that showed no appreciable binding affinity in the FA assays (>100 μ M).

	Structure			Structure	
S1	F HO N _{2N} HO O NHFmoc	hDM2	S6	F-N-N O NH O	hDMX
S2	AcHN OBn OBn OBn	hDM2	S7	HO N=N O NH O	hDMX
S3	HO H	hDM2	S8	HO N = N O OH OH	hDMX
S4	HO N.N.N. CF3	hDM2	S9	BnO OBn OBn BnO	hDMX
S5	N=N N=N N=N N=N N=N O NH O	hDMX	S10	F N=N O NH O NH O	hDMX

4. Binding Assays

Fluorescence Anisotropy

Fluorescence anisotropy was carried out on a BMG Labtech clariostar microplate reader with a fluorescence anisotropy optic measuring at 482/530 nm. Low-binding, Corning 96-well black plates were used and all reagents used in the assay were biological grade and purchased from Sigma Aldrich and Novabiochem. All solutions were made using MilliQ water.

Anisotropy values were automatically calculated using the following formula

$$r = \frac{l_a - l_b}{l_a + 2l_b}$$
$$I = l_a + 2l_b$$

Where r = anisotropy, $l_a =$ parallel emission light, $l_b =$ perpendicular emission light and I = total intensity.

*K*_D was determined using GraphPad Prism Version 6.0 software, following the method presented in *Nature Protocols*, **2011**, *6*, 365-387.

For ideal conditions, the concentration of receptor should be fixed at between K_D and 80% B_{max} .

IC₅₀ was determined using GraphPad Prism Version 6.0 software, using a four-parameter logistic model, according to precedent from Ottmann and co-workers.^[3] Errors are the transformed greater extreme of the standard error.

$$y = r_{min} + \frac{(r_{max} - r_{min})}{1 + 10^{((logIC_{50} - x) \times Hill Slope)}}$$

All experiments were performed at least three times. Triplicate implies independently expressed protein stocks, independently prepared compound stock solutions and different days. Each replicate included a triplicate of samples on that day.

hDMX

For binding each well contained 10 μ L of hDMX protein in 5-fold dilutions (82 pM to 8.22 μ M) and 90 μ L of 10 nM fluorescently-tagged high affinity binding peptide FAM-p4. Peptide and protein were dissolved in PBS: 0.05% Tween-20 buffer at pH 7.4. Reagents were incubated for 30 min at room temperature prior to reading. Bubbles were removed and 8 data points were generated. Nonlinear regression was then used to process the data, giving the K_D of the fluorescently-tagged peptide to be 334 nM. Performed in triplicate.

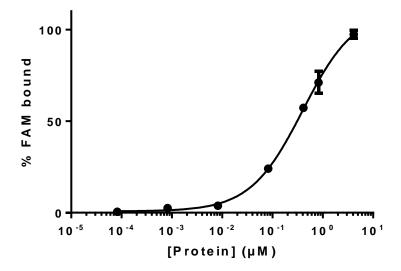


Figure S 1: Fluorescence Anisotropy direct titration. Fraction bound of FAM-p4 on hDMX

A titration of 100 nM hDMX with FAM-p4 suggested that with a 100 μL well volume 10 nM FAM-p4 peptide produced the greatest change in polarisation.

Inhibition screens were carried out using 250 nM hDMX, 10 nM fluorescently-tagged peptide and 100 μ M of inhibitor. Compounds were dissolved in DMSO. A positive control (wt p53 peptide) and negative control (DMSO) were used to ensure the assay was performing adequately. Compounds showing inhibition were then diluted in 10-fold dilutions (from 100 μ M to 10 pM well concentration) to generate IC₅₀ values, in triplicate. The IC₅₀ of wt p53 peptide was calculated to be 1.54 μ M.

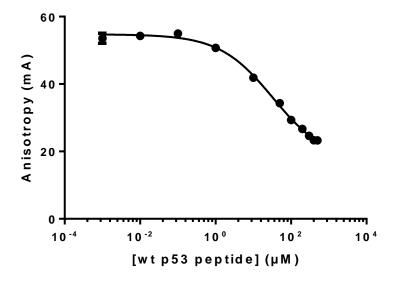


Figure S 2: Fluorescence Anisotropy dose response titration of wt p53 peptide vs FAM-p4 on hDMX

hDM2

For binding each well contained $10 \mu L$ of hDM2 protein in 10-fold dilutions (0.50 pM to 5.0 μ M) and 90 μ L of 10 nM fluorescently-tagged high affinity binding peptide FAM-LTFEHYWAQLTSCONH₂. Peptide and protein were dissolved in PBS: 0.05% Tween-20 buffer at pH 7.4. Reagents were incubated for 30 min at room temperature prior to reading. Bubbles were removed and 8 data points were generated. Nonlinear regression was then used to process the data, giving the K_D of the fluorescently-tagged peptide to be 1.44 nM. Performed in triplicate.

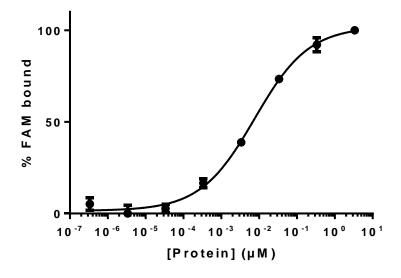


Figure S 3: Fluorescence Anisotropy direct titration. Fraction bound of FAM-p4 on hDM2. Normalized

A titration of 100 nM hDM2 with FAM-p4 suggested that with a 100 μL well volume 10 nM FAM-p4 peptide produced the greatest change in polarisation. Inhibition screens were carried out using 10 nM hDM2, 10 nM fluorescently-tagged peptide and 100 μM of inhibitor. Compounds were dissolved in DMSO. A positive control (wt p53 peptide) and negative control (DMSO) were used to define the minimum and maximum values and ensure the assay was performing adequately.

Compounds showing inhibition were then diluted in 10-fold dilutions (from 100 μ M to 10 pM well concentration) to generate IC₅₀ values, in duplicate. The IC₅₀ of p53 peptide was calculated to be 14.45 μ M.

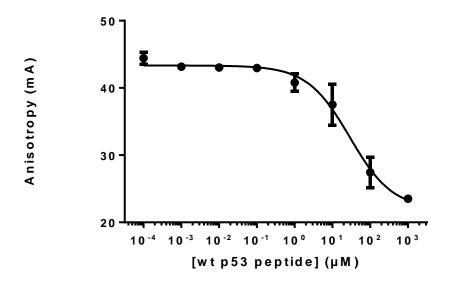


Figure S 4: Fluorescence Anisotropy dose response titration of wt p53 peptide vs FAM-p4 on hDM2

Differential Scanning Fluorimetry

A solution of protein (10 μM) and detection dye SYPRO orange (5X) and compound (30 μM) or vehicle (10% final DMSO concentration) were added to rotor gene-Q RT-PCR sample tubes, in the presence of buffer (final volume 20 μL). A buffer screen was performed to determine the most ideal buffer to observe a thermal stability effect between p53 and DMSO. The screened buffers included sodium acetate pH 5.0, pyridine pH 5.5, sodium cacodylate pH 6.0, MES pH 6.5, sodium citrate pH 6.5, imidazole pH 7.0, bis-tris pH 7.2, MOPS pH 7.2, HEPES pH 7.5, sodium phosphate pH 7.5, tricine pH 8.0, tris pH 8.0, glycine pH 9.0, borax pH 9.0, CHES pH 9.5, ethanolamine pH 9.5. buffers contained 50 mM of buffer compound, 1 mM TCEP and 150 mM NaCl. Sodium cacodylate was highlighted as the most appropriate buffer. Samples were incubated for 30 mins at 25°C then scanned at a ramp of 0.5°C/min from 25°C to 95°C. The fluorescence intensity at an excitation of 465 nm and emission of 580 nm was recorded. All measurements were run in triplicate. The first derivate of the melting curve was analysed to identify the inflexion point, which correlated to the melting temperature.

5. Molecular Docking

The X-ray crystal structures of hDMX with a modified p53 ligand (PDB ID 2GV2), and hDM2 with the same p53 ligand (3FEA) was utilized for docking calculations. The ligand was removed and the protein was prepared using the Protein Preparation application from the Schrodinger Suite 2015-2. The bound peptide was modified using Maestro drawing tools, and the resulting receptor was again prepared using Protein Preparation. Ligands were taken from the Sigma-Aldrich virtual library (896 alkynes and 214 azides) and prepared using the LigPrep application from the Schrodinger Suite 2015-2. Binding site grid generation was preformed using the Receptor Grid Generation application from the Schrodinger 2015-2 suite using the using the pocket left vacant by the removed section of the peptide, using the centre point of coordinates X 12.4 Y 12.6 Z 13.2 for

the alkyne ligands, or the centre point of coordinates X 13.4 Y 20.5 Z 12.8 for the azide ligands for hDM2, or the centre point of coordinates X 21.3 Y 19.0 Z 5.0 for the alkyne ligands, or the centre point of coordinates X 14.1 Y 21.3 Z 4.7 for the azide ligands for hDMX. Covalent docking was performed using the CovDock tool from the Schrodinger Suite 2015-2,^[5] using the following custom reaction code to generate the desired azido-alkynyl condensation.

Alkyne receptor, azide ligand:

```
LIGAND_SMARTS_PATTERN 4,CN=[N+]=[N-]
RECEPTOR_SMARTS_PATTERN 2,C#CCC=O
CUSTOM_CHEMISTRY ("<1>|<2>",("bond",1,(1,2)))
CUSTOM_CHEMISTRY ("<1>#C",("bond",2,(1,2)))
CUSTOM_CHEMISTRY ("<1>[N-]=[N+]=NC",("bond",1,(3,4)))
CUSTOM_CHEMISTRY ("<1>(=C)[N-]=[N+]N",("bond",1,(2,5)))
```

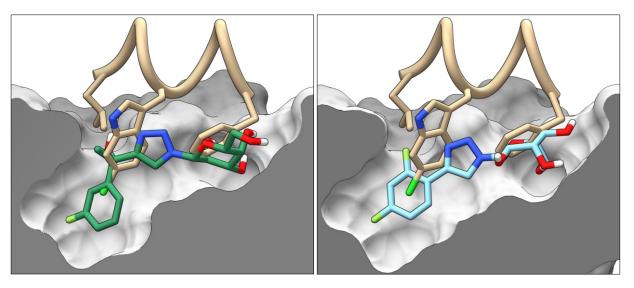
Azide receptor, alkyne ligand:

```
RECEPTOR_SMARTS_PATTERN 4,CN=[N+]=[N-] LIGAND_SMARTS_PATTERN 2,C#C CUSTOM_CHEMISTRY ("<1>",("charge", 0, 1)) CUSTOM_CHEMISTRY ("<1>=[N+]",("charge", 0, 2)) CUSTOM_CHEMISTRY ("<1>|<2>",("bond",1,(1,2))) CUSTOM_CHEMISTRY ("<2>#C",("bond",2,(1,2))) CUSTOM_CHEMISTRY ("<1>=[N]=[N]",("bond",1,(2,3))) CUSTOM_CHEMISTRY ("<1>[N]=[N]",("bond",2,(1,2))) CUSTOM_CHEMISTRY ("<1>[N]",("bond",2,(1,2))) CUSTOM_CHEMISTRY ("<1>[C]=[C])=[N][N]",("bond",1,(3,5)))
```

Induced fit docking was performed with protein flexibility using the Induced Fit^[6] application from the Schrodinger Suite 2015-2. A total of 100 possible binding conformations were generated and grouped into clusters using a 1.0 Å root-mean-square tolerance. Conformations were then ranked based on their Glide e-model scores.^[7] For induced fit docking, conformations with the lowest glide-emodel score were docked again using Induced Fit to generate the lowest energy conformation. Figures were generated using the Chimera package from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH P41 RR-01081).

Docking Poses

Representative binding poses of the most potent compounds, 2 & 3, are displayed. Poses are presented to provide insight in to possible binding of designed PPI modulators.



LEFT: Binding pose of **2** (green) to *h*DMX in the p53 binding site, with an overlay of peptide **1** (beige) showing the binding residues of Phe and Cl-Trp. The surface of *h*DMX is cut along the plane of the centroid of the aromatic rings of compound **2**. **RIGHT**: Binding pose of **3** (blue) to *h*DMX in the p53 binding site, with an overlay of peptide **1** (beige) showing the binding residues of Phe and Cl-Trp. The surface of *h*DMX is cut along the plane of the centroid of the aromatic rings of compound **3**.

6. Cell Culture

The human SJSA-1, A549, HCT116, MCF-7 and JEG3 cell lines were purchased from the European Collection of Cell Cultures (ECACC, Porton Down, UK). SJSA-1 and A549 cells were cultured in RPMI-1640 media with 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% FCS. MCF-7 cells and HCT-116 cells were cultured in DMEM media containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% FCS. JEG3 cells were cultured in EMEM media containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% FCS. Cells were sub-cultured twice weekly and maintained at 37 °C and 5% CO₂.

7. Cellular assays

MTS Cytotoxicity Assay

The anti-proliferative activity of the compounds studied was assessed by MTS assay (Promega) following the manufacturer's instructions. Cells were seeded at 5×10^3 / $100~\mu L$ in a 96-well plate and left untreated or treated with DMSO (vehicle control), or compounds ($100~\mu M - 10~pm$, in well concentration) in triplicate for 72 h at 37 °C with 5% CO₂. Following this, MTS assay reagent was added to each well and the plates incubated for 4 h at 37 °C with 5% CO₂. The absorbance was measured at 492 nm using the BMG Labtech POLARstarOPTIMA microplate reader. IC₅₀ values were calculated using GraphPad Prism Version 6.0 software, using a four-parameter logistic non-linear regression model.

All experiments were performed at least three times. Triplicate implies different cell passages, fresh compound stock solutions and different days. Each replicate included a triplicate of samples on that day.

Caspase 3/7 activation assay

Caspase 3/7 activation was determined using the chemiluminescent Caspase-Glo 3/7 reagent (Promega) in the cell lines A549 and JEG3. Cells were seeded in 96 well, white plates at 1×10^4 cells/well and treated with compounds at the determined IC₅₀ for 4 h. Cells were subsequently

treated according to the manufacturer's protocol. Samples were analysed using a BMG Labtech clariostar microplate reader. Data was analysed using GraphPad Prism Version 6.0 software.

Quantitative RT-PCR

JEG3 cells were seeded at 1×10^6 in 6 well plates and treated with compounds at 100 μ M in triplicate. DMSO was used as a vehicle control. Cells were incubated for 6 hr at 37 °C. RNA was isolated using Trizol (1 mL/well) and precipitated using 1-bromo-3-chloropropane. Purification was performed with isopropanol followed by 75% ethanol. 1 μ g of RNA was reverse transcribed to cDNA using Applied Biosystems high-capacity reverse transcription kit. The derived cDNA was used as the substrate to measure relative transcript levels by qRT-PCR on a Rotor Gene-Q system using SYBR green PCR master mix (Sigma-Aldrich). Primers specific for p53, hDM2, p21, MIC-1 and the 18S control were employed.

Primers for quantitative real-time RT-PCR:

Human p21 forward primer – GAGGCCGGGATGAGTTGGGAGGAG

reverse primer - CAGCCGGCGTTTGGAGTGGTAGAA

Human p53 forward primer – CCCCTCCTGGCCCCTGTCATCTTC

reverse primer – GCAGCGCCTCACAACCTCCGTCAT

Human HDM2 forward primer – TAGTATTTCCCTTTCGATGA

reverse primer – CACTCTCCCCTGCCTGATAC

Human MIC-1 forward primer – CGCGCAACGGGGACGACT

reverse primer - TGAGCACCATGGGATTGTAGC

Human 18S forward primer – GCCGCTAGAGGTGAAATTCTTG

Reverse primer – CATTCTTGGCAAATGCTTTCG

Reactions were performed with at least three triplicates. Threshold cycle (C_t) values were automatically calculated for each replicate and used to determine the relative expression of the gene of interest relative to I8S for both treated and untreated sampled by the $2^{-\Delta\Delta Ct}$ method.

8. STD NMR spectroscopy

The NMR spectra were recorded at 25 °C in PBS buffer pH 7.4, d₆-DMSO (8%) and D₂O as solvent, on an Ultra-Compact 800 MHz Bruker Avance III NMR spectrometer equipped with an inverse triple resonance (H/C/N) z-gradient probe head. For sample preparation, ligand was lyophilized twice with 99 % D₂O and once final in 99.99 % D₂O from Sigma-Aldrich. Final concentrations were 28 µM hDM2/X and 2 mM of compound 4. Chemical shift assignments were obtained at 400 MHz by means of 2D NMR experiments COSY and HSQC. STD NMR experiments were carried out by a pseudo 2D pulse sequence including spoil pulses to destroy residual magnetization during the relaxation delay (two trim pulses of 2.5 and 5 ms, followed by a 3 ms gradient pulse on the Zaxis). For selective saturation of hDM2/X, cascades of 49 ms Gaussian-shaped pulses were used with a 1 ms delay between successive pulses. Total saturation times for STD build-up curves were: 0.5, 0.75, 1, 1.5, 2, 3, 4 and 5 seconds. The selective saturation of the protein was achieved by setting the on-resonance frequency at 0.6 ppm, to produce direct saturation of most of the aliphatic side chains of the protein. The irradiation frequency was shifted to 40 ppm for the reference (offresonance) spectrum. The absence of direct irradiation of ligand 1H signals was verified by blank STD NMR experiments (without protein). STD NMR experiments were typically carried out with 128 scans, increasing to 512 at shorter saturation times. To obtain the binding epitope for verification of the ligand pharmacophore, the initial growth rates of the STD build-up curves were determined by mathematical fitting of the curves to a monoexponential curve $STD(t) = STD_{max} \cdot (1 - t)$

 $exp(-k_{sat} \cdot t)$). STD_{max} and k_{sat} are the two parameters to be determined by mathematical fitting, and represent the asymptotic maximum of the STD build-up curve, and a measure of how the chemical exchange and the relaxation properties of a given proton contribute to the STD accumulation in solution. After the mathematical fitting, the initial growth rate of the curve is obtained by the product $STD_{max} \cdot k_{sat}$. With these values, the binding epitope of the ligand was determined by assigning 100 % relative value to the most intense proton, and normalizing the values of the remaining ligand's proton against it.

Protein-Ligand docking calculations.

The crystal structure of hDM2 (PDB 2GV2) was imported into Schrödinger Maestro and was prepared with the Protein Preparation Wizard. All buffer atoms and non-bridging waters were removed. Protons were then added to the model, using PROPKA to predict the protonation state of polar sidechains at pH 7. The hydrogen-bonding network was automatically optimised by sampling asparagine, glutamine and histidine rotamers. The model was then minimised using the OPLS3 force field and a heavy atom convergence threshold of 0.3 Å. Docking of compound 4 to hDM2 was then performed using Glide. A cubic grid was generated, centered on the hydrophobic binding pocket of hDM2, with dimensions of 20 Å. The ligand was docked using the flexible SP algorithm, using enhanced sampling (x4). The fluorinated ring was constrained to the position of the ligand tryptophan in the crystal structure. The lowest energy complex was selected before refinement of all sidechains within 5 Å of the ligand using Prime.

CORCEMA calculations

Protein chemical shifts were calculated using the SHIFTX2 webserver according to experimental conditions. All protein protons within 10 Å of the ligand were considered in the calculation. The instrument field strength, solvent type, ligand concentration, and protein concentration were set according to experimental values. The free and bound ligand correlation times were estimated to be 0.1 ns and 50 ns respectively, based on reasonable values for a small molecule binding to a 14 kDa (hDM2) or 73 kDa (hDMX) protein. The non-specific leakage was also optimised to 0.4 Hz. The internal correlation time was set to 10 ps and the methyl-X order parameter was set to 0.85. All protein protons with resonances between 0.4– 0.8 ppm were considered to be instantaneously saturated to account for line broadening. The equilibrium constant and k_{on} were optimised to 10000 M⁻¹ and 10⁵ M⁻¹ s⁻¹ respectively.

STD NMR Initial Rates Method

In determining the STD binding epitope, the initial rates of STD build up (STD0) are used to eliminate any effects of ligand rebinding. STD0 can be calculated from:

$$STD_0 = STD_{max} \cdot k_{sat}$$

Where STD_{max} is the equilibrium STD intensity and k_{sat} is the rate constant for saturation transfer, both of which can be calculated from least squares fitting of the raw STD data to:

$$STD(t_{sat}) = STD_{max} \cdot (1 - \exp(-k_{sat} \cdot t_{sat}))$$

Where t_{sat} is the saturation time and STD(t_{sat}) is the STD intensity at a given saturation time. Each STD0 value is then normalised against the largest STD0 value.

STD NMR Data

hDM2

(s)	STD Intensity (%)							
				Satura	tion Time	(s)		
δ 1H (ppm)	0.5	0.75	1	1.5	2	3	4	5
7.62	4.32	6.02	7.32	9.16	10.91	12.51	13.40	13.53
7.18	4.24	5.62	6.90	9.34	10.91	12.51	13.14	13.27
7.15	4.58	6.26	7.68	9.9	11.57	13.14	14.34	14.48
6.95	4.95	6.70	8.14	10.7	12.27	14.34	15.21	15.81
5.71	3.66	4.49	5.15	6.02	5.90	6.64	6.64	7.10
1.88	3.55	4.19	4.45	4.95	5.05	5.20	5.20	5.20

δ 1H (ppm)	k _{sat} (s ⁻¹)	STD _{max} (%)	STD0 (% s ⁻¹)	STD0 (normalised)
7.62	0.74	14.01	10.33	0.90
7.18	0.73	13.88	10.09	0.88
7.15	0.72	14.98	10.85	0.94
6.95	0.71	16.23	11.52	1
5.71	1.46	6.73	9.82	0.85
1.88	2.21	5.16	11.40	0.99

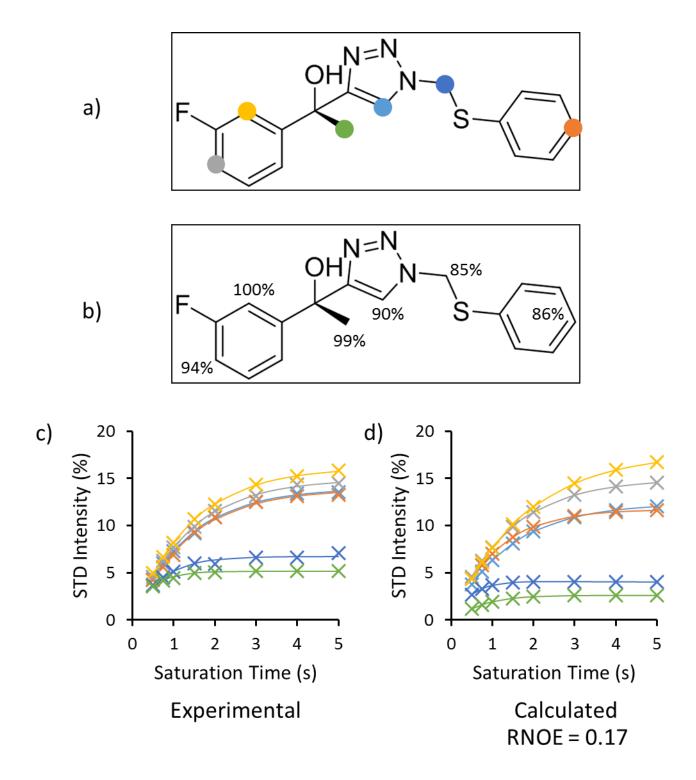
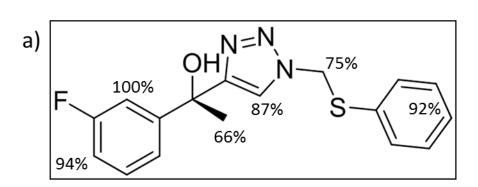


Figure S5: (a) Structure of compound 4 with protons analysed by STD NMR in coloured circles, corresponding to colours in c and d. (b) Ligand binding epitope of compound 4 in the presence of hDM2. Normalised values of STD0 used. (c) Experimental STD build up curve showing STD intensity as a function of time. Colours correspond to protons in a. Crosses show raw data, while curves show fitted data. (d) STD build up curve calculated from CORCEMA. Colours correspond to protons in a.

hDMX

STD intensities for compound 4 in the presence of hDMX as a function of saturation time								
	(s) STD Intensity (%)							
				Saturati	on Time (s	s)		
δ 1H (ppm)	0.5	0.75	1	1.5	2	3	4	5
7.62	28.68	42.79	52.52	60.20	61.99	63.22	63.22	64.46
7.18	29.53	44.93	54.61	62.60	65.09	65.09	67.03	64.46
7.15	31.01	45.81	55.68	63.83	66.38	67.03	68.35	67.03
6.95	32.88	48.10	57.90	65.73	67.68	68.35	69.69	68.35
5.71	25.02	36.96	44.49	50.51	53.03	53.55	54.08	55.14
1.88	22.25	31.31	36.96	41.15	42.37	43.21	43.63	43.63

Parameters used to determine STD0, calculated by least squares fitting to raw STD data							
δ 1H (ppm)	k _{sat} (s ⁻¹)	STD _{max} (%)	STD0 (% s ⁻¹)	STD0 (normailsed)			
7.62	1.44	64.93	93.73	0.87			
7.18	1.47	67.07	98.70	0.92			
7.15	1.46	68.87	100.79	0.94			
6.95	1.54	70.10	107.69	1			
5.71	1.45	55.25	80.38	0.75			
1.88	1.63	44.02	71.57	0.66			



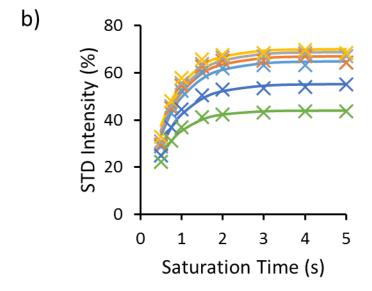
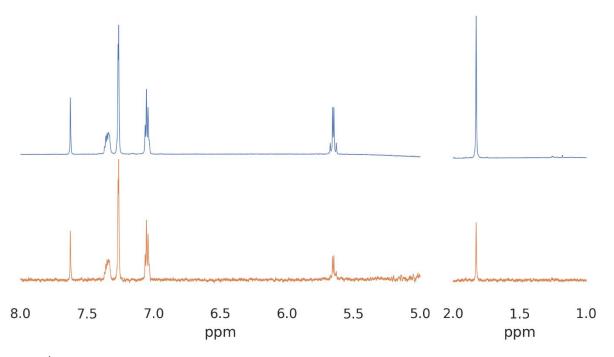
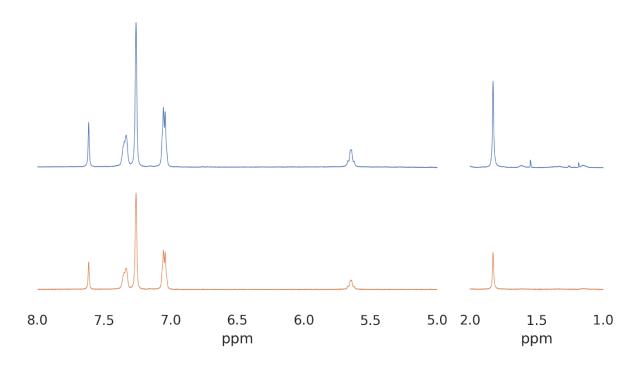


Figure S6: (a) Ligand binding epitope of compound 4 in the presence of hDMX. Normalised values of STD0 used. (b) Experimental STD build up curve showing STD intensity as a function of time. Colours correspond to protons in Fig S6a. Crosses show raw data, while curves show fitted data.

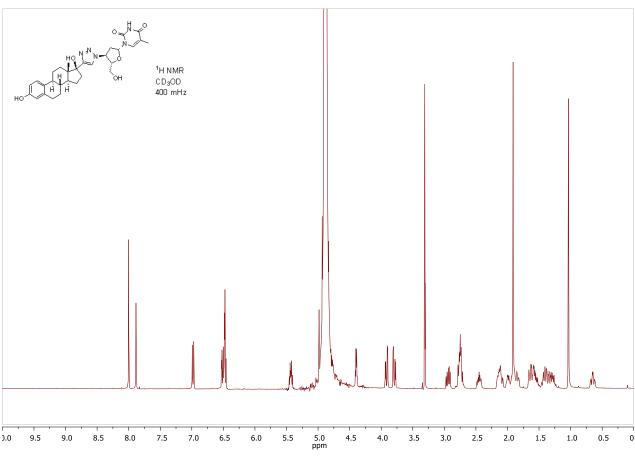


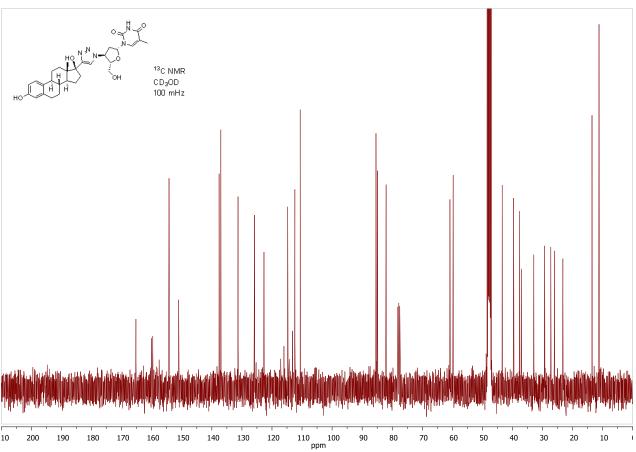
Reference 1H NMR spectrum (blue, top) of 2 mM compound **4** in the presence of 28 μ M hDM2 in PBS buffer pH 7.4, d₆-DMSO (8%) and D₂O as the solvent. Region between 2-5 ppm omitted for clarity as no compound **4** resonances occur in this region. STD NMR difference spectrum for a saturation time of 2 s (orange, bottom) is magnified 8x for clarity and shows the presence of compound **4** resonances, indicating binding.

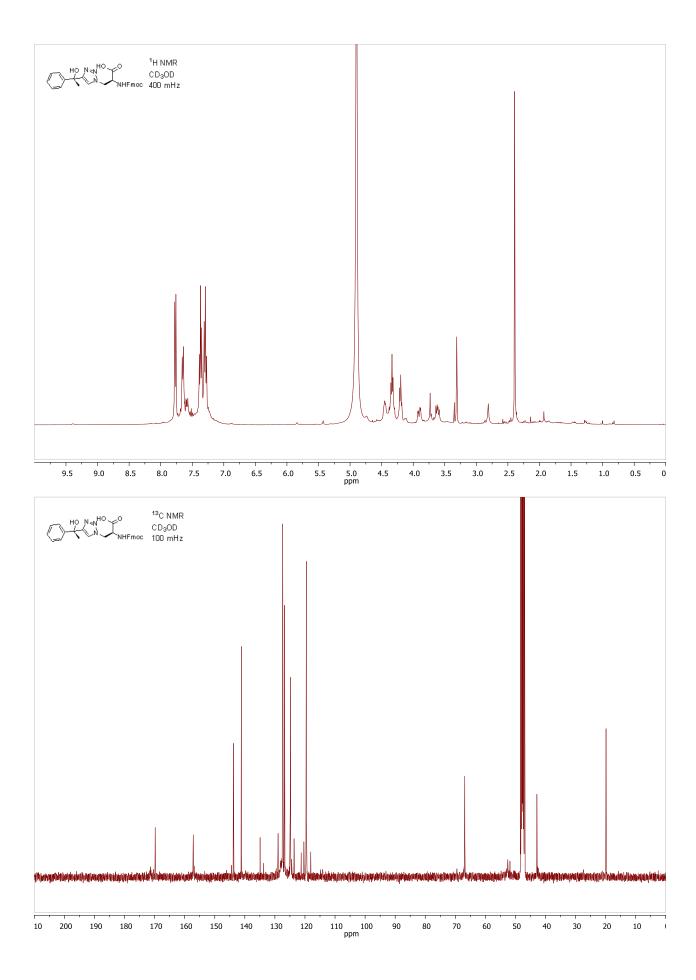


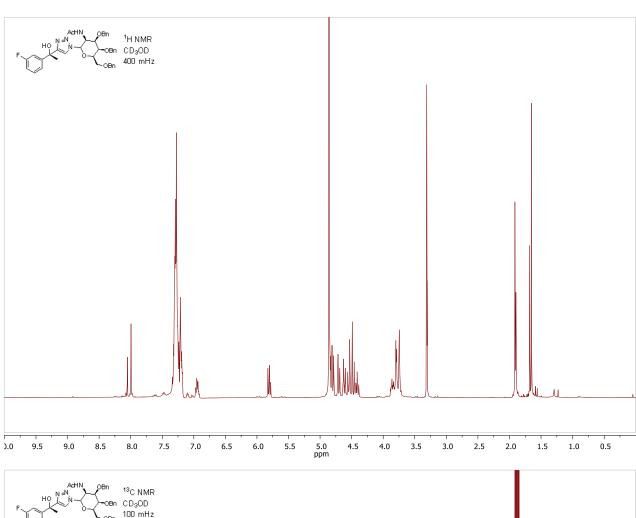
Reference 1H NMR spectrum (blue, top) of 2 mM compound 4 in the presence of 28 μ M hDMX in PBS buffer pH 7.4, d₆-DMSO (8%) and D₂O as the solvent. Region between 2-5 ppm omitted for clarity as no compound 4 resonances occur in this region. STD NMR difference spectrum for a saturation time of 2 s (orange, bottom) is shown 1:1 compared to reference. Larger intensities of compound 4 resonances indicate stronger binding to hDMX relative to hDM2. This is further confirmed by some degree of broadening in the presence of hDMX.

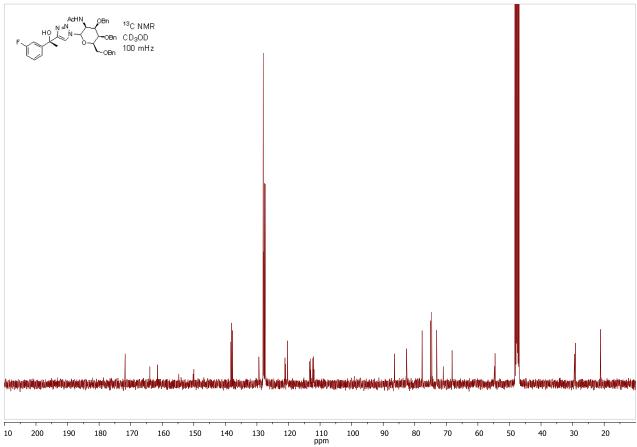
8. NMR Spectra

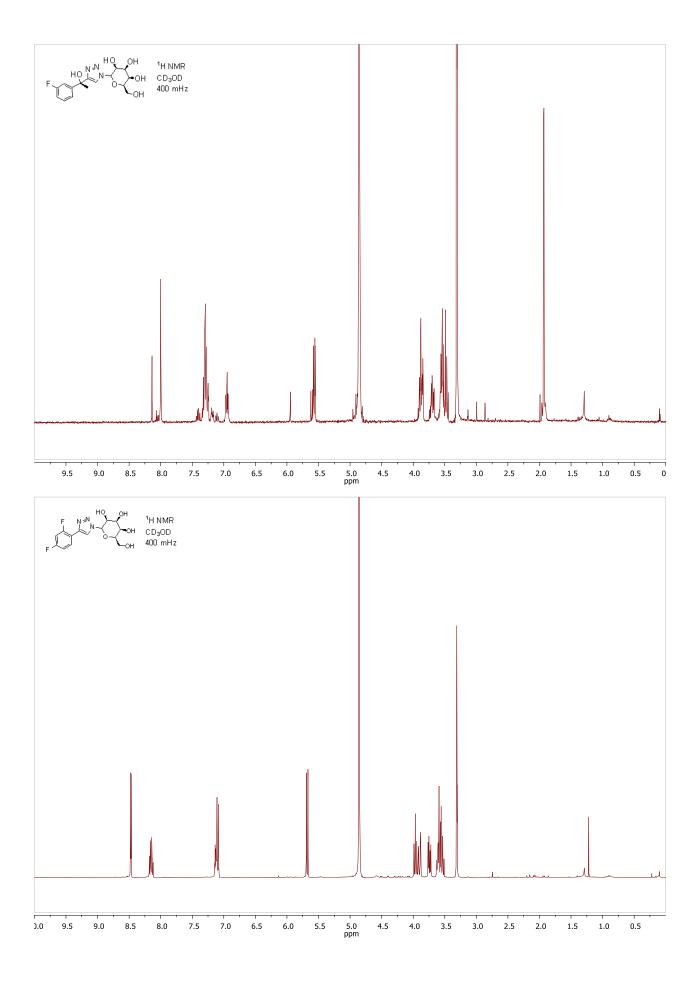


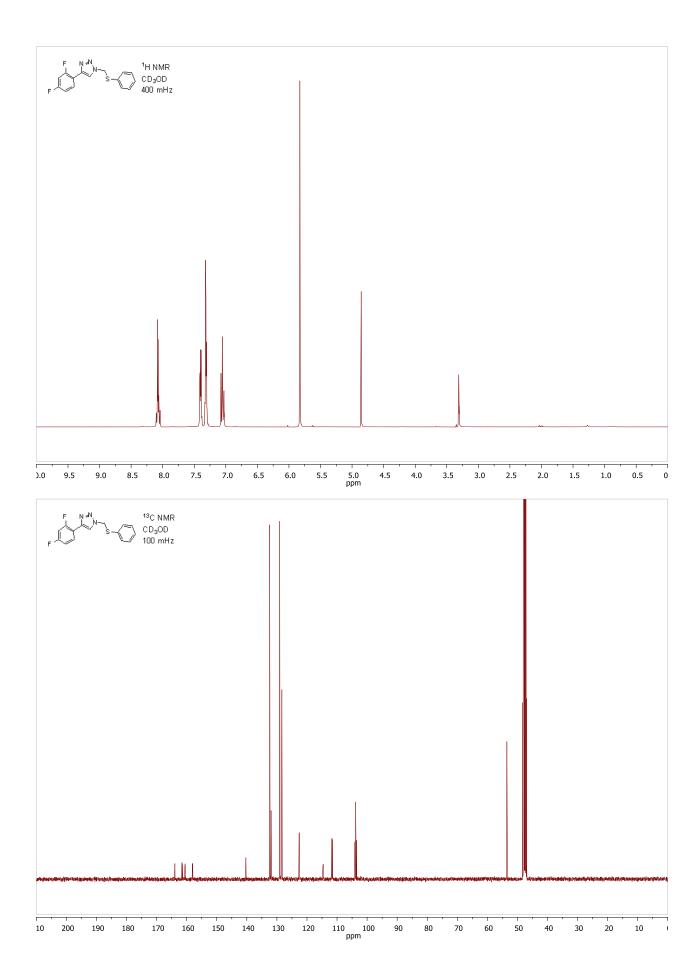


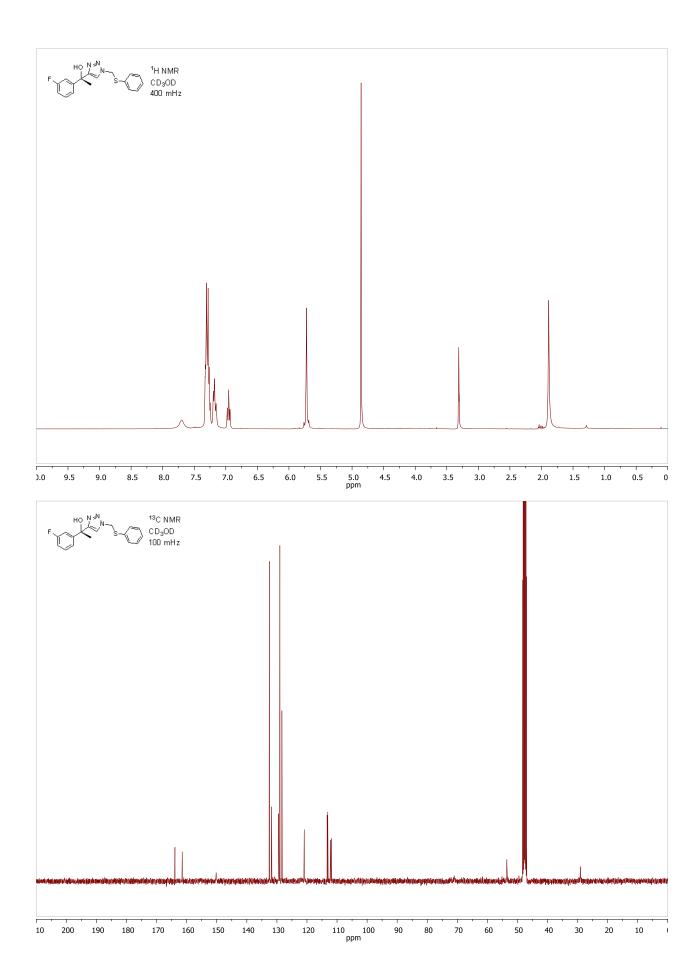


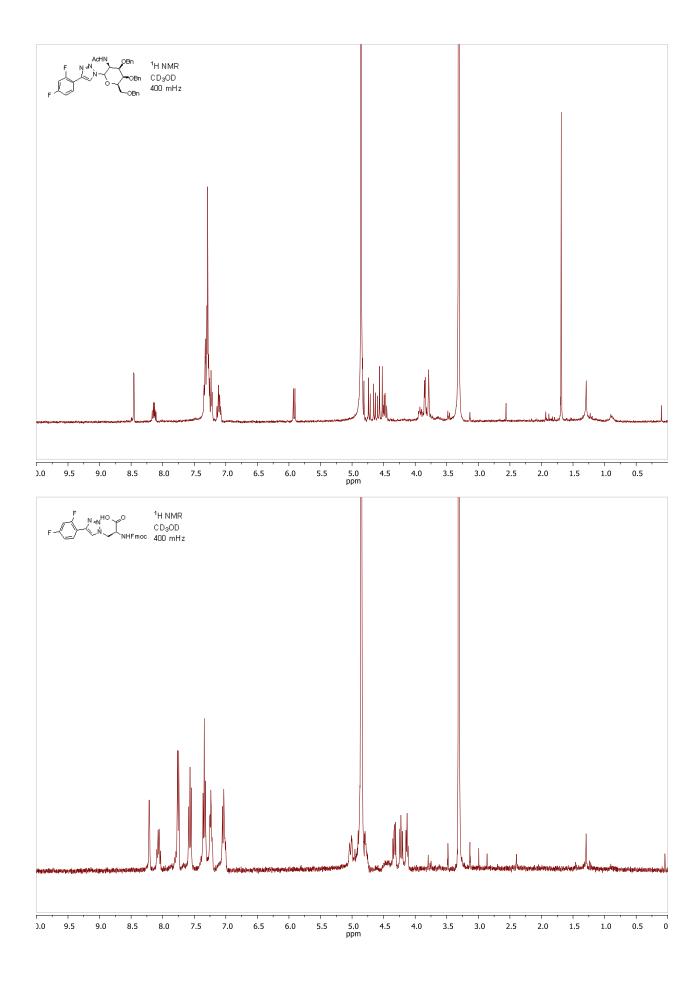


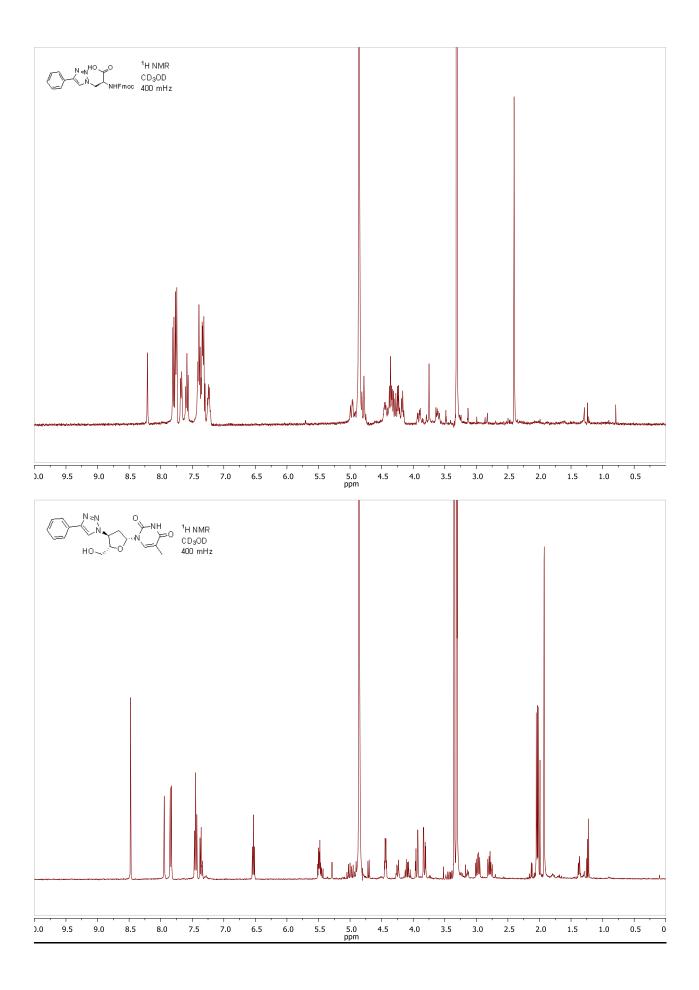


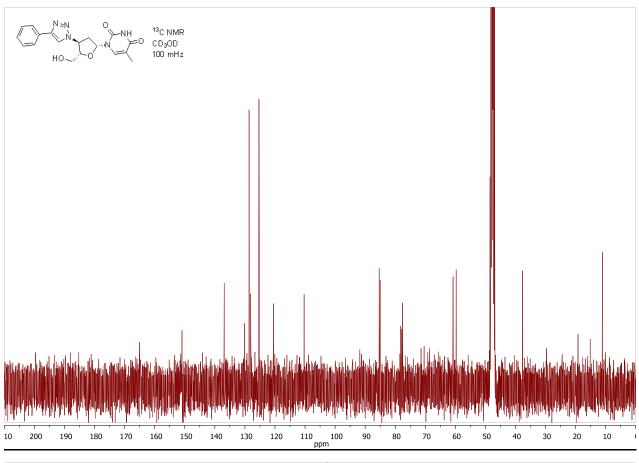


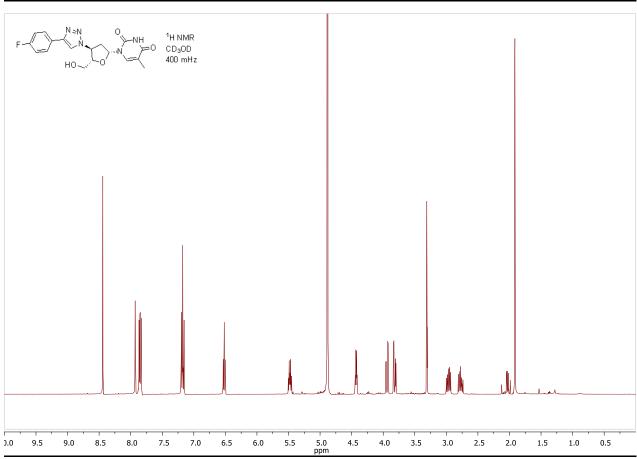


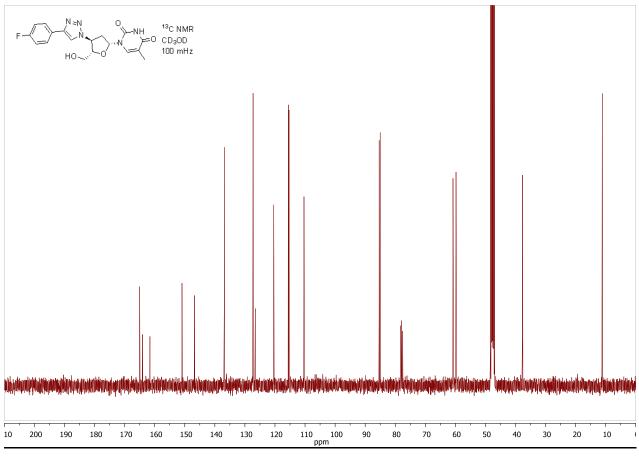


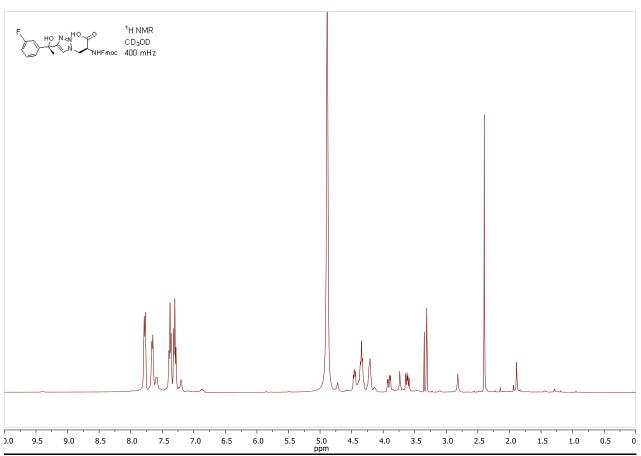


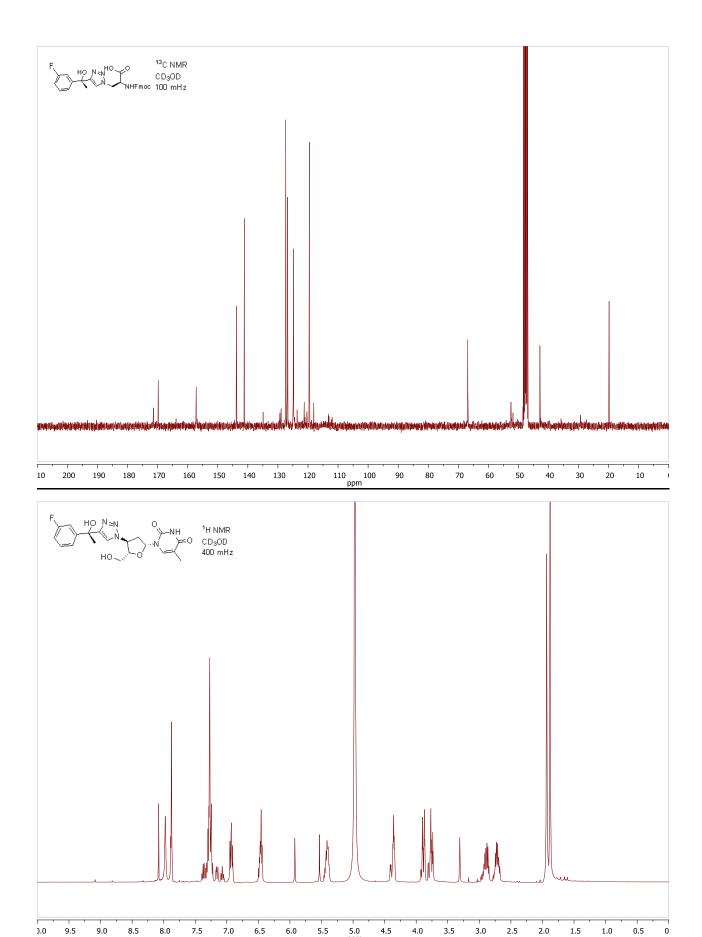


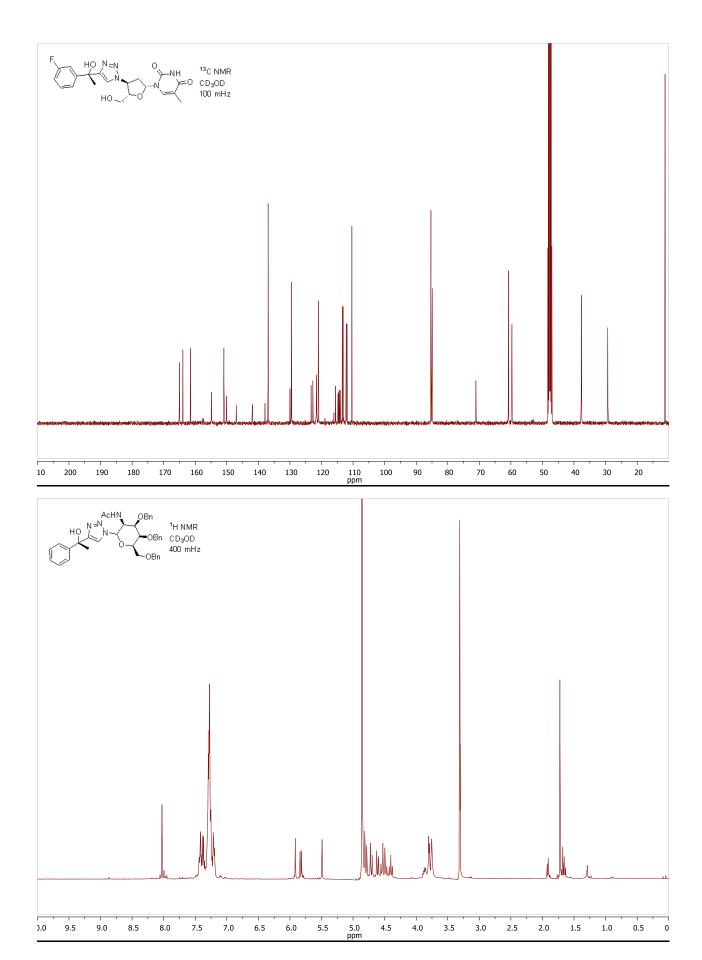


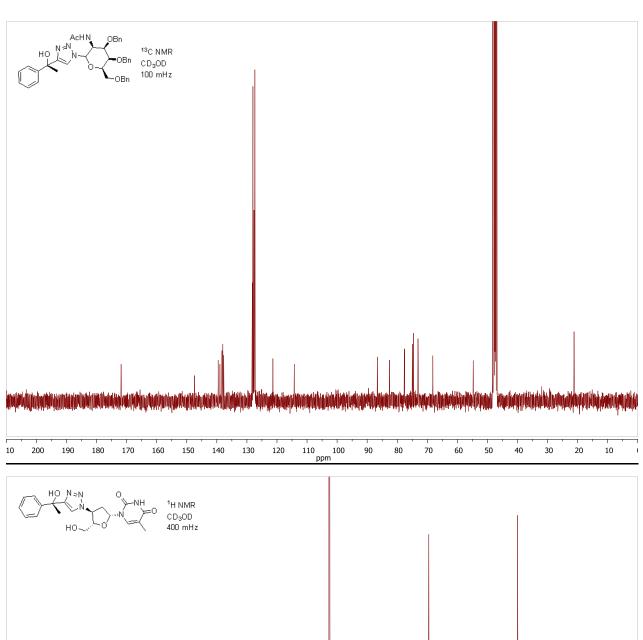


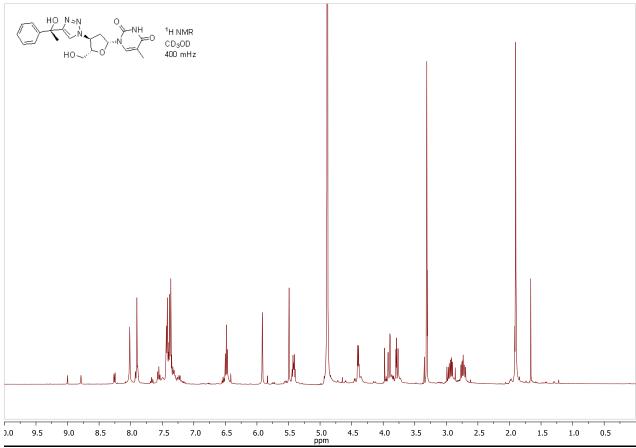


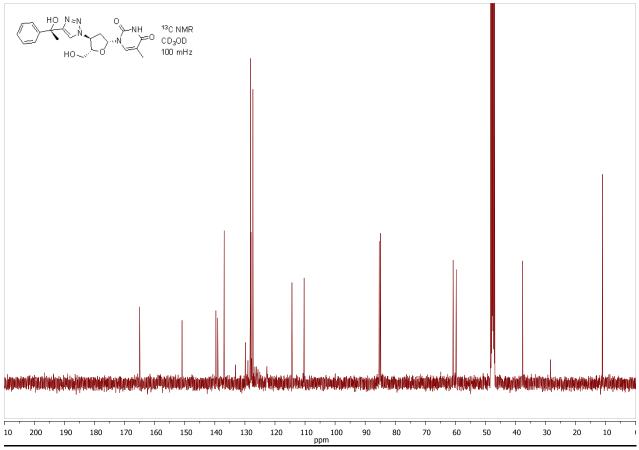


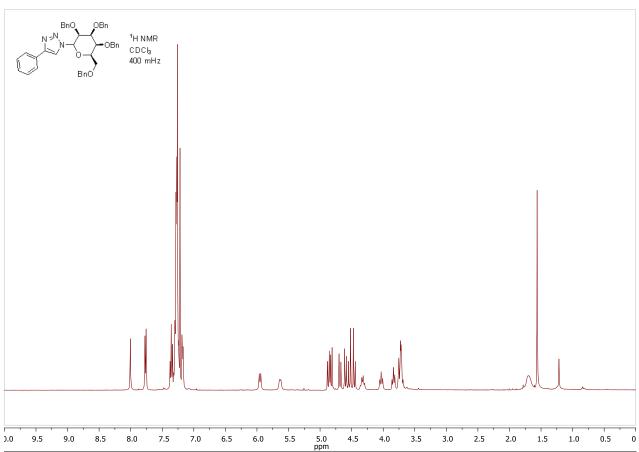


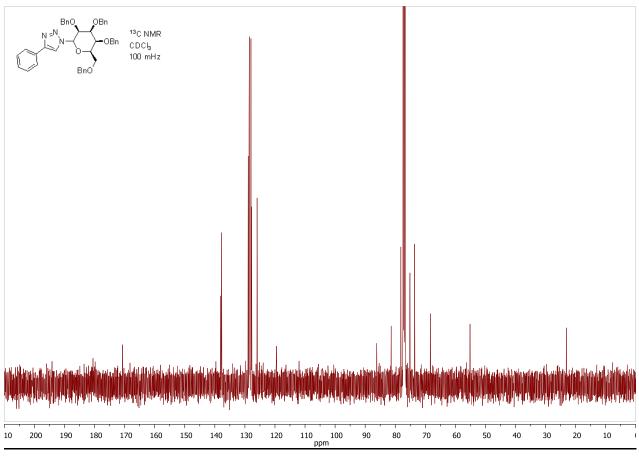


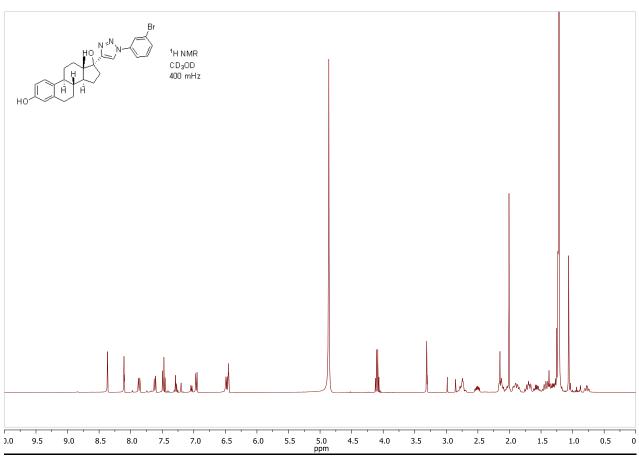


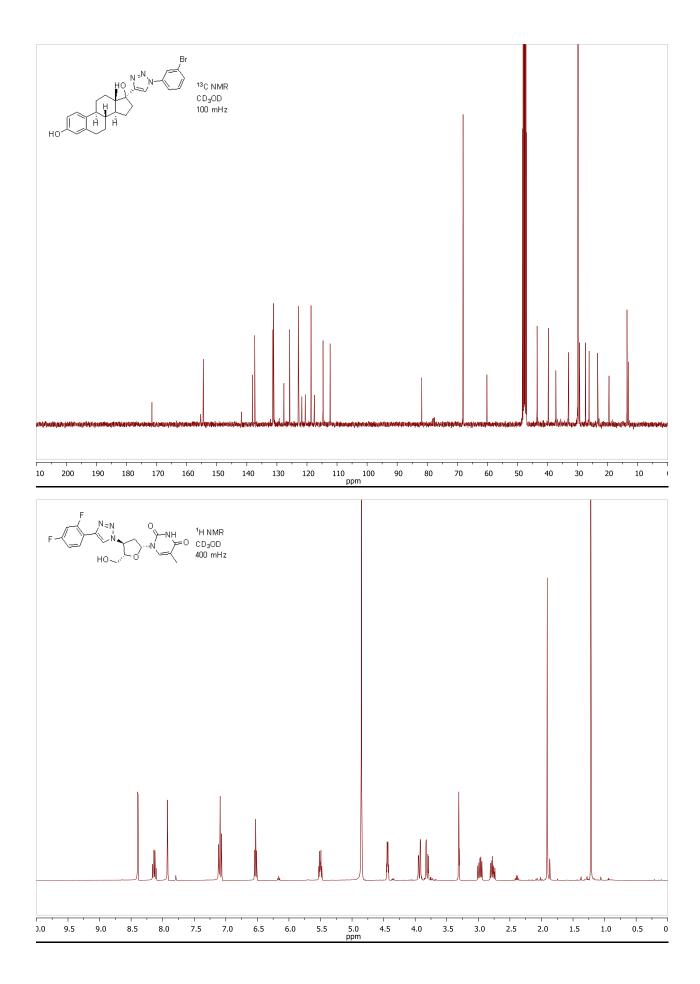


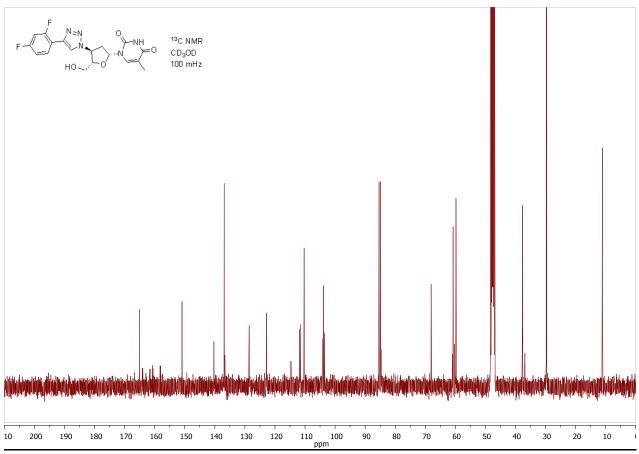


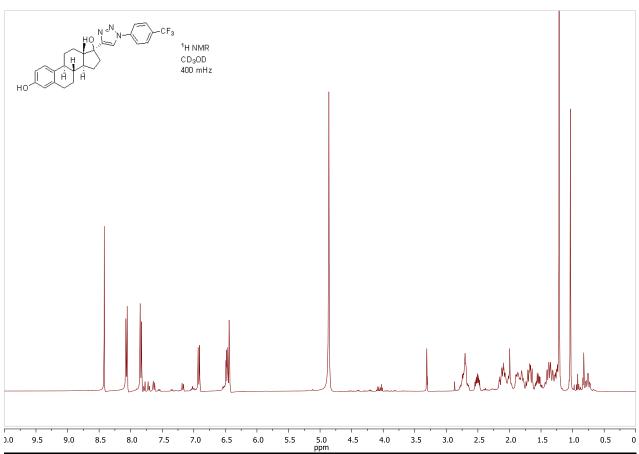


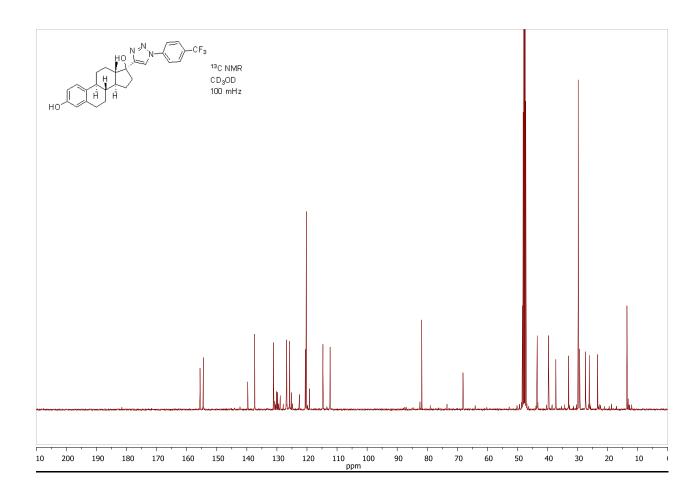












9. hDM2 Fluorescence Anisotropy Assay

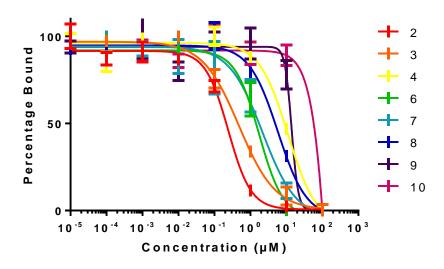


Figure S 7: Fluorescence Anisotropy dose response titration of compounds AB3018-AB3079 & vs FAM-p4 on hDM2. Normalized

10. hDMX Fluorescence Anisotropy Assay

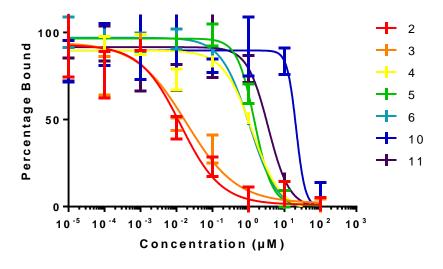


Figure S 8: Fluorescence Anisotropy dose response titration of compounds AB3043-AB3078 vs FAM-p4 on hDMX. Normalized

11. Differential Scanning Fluorimetry

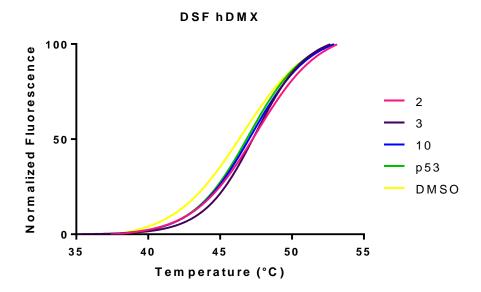


Figure S 9: Normalized fluorescence data for the thermal denaturation of hDMX in the presence of compounds AB3043-AB3070, p53 and DMSO.

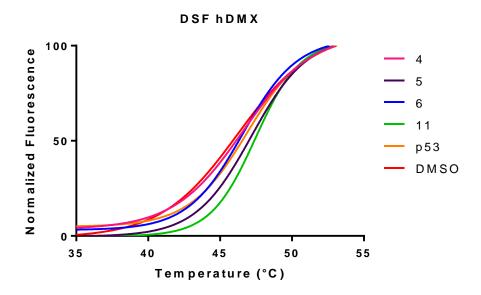


Figure S 10: Normalized fluorescence data for the thermal denaturation of hDMX in the presence of compounds AB3072-AB3078, p53 and DMSO.

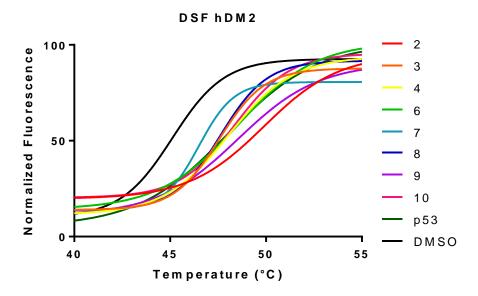


Figure S 11: Normalized fluorescence data for the thermal denaturation of hDM2 in the presence of compounds AB3018-AB3079, p53 and DMSO.

Table S2: DSF analysis of compounds which demonstrated binding in the FA assay

hDM2			hDMX		
Compound	T_m	ΔT_m	Compound	T_m	ΔT_m
2	49.89	4.77	2	46.41	1.2
3	47.56	2.44	3	46.38	1.17
4	48.03	2.91	4	46.59	1.38
6	48.50	3.38	5	47.17	1.96
7	46.50	1.38	6	46.57	1.36
8	47.62	2.50	8	45.23	0.02
9	48.53	3.41	9	45.15	-0.06
10	48.35	3.23	10	45.62	0.41
11	45.07	-0.05	11	47.46	2.25
p53	47.97	2.85	p53	46.03	0.82
DMSO	45.12	0	DMSO	45.21	0

12. Cell growth inhibition assay

5

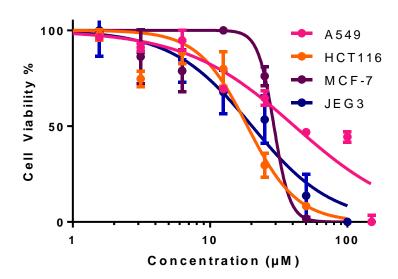


Figure S 12: MTS assay dose response of compounds 5 towards A549, HCT116, MCF-7 and JEG3 cell lines. Normalized

10

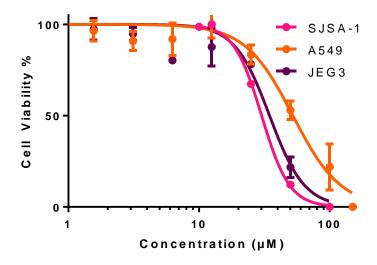


Figure S 13: MTS assay dose response of compounds 10 towards SJSA-1, A549 and JEG3 cells. Normalized

13. References

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