Supporting Information

Discovery, X-ray structure and CPP-conjugation enabled uptake of p53/MDM2 macrocyclic peptide inhibitors

Anselm F. L. Schneider,[a] Joerg Kallen,[b] Johannes Ottl,[b] Patrick C. Reid,[c] Sebastien Ripoche,[b] Stephan Ruetz,[b] Therese-Marie Stachyra,[b] Samuel Hintermann,[b] Christoph E. Dumelin,[b] Christian P. R. Hackenberger,*[a,d] Andreas L. Marzinzik*[b]

* Corresponding authors

a Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Robert-Rössle-Strasse 10, 13125 Berlin (Germany)

E-mail: hackenbe@fmp-berlin.de

b Novartis Institutes for BioMedical Research, Novartis Campus, CH-4056 Basel, Switzerland

E-mail: andreas.marzinzik@novartis.com

c PeptiDream, 3-25-23 Tonomachi, Kawasaki-Ku, Kanagawa, Japan 210-0821

d Humboldt Universität zu Berlin, Institut für Chemie, Brook-Taylor-Str. 2, 12489 Berlin, Germany

Table of Contents

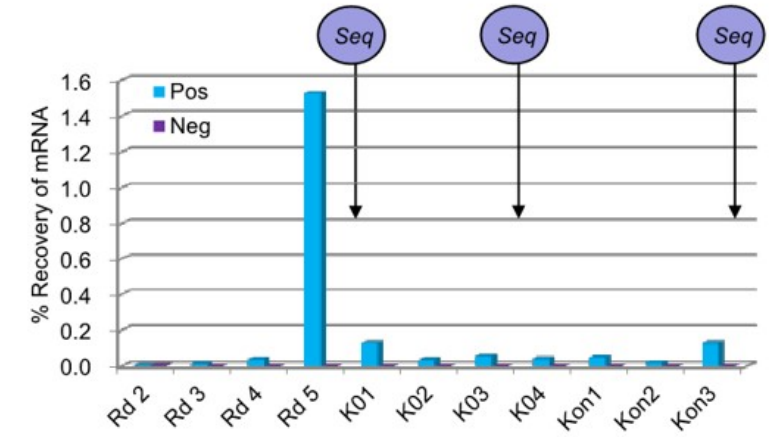
1. Library composition and selection

The peptide coding region of the naïve mRNA template for the MDM2 selection campaign contained a 10-13 NNU codon region to encode the random sequence which is flanked by the 5' initator AUG codon and a 3' eight codon region to encode CGSGSGS followed by an amber stop codon (TAG).

The in vitro translation system was reprogrammed through Flexizyme-mediated genetic code reprogramming as previously described.[1-3] Briefly, *N*-chloroacetyl L-Phe was used in place of the initiator methionine. For elongator positions, the natural amino acids Ser, Tyr, Pro, His, Arg, Thr, Asn, Val, Asp, Gly and Cys along with the non-natural elongators *N*-Methyl-Ser, *N*-Methyl-Phe, *N*-Methyl-Gly, *N*-Methyl-Ala and biphenylalanine were used. Applying this reprogrammed translation system to the aforementioned mRNA template affords a 12 to 15 amino acid thioether macrocyclic peptide library with each peptide containing a C-terminal Gly-Ser linker. Starting with an initial round containing > 10^{13} unique cyclic peptides, MDM2-specific binders were enriched using biotinylated-avitagged MDM2 bound streptavidin conjugated magnetic beads and non-specific binders were removed using the streptavidin conjugated magnetic beads only. Library-MDM2 binding steps were performed in an iterative manner while the binding-stringency was increased in latter rounds through temperature, incubation times and/or wash steps. Selections rounds marked as "Seq" were used for NGS to analyze the peptides sequences that were enriched.

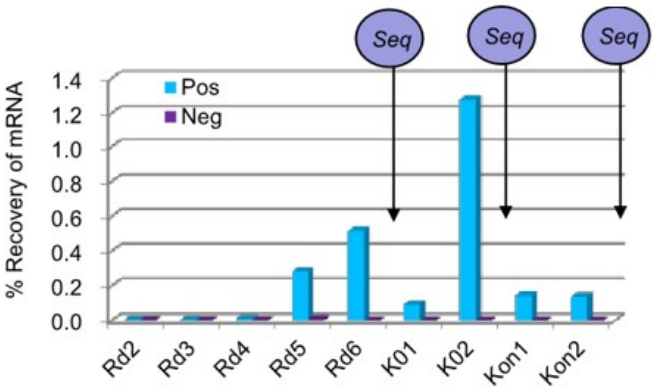
	U	C	A	G	
U	MePhe	Ser	Tyr	Bph	U C A G
C	MeSer	Pro	His	Arg	U C A G
A	MeGly	Thr	Asn	Ser	U C A G
	ClAcX				
G	Val	MeAla	Asp	Gly	U C A G

Supplementary Fig. 1 Cys-less NNU library composition. This library has filled the vacant TGT codon with biphenylalanine. The initiation start and C-terminal C are inserted via ATG codon.



Positive selection condition

	Temp.	Time	Conc.
Rd2	4C	1hour	250nM
Rd3	RT	30min	100nM
Rd4	RT	30min	100nM
Rd5	RT	30min	100nM
K1	37C	30min,wash 30min	100nM
K2	37C	30min,wash 30min	100nM
K3	37C	30min,wash 30min	100nM
K4	37C	30min,wash 30min	100nM
Kon1	37C	5min,wash 30min	100nM
Kon2	37C	5min,wash 30min	100nM
Kon3	37C	30min	100nM



Positive selection condition

	Temp	Time	Conc.
Rd2	4C	1hr	250nM
Rd3	RT	30min	100nM
Rd4	RT	30min	100nM
Rd5	RT	30min	100nM
Rd6	RT	30min	100nM
K1	37C	30min	100nM
K2	37C	30min, wash 30min	100nM
Kon1	37C	5min, wash 30min	100nM
Kon2	37C	5min, wash 30min	100nM

Supplementary Fig. 2 qPCR selection result of targeting MDM2 with the rapid display system. Graph shows recovered cDNA per round of selection against MDM2 obtained from the macrocyclic thioether peptide library initiated by *N*-chloroacetyl *L*-Phe. Blue bars (Pos) represent the fraction corresponding to cDNA bound to the complex, while purple bars (Neg) indicate non-specific binders, including the peptide conjugates binding to streptavidin loaded magnetic beads. The tables provide temperature, incubation time and target concentration per round.

2. General synthesis information

Reagents and Solvents

All chemicals were reagent grade and were purchased from Sigma Aldrich and VWR. Fmoc-amino acids and coupling reagents were purchased from Novabiochem. Peptide synthesis was performed on a microwave synthesizer (CEM LibertyBlue).

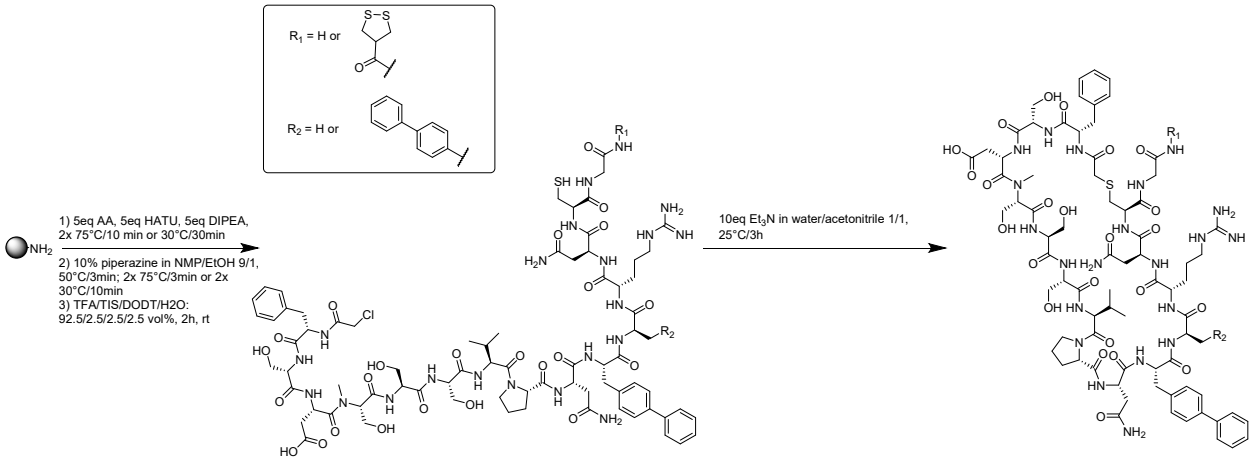
Chromatographic Techniques

Analytical RP-HPLC was performed on a Waters ACQUITY UPLC® UltraPerformance Liquid Chromatography system coupled to a Xevo G2-S QTof MS (ESI). The analysis were performed on ACQUITY UPLC® CSH™ C18 1.7µm column from Waters using either 0.05% TFA in water and 0.04% TFA in acetonitrile (ACN method) or 0.05% TFA in water and 0.04% TFA in Isopropanol (IPA method) as the two mobile phases.

Semi-preparative RP-HPLC was performed on either a High Performance Liquid Chromatography (HPLC) Waters AutoPurification® system, coupled to a Waters 2489 UV/Visible detector or Isco Combiflash® Rf+. Purification was performed using 0.1% trifluoroacetic acid in water and acetonitirle as the two mobile phases on XSelect Peptide CSH C18 130A 5um 250x30 columns for HPLC purification and on RediSep Reversed-phase C18 columns for the Isco combiflash® Rf+.

3. Preparation of compounds

3.1 General procedure for the synthesis of macrocyclic peptides



3.1.1 Solid phase synthesis

Microwave assisted solid phase peptide synthesis was performed using standard Fmoc/tert-butyl chemistry and using the CEM LibertyBlue microwave synthesizer starting with a NovaPEG Rink-Amide resin (0.46 mmol/g). A typical synthesis was done on a 0.1 mmol scale. For each amino acid, double couplings were performed at 75 °C for 10 min (except for the coupling of Fmoc-Cys(Trt)-OH and Fmoc-Arg(Pbf)-OH which was performed at 30 °C for 30 min) using 5 fold molar excess of each Fmoc-L-aa (0.2 M DMF

solution), HATU (0.5 M in DMF) and DIPEA (1 M in DMF). Fmoc groups were deprotected with either one or two consecutive treatments of the peptide with 10% piperazine in NMP/EtOH 9/1 vol% at either 50 °C or 75 °C for 3 min or 30 °C for 10 min.

3.1.2 Final cleavage of the peptides from the resin

The resin was suspended in a mixture of TFA/TIS/DODT/H_2O: 92.5/2.5/2.5/2.5 vol% (5 ml). After 2 h, the solution was filtered and poured into cold Et_2O (40 ml) to precipitate the crude peptides. The solids were separated by centrifugation, washed with cold Et_2O (30 ml) and dried at room temperature to obtain crude linear peptides.

3.1.3 Cyclization and purification

The crude linear peptides were dissolved in water/acetonitrile 1/1 (30 ml) and treated with Et_3N (10 eq, 0.170 ml). The reaction solutions were stirred at 25 °C for 3 h and then concentrated to dryness under reduced pressure.

Crude peptides were purified by a High Performance Liquid Chromatography (HPLC) Waters AutoPurification® system, coupled to a Waters 2489 UV/Visible detector. Purification were performed using 0.1% trifluoroacetic acid in water (A) and acetonitrile (B) as the two mobile phases on a XSelect Peptide CSH C18 130A 5um 250x30 column from Waters. Gradients were adjusted based on the polarity of the peptides.

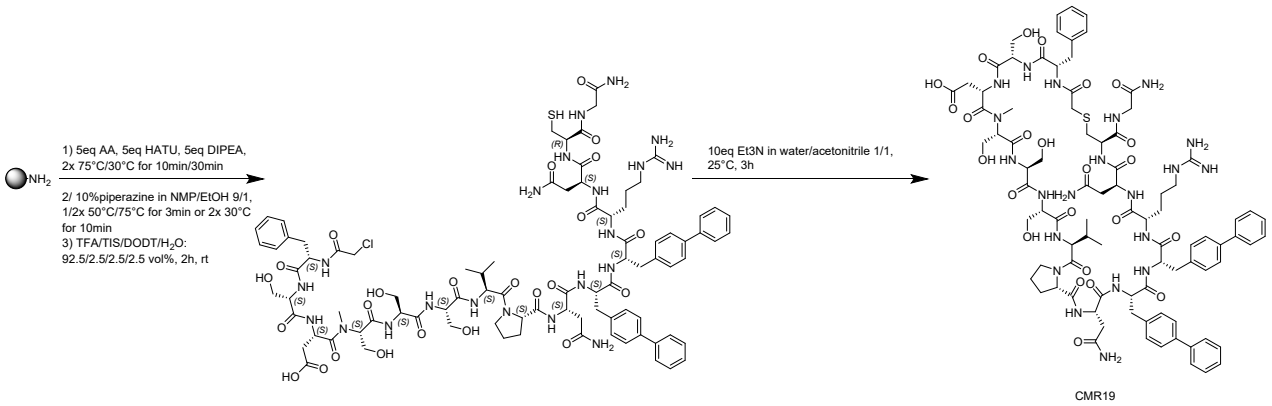
An example of gradient is given here:

Time (min)	**Flow (ml/min)**	**%A**	**%B**
-	30	90	10
1.75	30	90	10
31	30	60	40
32	30	0	100
35	30	0	100
35.5	30	95	5

For all peptides, fractions were automatically collected using the UV signal as trigger. Fractions were individually analyzed using Waters ACQUITY UPLC® UltraPerformance Liquid Chromatography coupled to a Xevo G2-S QTof MS (ESI). The analysis were performed on an ACQUITY UPLC® CSH™ C18 1.7 µm column from Waters using 0.05% TFA in water and 0.04% TFA in acetonitrile (ACN method) or 0.05% TFA in water and 0.04% TFA in isopropanol (IPA method) as the two mobile phases. The fractions containing pure product were combined and the solvents removed by lyophilization to obtain the pure macrocyclic peptides.

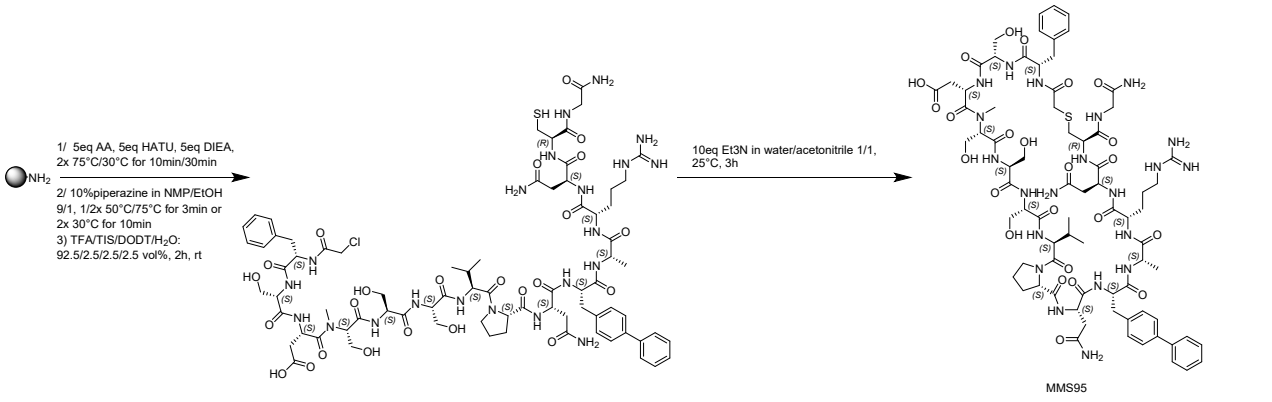
3.2 Synthesis of cyclic peptides and CPP-conjugates

Synthesis of CMR19



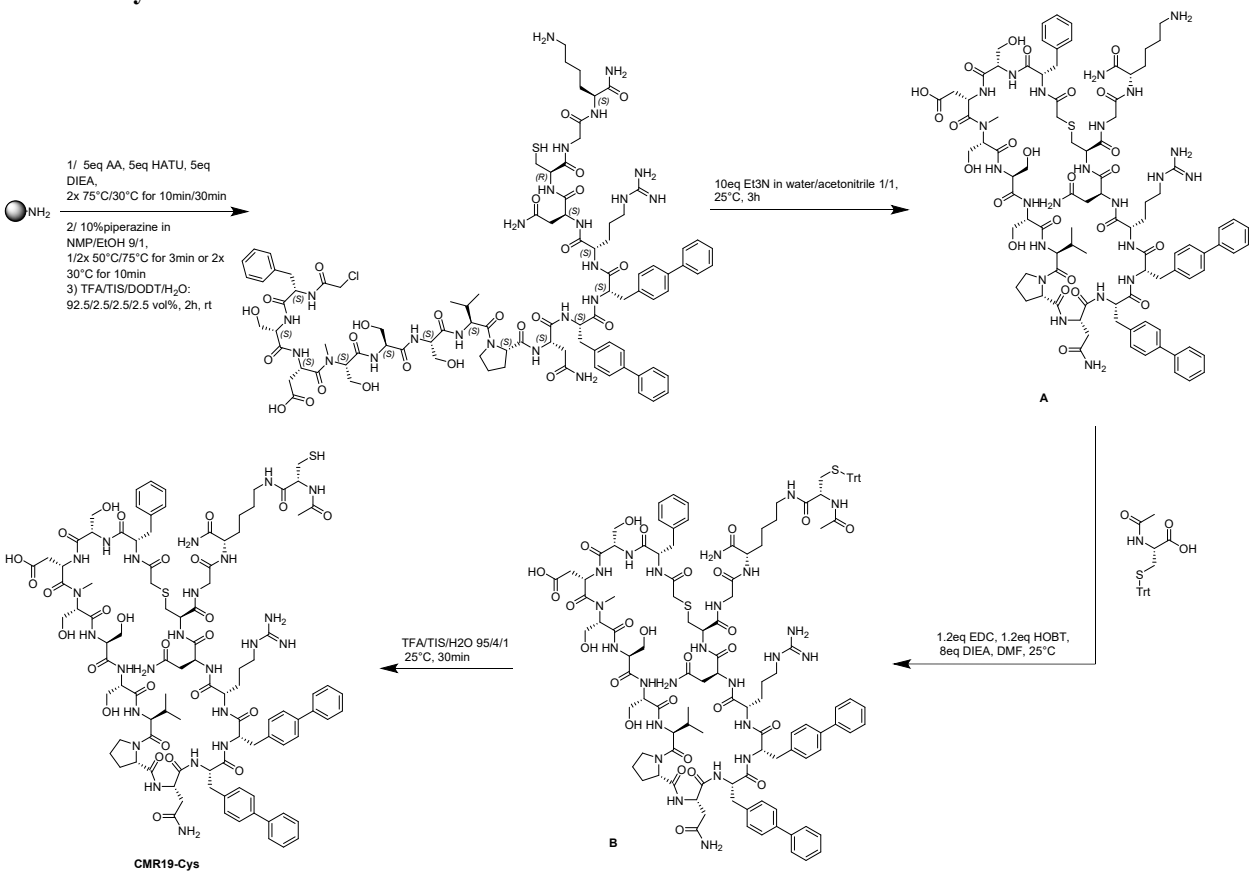
CMR19 was synthesized as described in the general method to give 7.4 mg (purity 90%; yield 3%) of a white solid. UPLC-QTof (ACN method): R_t 4.48 min; MS m/z 2129.7 $(M+H)^+$; UV-area (TIC) 90%.

Synthesis of MMS95



MMS95 was synthesized as described in the general method to give 8.8 mg (Purity 100%; Yield 4%) of a white solid. UPLC-QTof (ACN method): R_t 3.47 min; MS m/z 1978.6 $(M+H)^+$; UV-area (TIC) 100%.

CMR19-Cys



a) Solid phase synthesis and cyclization:

The intermediate **A** was synthesized as described in the general method (2 x 0.1 mmol scale) to obtain 54 mg (purity 95%; yield 12%) of a white solid. UPLC-QTof (ACN method): R_t 3.75min; MS m/z 1997.7$(M+H)^+$; UV-area (TIC) 95%.

b) Ac-Cys(Trt)-OH coupling:

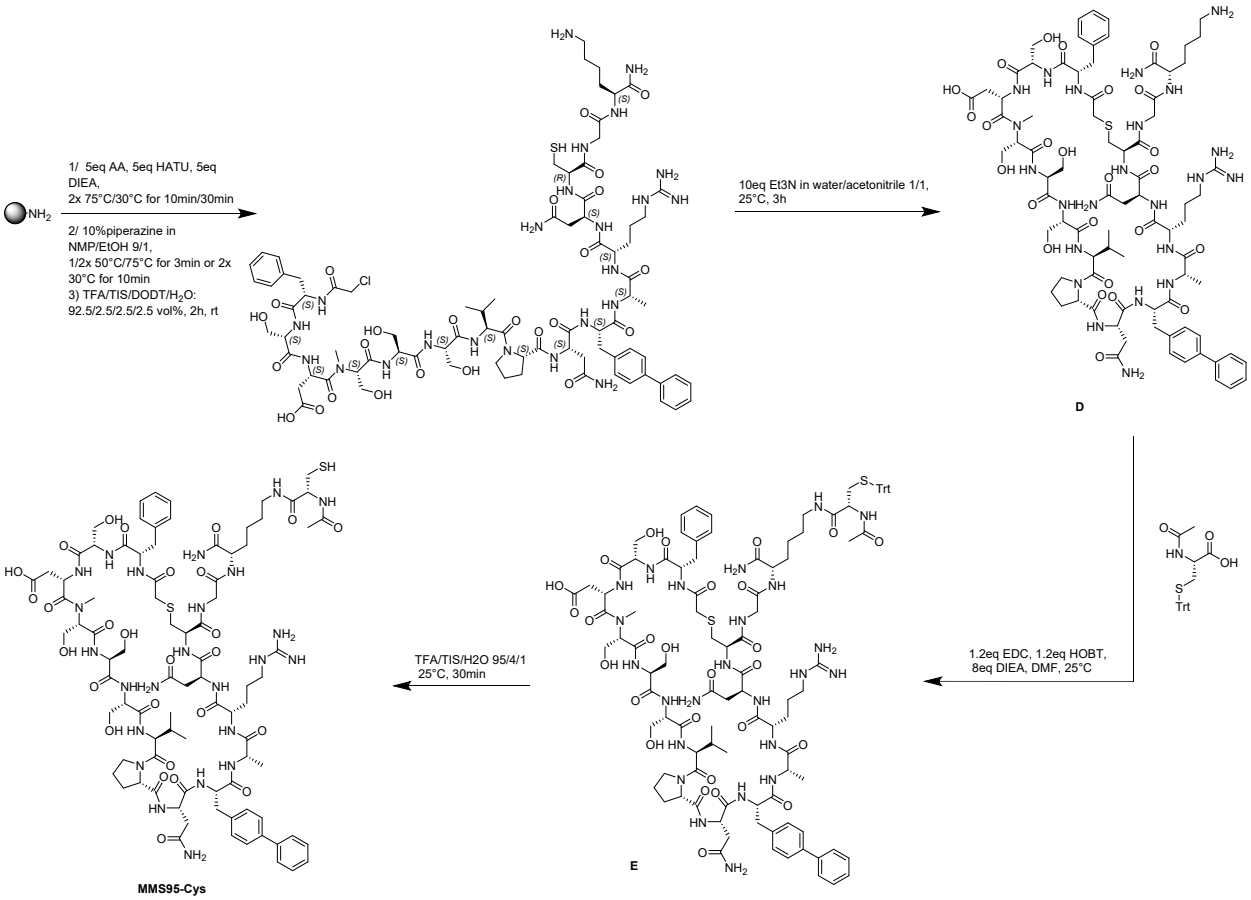
Ac-Cys(Trt)-OH (6.4 mg, 0.016 mmol) in DMF (0.1 ml) was treated with EDC (2.83 mg, 0.015 mmol), HOBt (2.26 mg, 0.015 mmol) and DIPEA (0.017 ml, 0.099 mmol). The mixture was stirred at 25 °C for 1h before it was added to the solution of **A** (27.4 mg, 0.012 mmol) in DMF (0.25 ml). The reaction solution was stirred at 25 °C for 3 h and then poured into cold Et_2O (14 ml) for precipitation. The solids were isolated by centrifugation to afford the crude **B** which was directly used for the Trityl-deprotection).

c) Trityl deprotection:

The crude **B** was treated with a mixture of TFA/TIS/H_2O: 95/4/1 vol% and stirred at 25 °C for 30 min. The crude reaction mixture was purified directly using a High Performance Liquid Chromatography (HPLC) Waters AutoPurification® system coupled to a Waters 2489 UV/Visible detector. Purification was performed using 0.1% trifluoroacetic acid in water (A) and acetonitrile (B) as the two mobile phases on a XSelect Peptide CSH C18 130A 5um 250x30 column from Waters. The product fractions were combined

and lyophilized to give **CMR19-Cys** (4.7 mg, purity 100%, yield 16%) as a white solid. UPLC-QTof (ACN method): R_t 4.22 min; MS m/z 2142.9 $(M+H)^+$; UV-area (TIC) 100%.

MMS95-Cys



a) Solid phase synthesis and cyclization:

Intermediate **D** was synthesized as described in the general method to give 18.5 mg (Purity 100%; Yield 10%) of a white solid. UPLC-QTof (ACN method): R_t 2.74 min; MS m/z 1845.7$(M+H)^+$; UV-area (TIC) 100%.

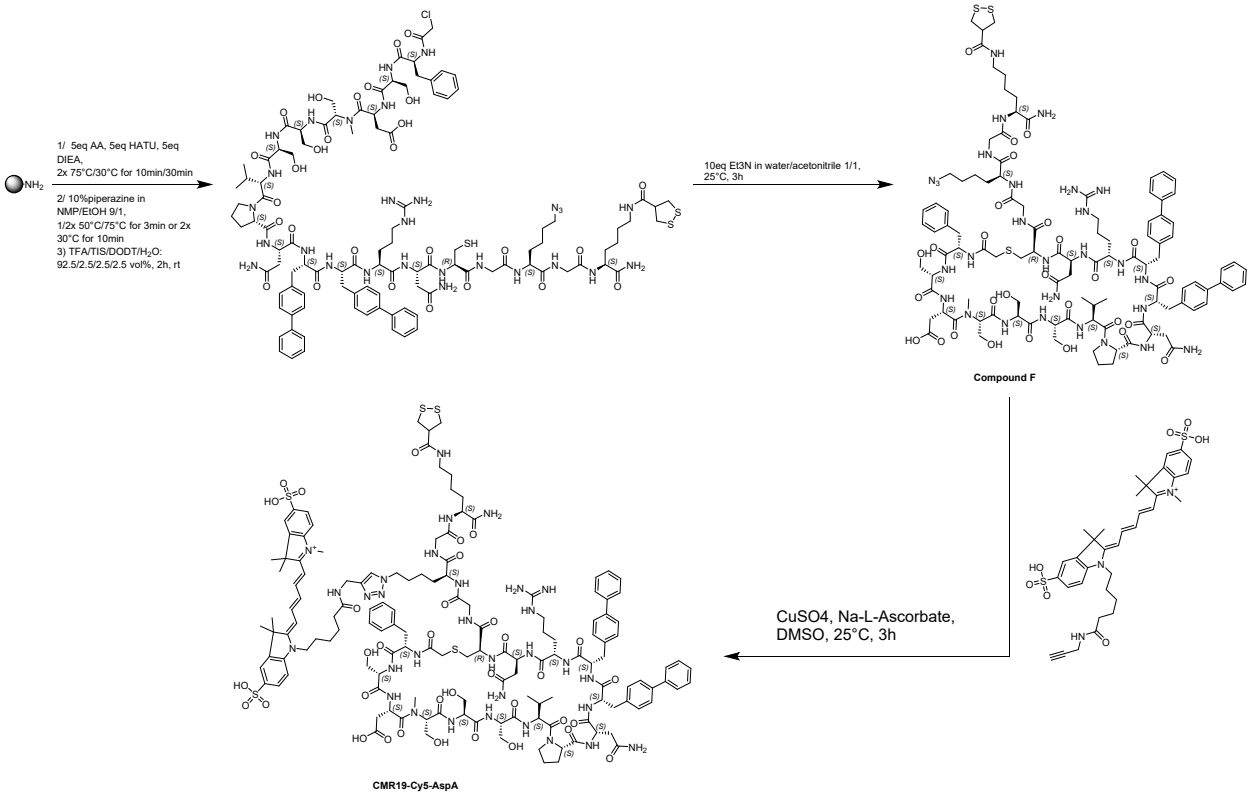
b) Ac-Cys(Trt)-OH coupling:

Ac-Cys(Trt)-OH (7.96 mg, 0.020 mmol) in DMF (0.1 ml) was treated with EDC (4.11 mg, 0.021 mmol), HOBt (3.28 mg, 0.021 mmol) and DIPEA (0.016 ml, 0.089 mmol). The mixture was stirred at 25 °C for 1 h before it was added to a solution of **D** (27.4 mg, 0.012 mmol) in DMF (0.25 ml). The reaction mixture was then stirred at 25 °C for 3 h. It was poured into cold Et_2O (14 ml) for precipitation. The solids were isolated by centrifugation to afford the crude **E** which was directly used for the Trityl deprotection.

c) Trityl deprotection:

The crude **E** was treated with a mixture of TFA/TIS/H2O: 95/4/1 vol% and stirred at 25 °C for 30 min. The product was purified directly on a High Performance Liquid Chromatography (HPLC) Waters AutoPurification® system coupled to a Waters 2489 UV/Visible detector. Purification was performed using 0.1% trifluoroacetic acid in water (A) and acetonitrile (B) as the two mobile phases on a XSelect Peptide CSH C18 130A 5um 250x30 column from Waters. The product fractions were combined and lyophilized to obtain **MMS95-Cys** (0.8 mg, purity 93%, yield 4% as a white solid. UPLC-QTof (ACN method): R_t 3.19 min; MS m/z 1990.3 $(M+H)^+$; UV-area (TIC) 93%.

Synthesis of CMR19-Cy5-AspA



a) Solid phase synthesis and cyclization:

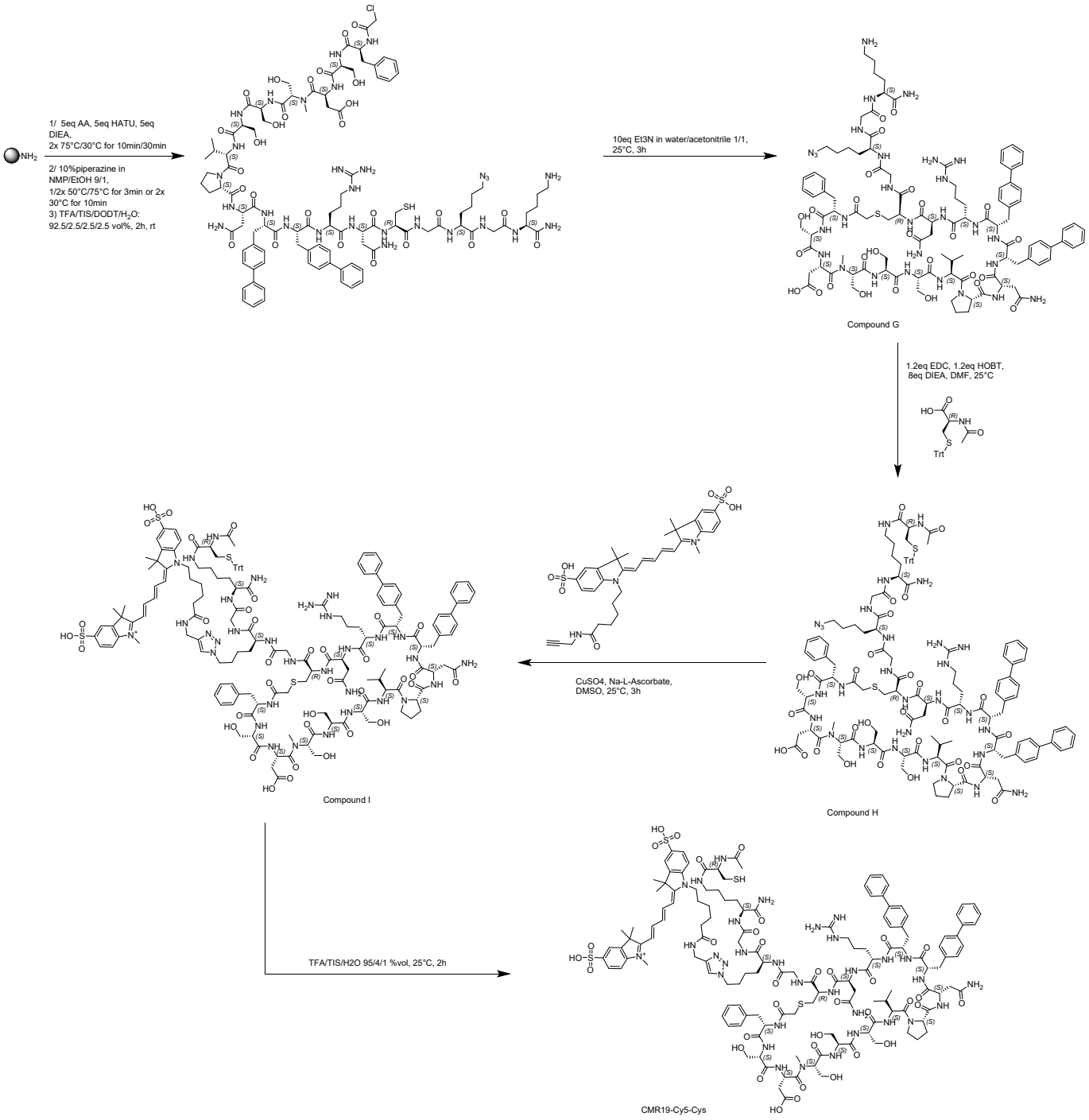
Intermediate **F** was synthesized as described in the general method to give 7.5 mg (purity 98%; yield 3%) of a white solid. UPLC-QTof (ACN method): R_t 4.65 min; MS m/z 2340.8 $(M+H)^+$; UV-area (TIC) 98%.

b) Sulfo-Cy5 alkyne click coupling:

Compound F (7 mg, 0.003 mmol) and 2-((1E,3E)-5-((E)-3,3-dimethyl-1-(6-oxo-6-(prop-2-yn-1-ylamino)hexyl)-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-5-sulfo-3H-indol-1-ium (Sulfo-Cy5 alkyne, 2.24 mg, 0.0033 mmol) were dissolved in DMSO (0.4 ml). The mixture was then treated with a 100 mM aqueous solution of $CuSO_4$ (60 µl, 0.006 mmol) and a 100 mM aqueous solution of sodium-L-ascorbate (60 µl, 0.006 mmol) and the mixture stirred at 25 °C for 3 h.

The crude reaction mixture was purified by reverse phase chromatography using a Isco Combiflash Rf+ coupled to a UV/Visible detector. Elution was performed using 0.1% trifluoroacetic acid in water (A) and acetonitrile (B) as the two mobile phases on a RediSep® Rf Reversed-phase C18 column (4.3 g). The product fractions were combined and lyophilized to obtain **CMR19-Cy5-AspA** (2.9 mg, purity 97%, yield 15%) as a blue solid. UPLC-QTof (ACN method): R_t 4.73 min; MS m/z 1511.5 $((M+2H)/2)^+$; UV-area (TIC) 97%.

Synthesis of CMR19-Cy5-Cys



a) Solid phase synthesis and cyclization:

Intermediate **G** was synthesized as described in the general method (2x 0.1 mmol scale) to give 80 mg (purity 96%; yield 17%) of a white solid. UPLC-QTof (ACN method): R_t 3.89 min; MS m/z 2209.0 $(M+H)^+$; UV-area (TIC) 96%.

b) Ac-Cys(Trt)-OH coupling:

Ac-Cys(Trt)-OH (9.08 mg, 0.022 mmol) in DMF (0.1 ml) was treated with EDC (5.28 mg, 0.028 mmol), HOBt (4.22 mg, 0.028 mmol) and DIPEA (0.024 ml, 0.138 mmol). The mixture was stirred at 25 °C for 1 h before it was added to a solution of **G** (40 mg, 0.017 mmol) in DMF (0.25 ml). The reaction solution was then stirred at 25 °C for 16 h and then poured into cold Et_2O (14 ml) for precipitation. The solids were isolated by centrifugation to afford the crude **H** which was directly used for the click coupling. UPLC-QTof (ACN method): R_t 5.47 min; MS m/z 2596.2 $(M+H)^+$; UV-area (TIC) 15%.

c) Sulfo-Cy5 alkyne click coupling:

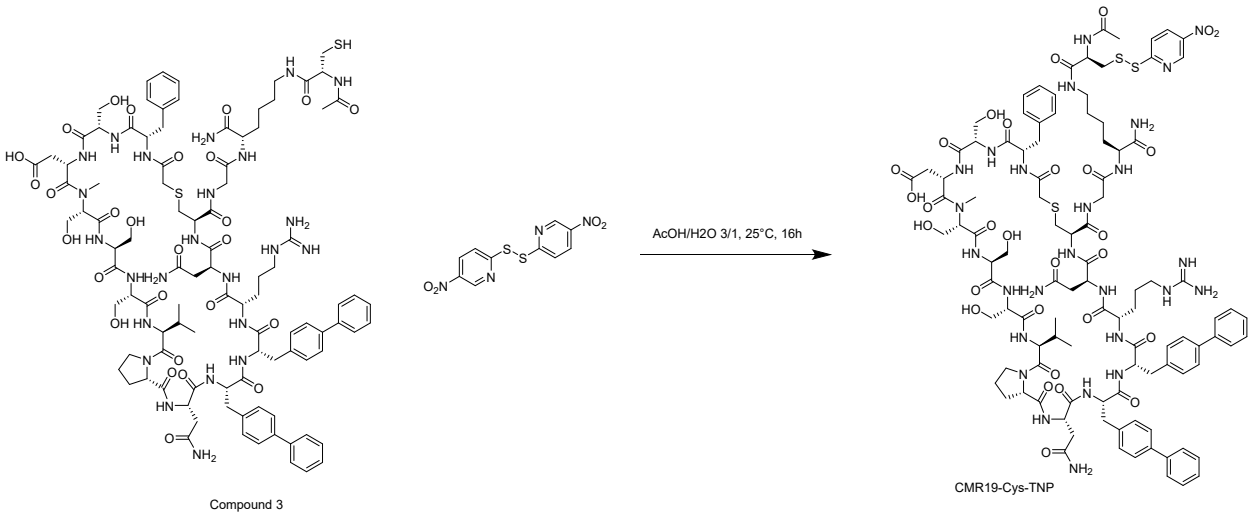
Crude intermediate **H** (0.017mmol) and 2-((1E,3E)-5-((E)-3,3-dimethyl-1-(6-oxo-6-(prop-2-yn-1-ylamino)hexyl)-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-5-sulfo-3H-indol-1-ium (sulfo-Cy5 alkyne, 11.71g, 0.017ol) were dissolved in DMSO (0.3l). The mixture was then treated with 100 mM aqueous $CuSO_4$-solution (0.15 ml, 0.015 mmol) and 100 mM aqueous sodium-L-ascorbate solution (0.15 ml, 0.015 mmol). The reaction mixture was then stirred at 25 °C for 16 h.

The crude reaction mixture was taken up in acetonitrile/water 9/1 (1 ml) and purified by reverse phase chromatography using an Isco Combiflash Rf+ coupled to a UV/Visible detector. Purification was performed using 0.1% trifluoroacetic acid in water (A) and acetonitrile (B) as the two mobile on a RediSep® Rf Reversed-phase C18 column (4.3 g). The product fractions were combined and lyophilized to obtain **I** (8 mg, purity 35%) as a blue solid. UPLC-QTof (IPA method): R_t 4.17 min; MS m/z 1638.7 $((M+2H)/2)^+$; UV-area (TIC) 35%.

d) Trityl deprotection:

The **compound I** (8 mg) was treated with a mixture of TFA/TIS/H_2O: 95/4/1 vol% and stirred at 25 °C for 2 h. The product was directly purified using a High Performance Liquid Chromatography (HPLC) Waters AutoPurification® system coupled to a Waters 2489 UV/Visible detector. Purification was performed using 0.1% trifluoroacetic acid in water (A) and acetonitrile (B) as the two mobile phases on XSelect Peptide CSH C18 130A 5um 250x30 column from Waters. The product fractions were combined and lyophilized to give **CMR19-Cy5-Cys** (1.4 mg, purity 85%, yield 2.2%) as a blue solid. UPLC-QTof (ACN method): R_t 4.56 min; MS m/z 1517.6 $((M+2H)/2)^+$; UV-area (TIC) 85%.

Synthesis of CMR19-Cys-TNP



Compound 3 (2.5 mg, 0.001 mmol) and 2,2-dithiobis(5-nitropyridine) (1.72 mg, 0.055 mmol) were dissolved in AcOH/Water 3/1 %vol (0.5 ml). The reaction mixture was stirred at 25 °C for 16 h and then the mixture was lyophilized. The crude solid was dissolved in H_2O (2 ml) and extracted with Ethyl acetate (8x). The water layer was then lyophilized to give **CMR19-Cys-TNP** (1.9 mg, purity 88%, yield 63%). UPLC-QTof (IPA method): R_t 3.79min; MS m/z 2296.9 $(M+H)^+$; UV-area (TIC) 88%.

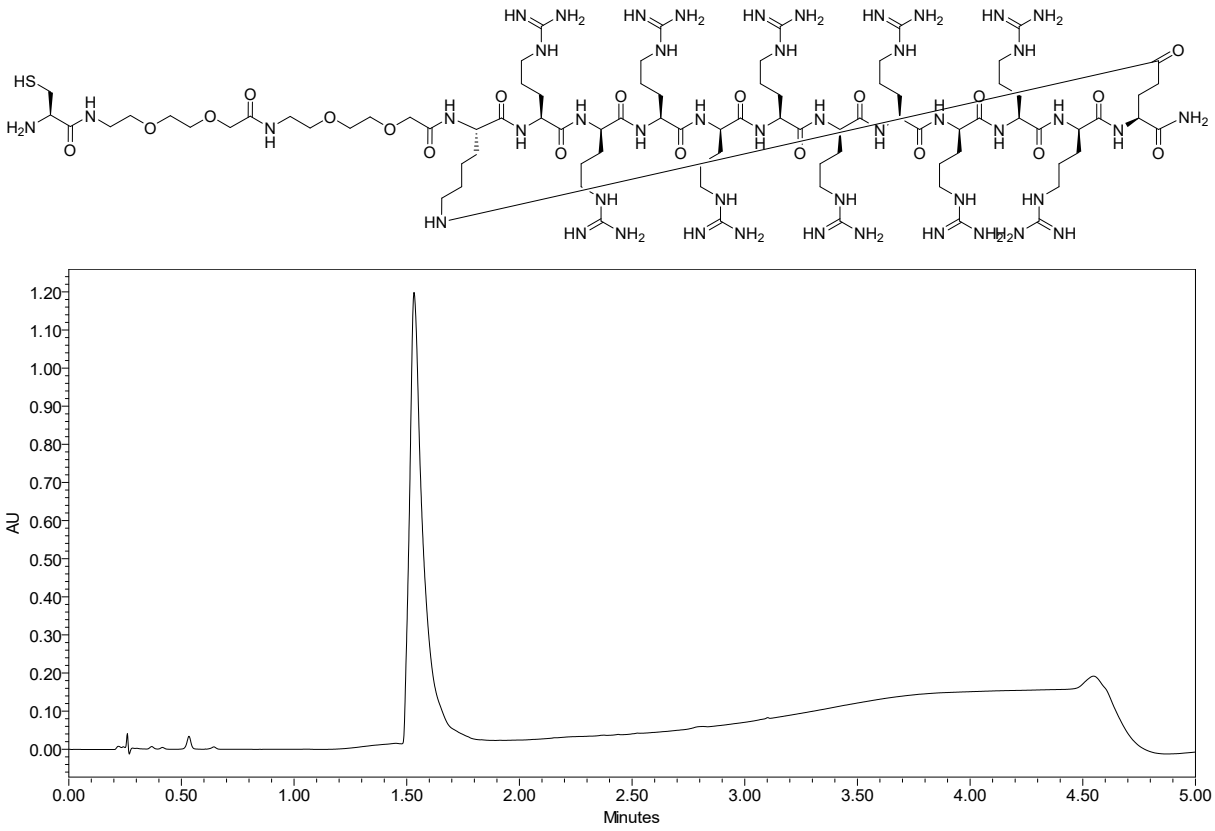
Peptide-CPP conjugation

The inhibitor peptides CMR19 and MMS95, as well as the Cy5-modified CMR19 peptide were dissolved in 1:1 water:acetonitrile at a 10 mM concentration. 10 equivalents of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) were added (100 mM) and the mixture was incubated for an hour while shaking. The solution turned bright yellow. Activated peptides were purified by preparative HPLC using a linear gradient (A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 10% B 0-10 min, 10-60% B 10-60 min, 60-99% 60-80 min). Purified peptides were lyophilized.

After purification, the activated peptides were redissolved in 1:1 water:acetonitrile at a 10 mM concentration. 2 equivalents of the cysteine-functionalized cR10 peptides were added to the solution and it was incubated at room temperature overnight. The next day, disulphide-conjugated peptide dimers were purified by preparative HPLC using a linear gradient (A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 5% B 0-10 min, 5-50% B 10-60 min, 50-99% 60-80 min). Obtained peptides were lyophilized.

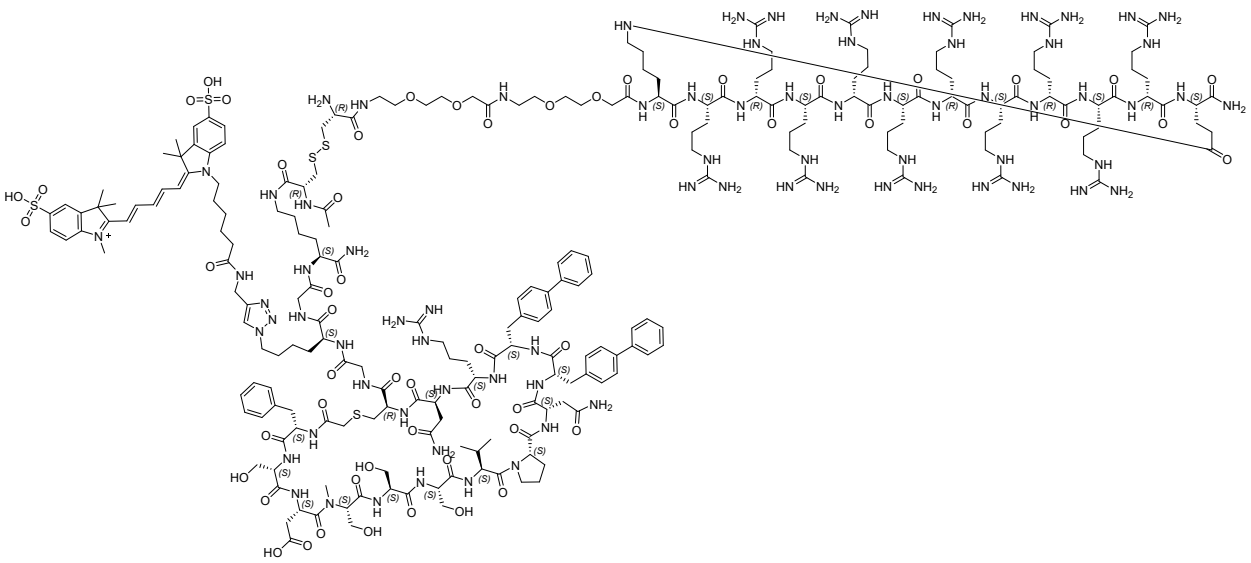
Peptide characterization

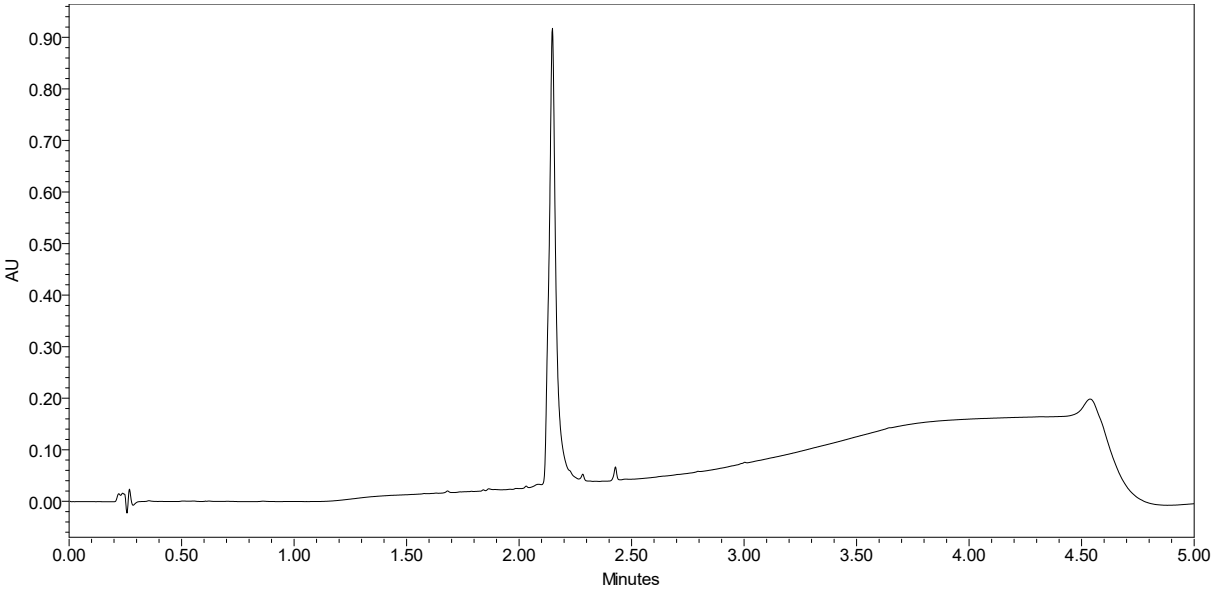
Cys-cR10 peptide



HRMS: Calc.: [M+3H]: 738.1156, Exp.: 738.1941.

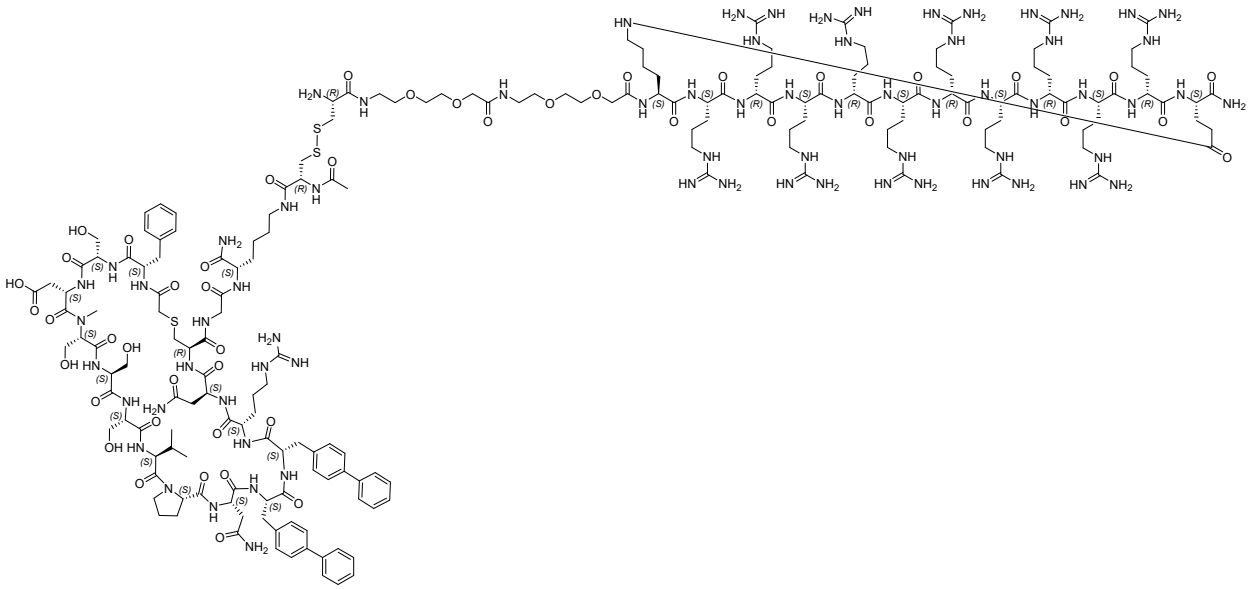
CMR19-Cy5-cR10 peptide

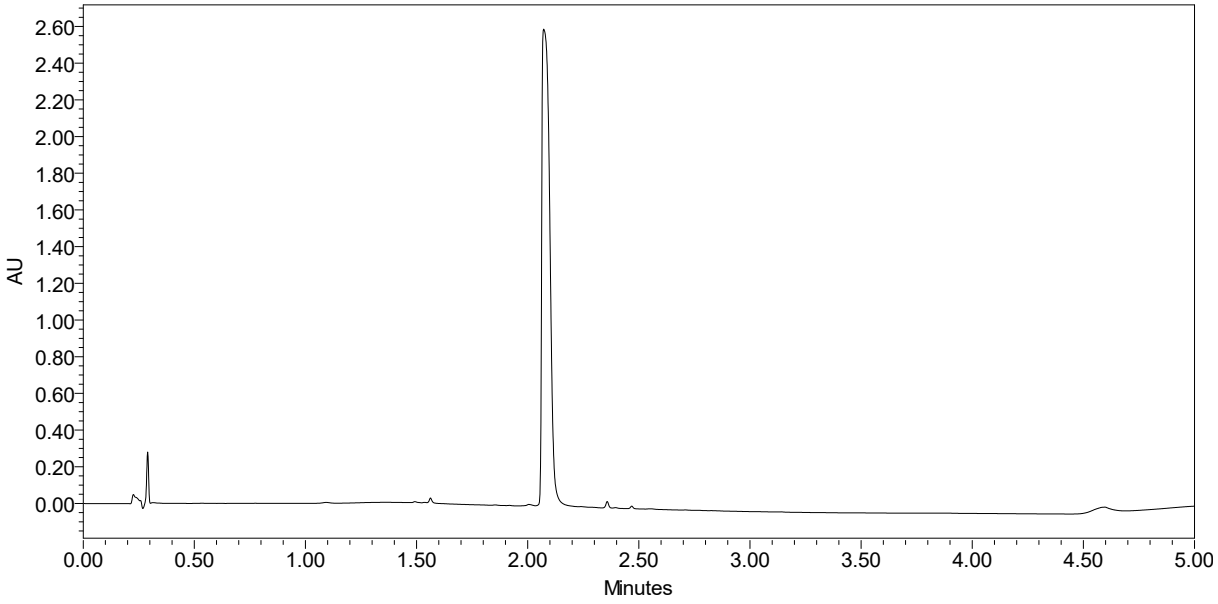




HRMS: Calc.: [M+7H]: 749.6599, Exp.: 749.6643.

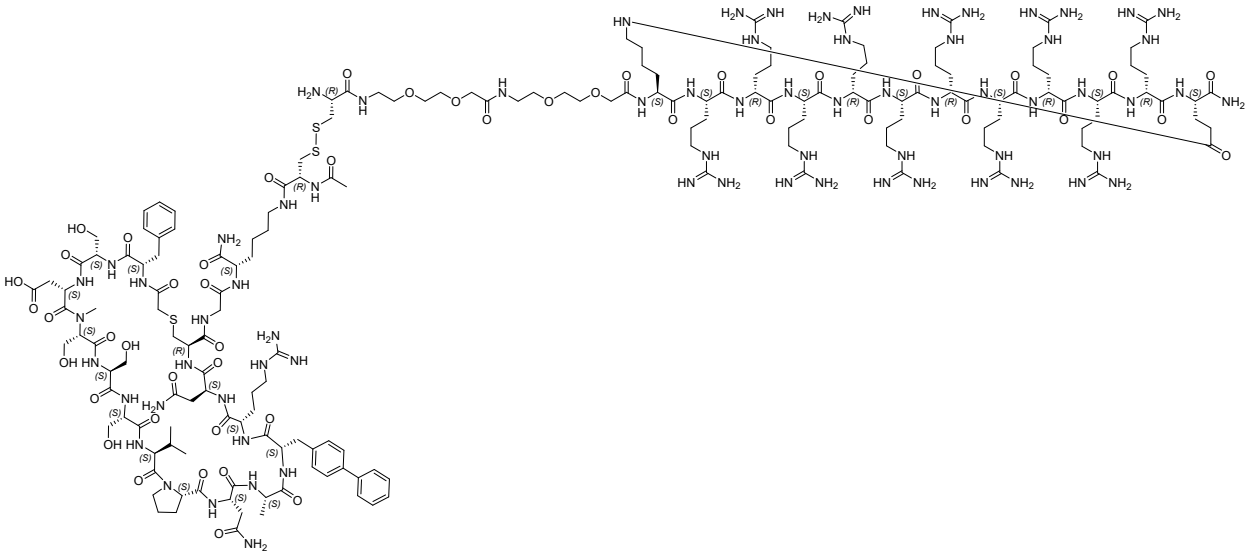
CMR19-cR10 peptide

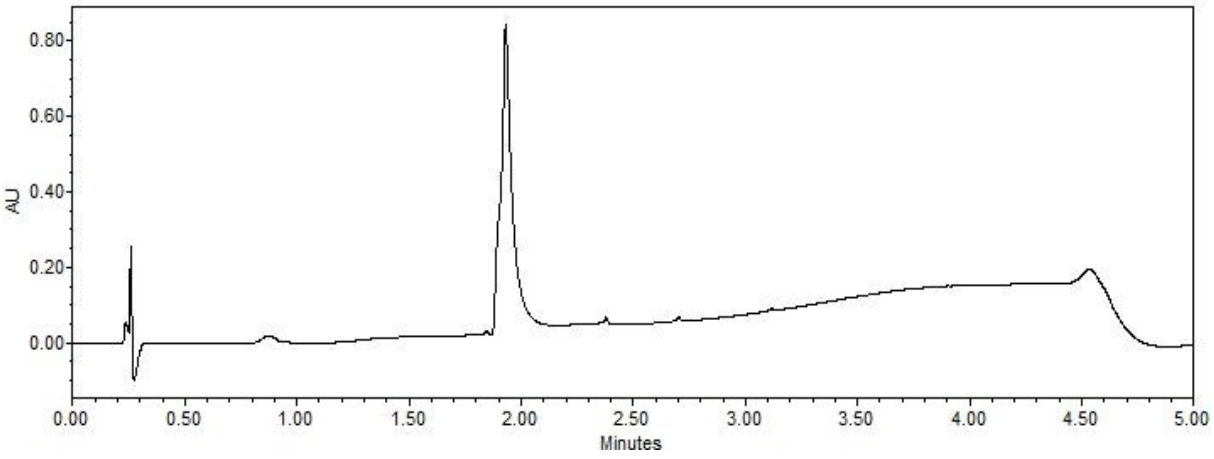




HRMS: Calc.: [M+6H]:726.2116, Exp.: 726.2087.

MMS95-cR10 peptide





HRMS: Calc.: [M+6H]:700.8679, Exp.: 700.8663.

4. X-ray crystallography

4.1 Protein expression and purification

MDM2 Gly-(S17-N111) (numbering according to Q00987) was cloned, expressed and purified as described previously.[4]

4.2 Crystallization, data collection, and refinement

The protein solution for MDM2 Gly-(S17-N111) (numbering according to Q00987) was 6-10 mg/mL in 50 mM TRIS pH 8.0, 200 mM NaCl, 1mM TCEP, 10% glycerol. Co-crystals of MDM2 Gly-(S17-N111) in complex with **CMR19** were obtained by adding a 3-fold molar excess of compound. Co-crystals were obtained at 20 °C and by sitting drop vapor diffusion. The drops were made up of 400 nL of protein solution and 400 nL of well solution. The reservoir solution consisted of 2.2 M $AmSO_4$, 0.2 M NaAcetate. All crystals were cryo protected in well solution supplemented with 20 % glycerol and flash frozen in liquid nitrogen.

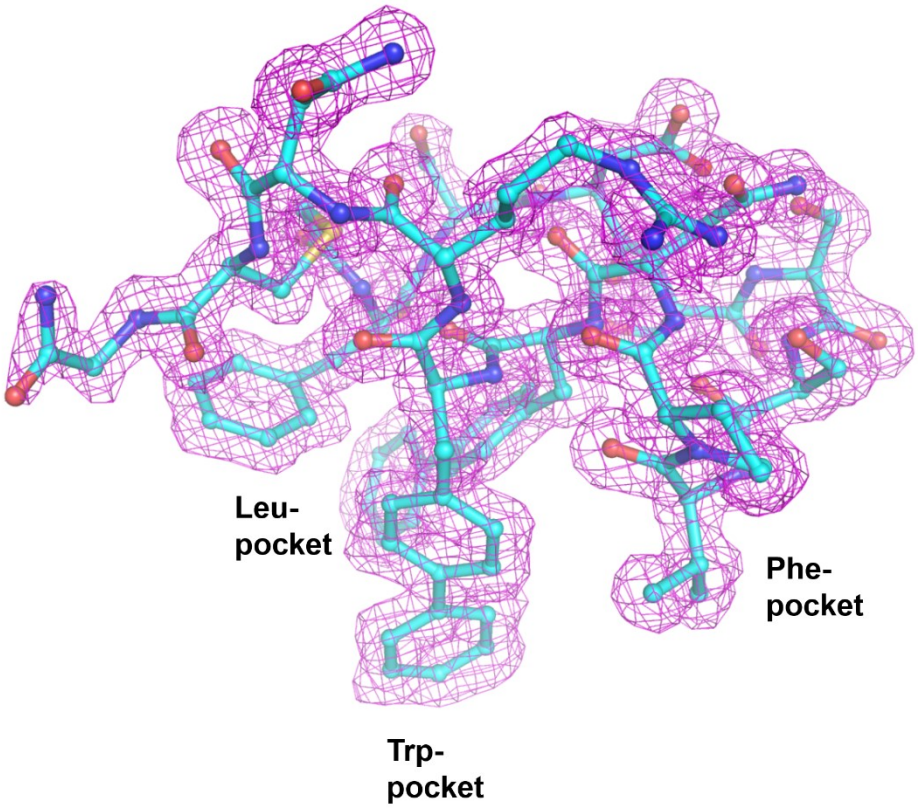
Data sets were collected at the Swiss Light Source Facility (SLS, Villigen, Switzerland). The data were processed with XDS.[5] The structure of the MDM2/**CMR19** complex was determined by molecular replacement (PHASER[6]) using pdb-entry 4ZYF[7] as the search model. Programs REFMAC[6] and COOT[8] were used for refinement and model (re)building. The final refined structure has R/R_{free} values of 0.177/0.196 and showed excellent geometry in the Ramachandran plot. All residues for the 3 protein chains (A,B,C) in the asymmetric unit are in the allowed regions for all X-ray structures, except for residue K70 (chain A) which is flexible and has unclear density. See Table S1 for details of the data collection and structure refinement. All molecular figures were prepared with PyMOL.[9]

The crystallographic data have been deposited at the RSCB Protein Data Bank (PDB, www.pdb.org) with the code 7NUS.

Table S1:

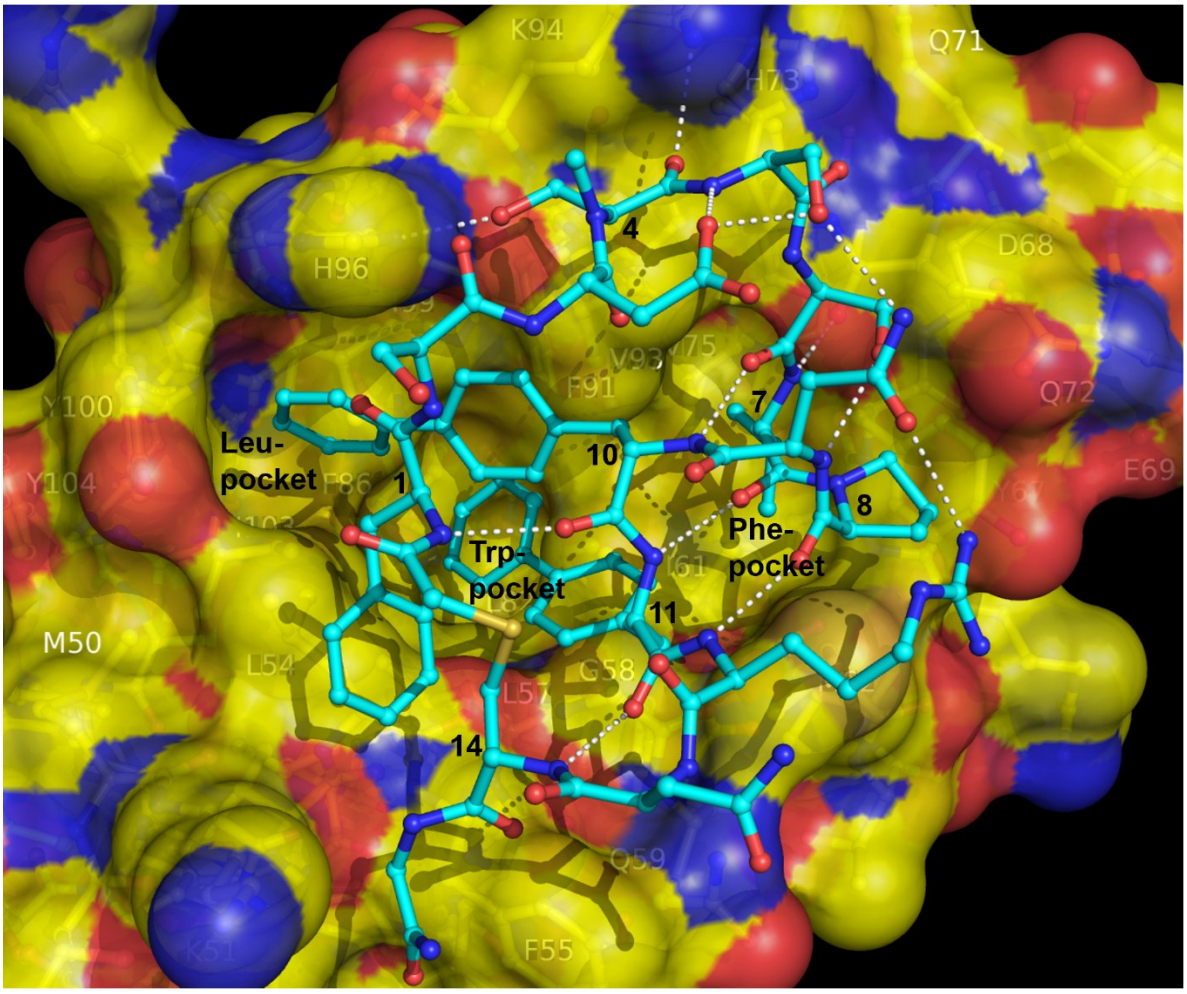
Data set	Complex of **CMR19** with MDM2(17-111)
PDB code	7NUS
Space group	C2
Cell parameters (Å/deg)	140.8, 40.3, 70.2 / 90, 112.2, 90
No. of complexes per ASU	3
Resolution (last shell) (Å)	1.45 (1.49-1.45)
Unique reflections	63733
R_{merge} (all/last shell)	0.078/0.473
I/Iσ (all/last shell)	11.8/3.2
Completeness (%) (all/last shell)	99.9/96.2
Redundancy (all/last shell)	3.4/3.4
Refinement	
Resolution (last shell) (Å)	1.45 (1.49-1.45)
Number of reflections used (last shell)	60547 (4343)
Fraction of test set for calculating R_{free} (%)	5.0
No. of reflections in the test set (last shell)	3187 (228)
R factor: R_{work} /R_{free} (last shell)	0.177/0.196 (0.304/0.306)
r.m.s.d. bond lengths (Å) / bond angles (^{o})	0.007/1.233
No. of atoms (protein/ waters/ligand)	2262/488/399

4.3 Electron density for CMR19 bound to MDM2



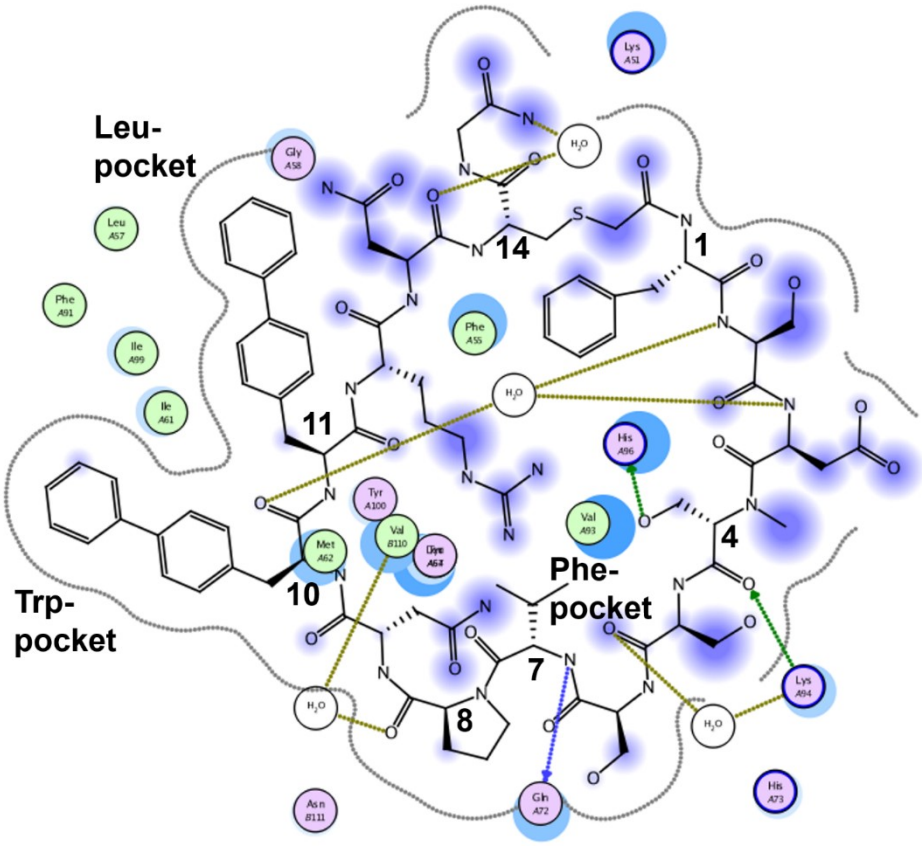
Supplementary Fig. 3 Electron density map (2Fo-Fc map contoured at 1σ) for **CMR19** bound to MDM2)**.**

4.4 Details of interactions for MDM2/CMR19



Supplementary Fig. 4 X-ray structure for MDM2/**CMR19** at 1.45 Å resolution. MDM2 is shown in a surface representation (carbons in yellow) and **CMR19** as a stick-model (carbons in cyan, nitrogens in blue, oxygens in red, sulfurs in brown). For **CMR19,** the C-alphas of the key residues 1, 4, 7, 8, 10, 11, 14 are indicated with the corresponding numbers in black. The non-canonical biphenyl side chains in positions 10 and 11 occupy the Leu- and Trp- pockets of MDM2. The Phe-pocket of MDM2 is occupied by the valine side chain in position 7 and proline 8 makes vdW-contacts with Y(67). The side chain of phenylalanine in position 1 makes vdW-interactions with L(54). Interestingly, the MDM2 side chain of F(55) is in a position as found e.g. for the complex with the p53 peptide and is not "flipped in" towards the ligand, as found e.g. for the clinical compound **CGM097**.[7] Selected intra- and intermolecular H-bonds are shown as white dotted lines. Water molecules are not shown. **CMR19** makes direct inter-molecular hydrogen bonds between the backbone NH of Val(7) and the backbone CO-Gln(72) (distance 2.9 Å), between the side chain OH of MeS(4) and the side chain of His(96) (distance 2.7 Å), and the backbone CO of MeS(4) and the side chain of K(94) (distance 2.9 Å). The coordinates for MDM2/**CMR19** have been deposited in the PDB databank (PDB access code = 7NUS).

4.5 Simplified LigPlot diagram for MDM2/CMR19



Supplementary Fig. 5 Simplified LigPlot[10] diagram for the X-ray structure of MDM2/**CMR19** at 1.45 Å resolution. Selected intra- and intermolecular H-bonds are shown as dotted lines. Selected water molecules are shown. For **CMR19** the C-alphas of the key residues 1, 4, 7, 8, 10, 11, 14 are indicated with the corresponding numbers in black.

5. Cell experiments and assay protocols

TR-FRET competition assay

Ability of peptides to selectively disrupt p53/HDM2 interaction (vs p53/HDM4 interaction) was measured in a competition assay. Inhibitory effect of peptides were measured by a Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET). Different concentrations of tested compounds were added to 0.1 nM Lance Europium streptavidin, 0.1 nM biotinylated HDM2(2-188) or HDM4(2-185) p53 binding domain, and 10 nM Cy5-p53 aa18-26. IC_{50}s are mean values of at least 2 independent experiments. Affinity of the compounds (K_i) was calculated from IC_{50} using the Cheng-Prusoff equations,[11] adapted for competition ligand binding assay:

$$K_i = IC_{50}/(1 + ([L]/K_D))$$

where

K_I = the inhibition constant, defined as the equilibrium concentration of competitive inhibitor that would occupy 50% of receptor sites if no competing labeled ligand was present

IC_{50}= the concentration of competitive inhibitor that displaces 50% of the specifically bound radiolabeled ligand,

K_D = the affinity constant, defined as the equilibrium concentration of fluorescent ligand that occupies 50% of receptor sites in the absence of competition.

[L] = the concentration of fluorescent ligand (Cy5-p53).

The concentration of the Cy5-p53 was used at 10 nM. The K_D used for p53(aa18-26)/MDM2(2-188) was 41 nM, it was 79 nM for p53(aa18-26)/MDM4(2-185), evaluated by TR-FRET saturation curve, confirmed by fluorescence polarization and ITC (data not shown). In these assay conditions, the measured IC_{50} values are close to the K_I values of tested compounds (see Table S2).

Table S2:

	MDM2		MDM4	
	IC_{50} ± SD in nM (n)	K_I in nM	IC_{50} ± SD in nM (n)	K_I in nM
NVP-CMR019 (2 different batches)	0.18 ± 0.05 (8)	0.15	835 ± 235 (2)	740
NVP-MMS395	>10000 (2)	>8060	>10000 (2)	>8880
CGM097 used as assay control	1.7 ± 0.1 (19)	1.37	2200 ± 800 (5)	1950

SD: Standard Deviation. n: number of independent experiments. IC_{50} coming close to sensitivity limit of the assay, (around 50 pM with 100 pM protein) for NVP-CMR019 - Hill coefficient remained around 1. CGM97 was used as assay control.

Cell culture

SJSA-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Cellular uptake of fluorescent peptides

10'000 SJSA-1 cells were seeded in each well of a 96-well glass bottom plate. The cells were incubated for 24 hours at 37°C with 5% CO_2. The next day, cells were washed twice with RPMI-1640, before addition of the peptides in RPMI-1640 medium. The cells were then incubated at 37°C for 2 hours. After cellular uptake, the cells were washed three times with RPMI-1640 containing 10% FBS. In the final washing step, 5 µg/mL Hoechst 33342 were included.

Microscopy

Confocal microscopy images were acquired on a Zeiss LSM 780 laser scanning microscope or a Nikon CSU spinning disc microscope with a CSU-X1 (Andor). Images were acquired using a PlanApo 100x (Zeiss) or 20x (Nikon) objective (Nikon). Brightfield images were aquired along with fluorescence images. Standard laser, a quad Dicroic (400-410,486-491, 560-570, 633-647, AHF) and Emission filters were used in the acquisition of confocal fluorescence images: Hoechst 33342, ex.: 405 nm em.:450/50:, Alexa Fluor 568, ex.: 561 em.:600/50 nm and Cy5 ex.: 640 em.:685/50 nm.

WST-1 cell viability assay

20'000 SJSA-1 cells were seeded in each well of a 96-well plate tissue culture plate. The cells were incubated with the inhibitor compounds for 24 hours at 37°C in RPMI-1640 medium containing 10% FBS. Then, 10 µl of the WST-1 solution (Roche) were added to each well. As a background sample, medium with the WST-1 solution (without cells) was used. Absorbance was measured at 440 nm with 650 nm as reference wavelength.

MDM2 immunofluorescence after treatment with inhibitors

10'000 SJSA-1 cells were seeded in each well of a 96-well glass bottom plate. The cells were incubated for 24 hours at 37°C with 5% CO_2. The next day, the medium was replaced by RPMI-1640 medium with 10% FBS containing the inhibitors at 0.4-10 µM concentration. The cells were then incubated for 4 hours at 37°C with 5% CO_2. The cells were then washed once with PBS and then fixed using 4% PFA in PBS for 20 minutes at room temperature. To remove PFA, the cells were washed thrice with PBS.

PBS was then removed from the wells and the cells were permeabilized and blocked with 10% FCS and 0.1% Triton X-100 in PBS for 30 minutes at room temperature. The MDM2 antibody (rabbit monoclonal anti-MDM2 (abcam, ab38618) was diluted 1:100 in the blocking solution. The blocking solution was removed and the antibody solution was added to each well. The cells were incubated at room temperature for 1 hour. The cells were washed with PBS twice, 5 minutes each.

Hoechst was diluted 1:2000 in 1% FCS in PBS. The secondary antibody (Alexa Fluor 568 goat anti rabbit (highly x-adsorbed, Invitrogen) was diluted 1:1000 in the same solution. PBS was removed from the wells and the secondary antibody solution was added to the cells. The cells were incubated for 1 hour at room temperature in the dark. The cells were then washed thrice with PBS, taken to the microscope and imaged.

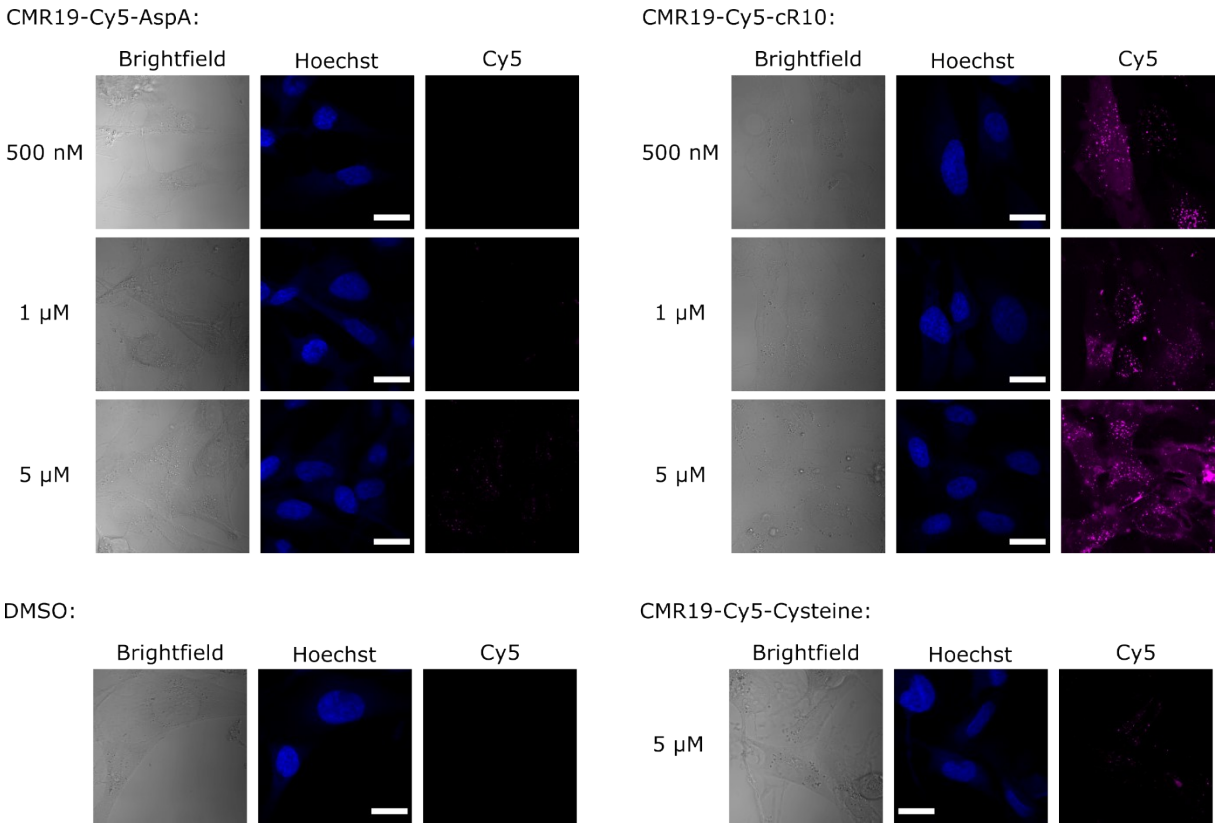
Quantification script to measure nuclear MDM2

The following quantification script for FIJI opens files in a given directory, then uses the Hoechst signal (in channel 2) to create a mask for the quantification. It then quantifies the fluorescent signal of the MDM2-immunofluorescence (in channel 3).

```
setBatchMode(true);

dir=getDirectory("Choose Source Directory ");

list = getFileList(dir);

for(i=0; i<list.length; i++) {

file=list[i];

run("Bio-Formats Importer", "open=["+dir+"/"+file+"] autoscale color_mode=Default concate-
nate_series open_all_series rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT");

getDimensions(width, height, channels, slices, frames);

{

run("Bio-Formats Importer", "open=["+dir+"/"+file+"] autoscale color_mode=Default rois_import=[ROI
manager] view=Hyperstack stack_order=XYCZT");

name=getTitle;

print(name);

run("Duplicate...", "duplicate channels=2");

run("Median...", "radius=5");

run("Auto Threshold", "method=Default white");

run("Fill Holes");

run("Erode");

run("Create Selection");

roiManager("Add");

run("Set Measurements...", "area mean modal integrated display redirect=None deci-mal=0");

selectWindow(name);

run("Duplicate...", "duplicate channels=3");

run("Subtract Background...", "rolling=100");

run("Subtract Background...", "rolling=100");

roiManager("Select", 0);

run("Measure");
```

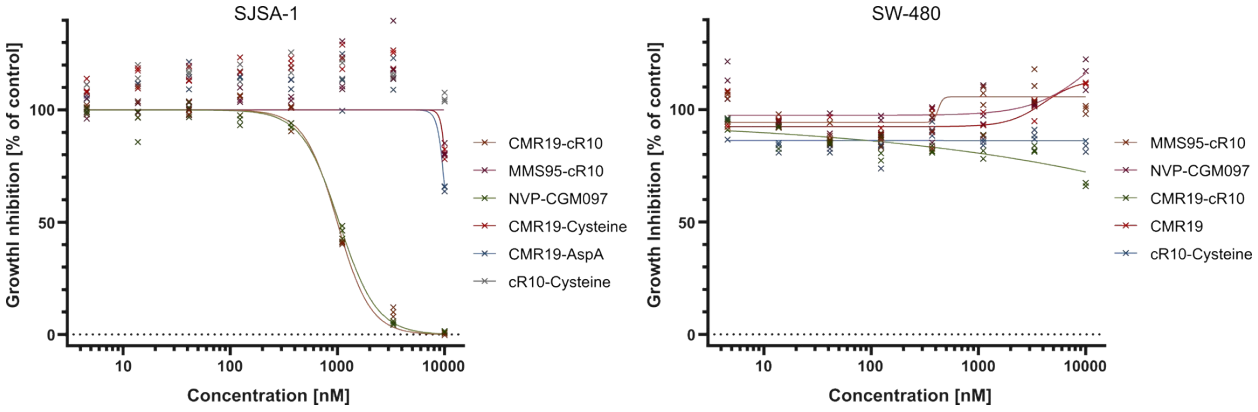
```
roiManager("Deselect");
roiManager("Delete");
run("Close All");
}
}
```

5.1 Confocal microscopy after cellular uptake



Supplementary Figure 6. Full dataset of confocal microscopy images after cellular uptake of Cy5-labelled CMR19 peptides. Scale bars 20 µm.

5.2 WST-1 assay in SW-480 cells



Supplementary Figure 7. WST-1 assay in SW-480 cells. As an additional control, we performed the WST-1 assay in SW-480 cells, in which we did not observe significant cytotoxicity.

6. References

1. Y. Goto, T. Katoh, H. Suga, *Nat. Protoc.* **2011**, *6*, 779–790.

2. K. Kashiwagi, P. C. Reid, P. Inc, P. *Rapid display method in translational synthesis of peptide*, **2013**, EP2492344.

3. T. Ishizawa, T. Kawakami, P. C. Reid, H. Murakami, *J. Am. Chem. Soc.* **2013**, *135*, 5433–5440.

4. J. Kallen, A. Goepfert, A. Blechschmidt, A. Izaac, M. Geiser, G. Tavares, P. Ramage, P. Furet, K. Masuya, J. Lisztwan, *J. Biol. Chem.* **2009**, *284*, 8812-8821.

5. W. Kabsch, *Acta Crystallogr. D Biol. Crystallogr*. **2010**, *66*, 125-132.

6. Collaborative Computational Project, Number 4, *Acta Crystallogr. D Biol. Crystallogr*. **1994**, *50*, 760-763.

7. J. Kallen, A. Izaac, S. Chau, E. Wirth, J. Schoepfer, R. Mah, A. Schlapbach, S. Stutz, A. Vaupel, V. Guagnano, K. Masuya, T.-M. Stachyra, B. Salem, P. Chene, F. Gessier, P. Holzer, and P. Furet, *ChemMedChem* **2019**, *14*, 1305-1314.

8. P. Emsley, K. Cowtan, *Acta Crystallogr. D Biol. Crystallogr*. **2004**, *60*, 2126-2132.

9. D. Warren, *The PyMOL Molecular Graphics System*, Version 1.3, Schrödinger, LLC.

10. A. C. Wallace, R. A. Laskowski, J. M. Thornton *Prot. Eng*. **1995**, *8*, 127-134.

11. Y. Cheng, W. H. Prusoff, *Biochem. Pharm*. **1973**, *22(23)*, 3099–3108.