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Supporting Information

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for

A Highly Potent and Cellular Active β-Peptidic Inhibitor of the p53/hDM2 Interaction

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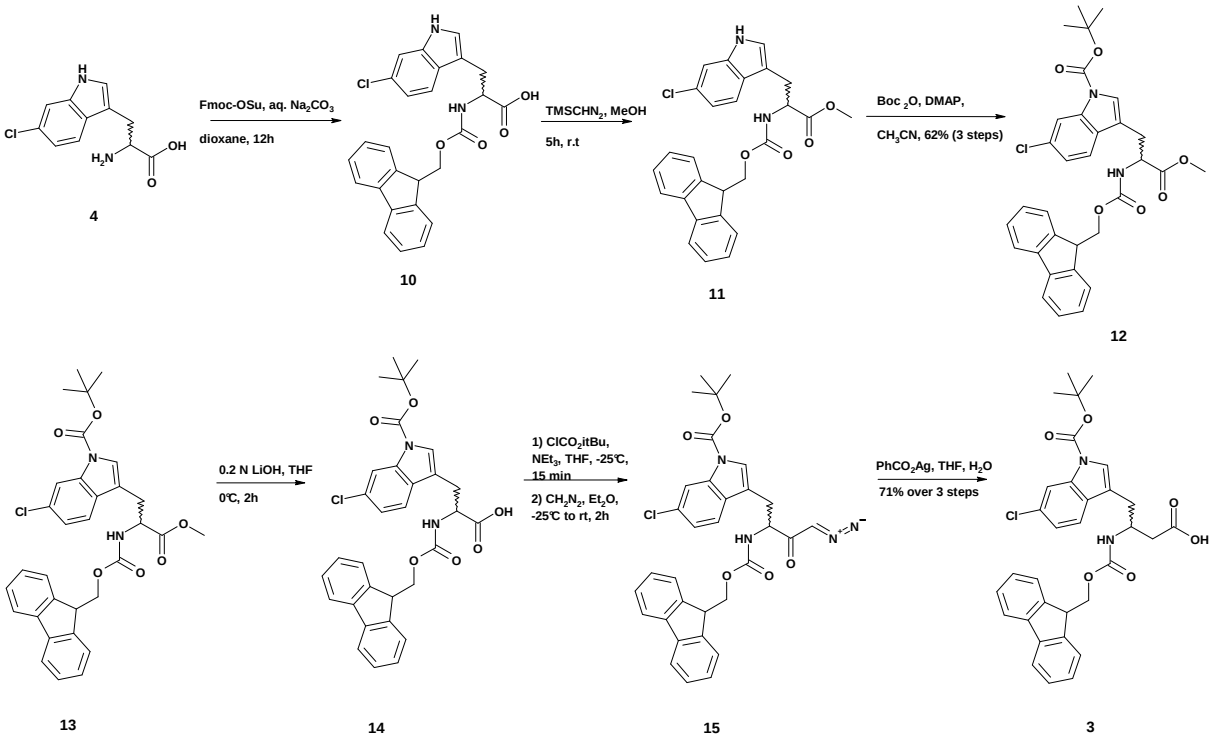

Scheme 1. Synthesis of β^3-homoamino acid Fmoc-(D,L)-β^3h(6-Cl) Trp(Boc)-OH (**3**). The new β^3-homoamino acid Fmoc-(D,L)-β^3h(6-Cl) Trp(Boc)-OH (**3**) was synthesized from the commercially available H_2N-(D,L)-(6-Cl)Trp-OH (**4**). First, Fmoc protection was carried out using FmocOSu, followed by esterification of the carboxylic acid functionality with $TMSCH_2N_2$. Boc protection of the indole nucleus was effected by treatment with Boc_2O and a catalytical amount of DMAP. This led to the indole derivative **12** in 62% yield. Finally, the homologation to the β^3-homoamino acid was performed via the Arnd-Eistert procedure developed by Seebach et al.[26]

Chemical Syntheses

Abbreviations: Boc (*tert*-butoxycarbonyl), Boc_2O (di-*tert*-butyl dicarbonate), DCM (dichloromethane), DIPEA (diisopropylethylamine), DMAP (4-(dimethylamino)pyridine), Fmoc (9-fluorenylmethoxycarbonyl), Fmoc-OSu (9-fluorenylmethoxycarbonyl-*N*-succinimidyl-carbonate), HATU (O-(7-azabenzotriazol-1-yl) *N,N,N,N*- tetramethyluronium hexafluorophosphate, HMBA (4-hydroxymethylbenzoic acid), HPLC (high-performance liquid chromatography), h.v. (high vacuum (0.01-0.1 Torr), MeIm (1-methylimidazole), MSNT (1-mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole), NMM (N-methylmorpholine), NMR (nuclear magnetic resonance), RT (room temperature), TFA (trifluoroacetic acid), TIS (triisopropylsilane), TLC (thin layer chromatography), FC (flash chromatographie), TMR (carboxy-tetramethylrhodamin), TNBS (2,4,6-trinitrobenzosulfonic acid), UV (ultra violet), equiv (equivalent)

Reagents: The Fmoc-protected α and β^3-homoamino acids were purchased from Fluka, AnandChem GmbH or Chem-Impex International. The HMBA and Wang resins were purchased from Rapp Polymere. All other reagents were used as received from Fluka or Aldrich.

Devices: Mass spectrometery: The mass spectra were recorded on a Waters MS-70.4000 micromass spectrometer, fragment ions are given in *m/z*.

Analytical HPLC**:** Analytical reversed phase HPLC was performed on a *Agilent* 1100 Series System (Quart pump G1311A, degaser G1322A, multiwavelength and fluorescence detector DAD G1315B and FLD G1321A). Column: *Zorbax* C_{18} (4.6 x 150 mm, 3.5 µm). Analyses were performed using a linear gradient of A (95% H_2O, 5% MeCN, 0.1% TFA) and B (95% MeCN, 5% H_2O, 0.1% TFA) at a flow rate of 0.8 mL/min with UV detection at 220 nm, and/or FLD detection at 555 nm for the TMR.

Preparative HPLC: Purification by preparative reversed phase HPLC was performed on a *Agilent* 1100 Series preparative system (Prep. pump G1361A, multiwavelength detector MWD G1365B). Column: Agilent prep. C_{18} (21.2 x 150 mm, 10 µm). Crude products were purified using the following linear gradient of A (95% H_2O, 5% MeCN, 0.1% TFA) and B (95% MeCN, 5% H_2O, 0.1% TFA) at a flow rate of 20 mL/min with UV detection at 220 nm.

General procedures: Anchoring of Fmoc-β^3hPra-OH on HMBA resin (GP 1): Esterification of the Fmoc-protected propargylglycine amino acid with the HMBA resin was

performed as follows: A solution of the Fmoc protected amino acid (5 equiv) in dry DCM (3 mL/mmol) was treated with MeIm (3.75 equiv) followed by MSNT (5 equiv) at RT. After complete dissolution of the MSNT the solution. was added to the preswollen resin (1 equiv) The suspension was mixed by Ar bubbling for 2h. Subsequently, the resin was filtered, washed with DCM (5 mL, 5 x 1min) and dried under h.v. for 24 h.

Solid-phase β-peptide synthesis on the HMBA resin (GP 2): The Fmoc group of the first amino acid attached to the resin was removed using 20% piperidine in DMF (3 mL, 4 x 10 min). After filtration, the resin was washed with DMF (2 mL, 4 x 1 min). Solid phase peptide synthesis was then continued for each resin part by sequential incorporation of *N*-Fmoc-protected β^3-homoamino acids. For each coupling step, the resin was treated with a solution of N-Fmoc-protected β^3-homoamino acid (5 equiv), HATU (4.8 equiv), DIPEA (10 equiv) in DMF (1 mL) and mixed for 45-60 min. Monitoring of the coupling reaction was performed with TMBS test. In case of a positive TMBS test (indicating incomplete coupling), the suspension was filtered off and the peptide resin was treated again with the same Fmoc-β^3-homoamino acid and coupling reagents. After the last Fmoc deprotection, the resin was Fmoc deprotected by treatment with 20% piperidine in DMF (3 mL, 4 x 10 min) and washed with DMF (1 mL, 5 x 1min), DCM (1 mL, 5 x 1min) and dried under h.v. for 12 h. The side chain protecting groups were then removed by treatment with 2 mL of a solution of TFA/ H_2O/TIS 95:2.5:2.5. After filtration, the resin was washed with DCM (1 mL, 3 x 1 min), with a solution of 10% DIPEA in DCM (1 mL, 3 x 10 min), and again with DCM (1 mL, 3 x 1 min). Finally, the resin was dried under h.v. and subsequently used for the labeling reaction or stored.

On-bead labeling and cleavage (GP 3): Several hundred beads are suspended in a mixture of 40 µL *t*BuOH and 35 µL H_2O and treated with 20 µL of a freshly prepared solution of $CuSO_4$ (3 mg) and ascorbic acid (1.5 mg) in 1 mL H_2O. 30 µL of a solution of TMR-azide **5** in MeOH (ca. 5 mm) are then added and the reaction mixture is left to stand at RT for 24 h. The solution is then removed and the beads are washed 5 times with 100 µL MeOH and 5-times with 50 µL H_2O. Finally, the labeled compound is cleaved from the bead by treatment of the resin with 120 µL of a cold (0° C) solution of NaOH (1n)-dioxane 1:3 for 15 min, followed by addition of 80 µL of HCl (1n). The solution is then purified by RP-HPLC.

Preparation of 9-[4-(3-Azido-propylcarbamoyl)-2-carboxy-phenyl]-6-dimethyl-amino-xanthen-3-ylidene}-dimethyl-ammonium (TMR-azide) (7).

5-Carboxy-tetramethylrhodamin-N-succinimidylester (20 mg, 0.46 µmol) in 2 mL DMF were added to a cold (0℃) solution of 3-azido prop ylamine (50 mg, 0.5 µmol). followed by 2 drops of DIPEA. The reaction mixture was stirred overnight and allowed to warm up to room temperature. The reaction mixture was then concentrated under reduced pressure to half of the volume and purified by RP-HPLC to yield the pure TMR-azide **5** (5-45% B in 20 min, 45-95 % B in 5 min, C_{18}, 555 nm). Anal. RP-HPLC: t_R 22.2 min (5-45% B in 20 min, C_4, 555 nm). MS (ESI positive mode): $[M+H]^+$ 513.3 (100%).

Preparation of Fmoc-(d,l)-(6-Cl)Trp(Boc)-OMe (12).

(2 g, 8.4 mmol) of commercially available H_2N-(dl)-(6-Cl)Trp-OH was suspended in 1M aq. Na_2CO_3 solution (50 mL) and treated with a solution of Fmoc-OSu (3.4 g, 10 mmol) in dioxane (30 mL). If necessary the pH was reajusted to 9-10 with additional aq. Na_2CO_3 solution and the mixture was stirred for 12h. The aqueous mixture was then extracted with Et_2O (2 x 20 mL). The aqueous phase was cooled to 0℃ and the pH adjusted to 2-3 with slow addition of 1m HCl. The aqueous phase was then extracted with AcOEt (3 x 40 mL) and the combined org. layers were dried ($MgSO_4$). Removal of solvent under reduced pressure yielded Fmoc-(dl)-(6-Cl)Trp-OH, which was used in the next step without further purification. To a solution of the crude Fmoc-(dl)-(6-Cl)Trp-OH in MeOH (40 mL) was slowly added 20 mL of TMS-CHN_2 (2M in hexane) and the mixture is stirred under Ar for 5h. The solvent is then evaporated under reduced pressure and the resulting crude material was dissolved in CH_3CN (40 mL). A solution Boc_2O (2 g, 9.17 mmol) and a catalytic amount of DMAP (12 mg, 0.01 mmol) was added and the mixture stirred for 5 h at RT. The solvent was removed under reduced pressure, and the crude product was purified by FC (cyclohexane/EE 5:1) to give Fmoc-(dl)-(6-Cl)Trp(Boc)-OMe (**12**; 3.1 g, 5.4 mmol, 62%). ^{13}C NMR (125 MHz, 25℃, $CDCl_3$): δ= 172.3 (C=O), 156.0 (NHCO, Fmoc), 149.5 (NHCO, Boc), 144.1, 141.7, 136.1, 131.1, 129.4 (C), 128.2, 127.5, 125.5, 125.0, 123.7, 120.4, 119.9, 116.1 (CH), 115.1 (C), 84.7 (C, OtBu), 67.6 (CH_2O, Fmoc), 54.5 (CH), 52.9 (CH_3, OMe), 47.5 (CH), 28.5 (CH_3, OtBu), 28.3 ppm (CH_2). ^1H (500 MHz, $CDCl_3$): δ= 1.67 (s, 9H, OtBu), 3.22 (m, CH_2Trp), 3.70 (s, OMe), 4.21 (t, *J*= 7.1 Hz, 1H, CH-Fmoc), 4.39 (d, *J*= 7.2 Hz, 2H, CH_2-Fmoc), 4.73 (m, 1H, NHCH-COOMe),

5.40 (d, *J*= 7.9 Hz, 1H, NH-Fmoc), 7.16-7.75 (m, 10H), ppm 8.17 (s, 1H, CHCCl, Trp). MS (ESI, positive mode): $[M+Na]^+$ 597.06 (100%).

Preparation of Fmoc-(d,l)-β^{3}h(6-Cl)Trp(Boc)-OH (3).

To a solution Fmoc-(d,l)-(6-Cl)Trp(Boc)-OMe (**12**) (1.37 g, 2.4 mmol) in 10 mL THF was slowly added 24 mL of a cold (0℃) solution of LiOH (0.2n) and the resulting mixture was stirred for 2 h. Et_2O (2 x 20 mL) was then added. The pH of the aqueous phase was adjusted to 2-3 with slow addition of 1m HCl. The aqueous phase was then extracted with AcOEt (3 x 40 mL) and the combined org. layers were dried ($MgSO_4$). The crude product was then dissolved in THF (20 mL) under Ar and cooled to -25℃. Et_3N (1.1 equiv, 385 µL) and $ClCO_2$*i*tBu (1.3 equiv, 430 µL) were then added to the solution and the suspension was allowed to warm to 0℃. A solution of CH_2N_2 in Et_2O was added until the intensive yellow color persisted over a longer period. The mixture was allowed to warm to RT, and stirred for 2h. Excess CH_2N_2 was destroyed by addition of a small amount of HOAc. After aq. workup by extraction with sat. $NaHCO_3$, NH_4Cl, the org. layer was separated, dried ($MgSO_4$) and the solvent was evaporated. The crude product was purified by FC (cyclohexane/EE 5:1) to give di-azoketone as a yellow foam (1.483 g, 2.53 mmol). MS (ESI, positive mode): $[M+Na]^+$ 607.13 (100%).

The diazoketone (1.48 g, 2.53 mmol) was dissolved in THF/H_2O (5: 1) at RT, silver benzoate (0.1 equiv, 58 mg) was added and the resulting mixture was ultrasonicated for 2h with the exclusion of light and the reaction monitored by TLC. After 2h, the solution was acidified to pH 2 with 1 m HCl and extracted with EtOAc. The combined organic layers were dried ($MgSO_4$) and evaporated. The crude product was purified by FC (cyclohexane/EE/AcOH 2:1:1%) to give Fmoc-(D,L)-β^{3}h(6-Cl)Trp(Boc)-OH (**3**) as a white solid (1 g, 1.7 mmol, 71 %). Analytical RP-HPLC: t_R 25.2 min (5-45% B in 20 min, C_{18}, 220 nm). ^{13}C NMR (125 MHz, DMSO): δ= = 172.5 (COOH), 155.6 (NHCO, Fmoc), 148.7 (NHCO, Boc), 143.8, 140.7, 135.2, 129.4, 129.0 (C), 127.6, 127.0, 125.2, 124.6, 122.8, 120.8, 120.2 (CH), 117.4 (C), 114.6 (CH), 84.1 (C, OtBu), 65.4 (CH_2O, Fmoc), 48.08 (CH), 46.74 (CHFmoc), 39.2 (CH2COOH), 29.5 (CH_2), 27.5 ppm (CH_3, O*t*Bu). ^{1}H (500 MHz, DMSO): δ= 1.52 (s, 9H, O*t*Bu), 2.45 (m, 2H, CH_2COOH), 2.82 (d, *J*= 6.7 Hz, 2H, CH_2-Trp), 4.05-4.22 (m, 4H, $NHCHCH_2COOH$, CH-Fmoc, CH_2-Fmoc), 7.22-7.28 (m, 3H, arom), 7.35-7.43 (m, 3H, 2H arom, NH), 7.51 (s, 1H, CCHN, Trp), 7.57 (d, *J*= 7.5 Hz, 2H, arom.), 7.67 (d, *J*= 8.4 Hz, 1H,

arom), 7.86 (d, J= 7.5 Hz, 2H, arom), 8.02 (s, 1H, arom), 12.2 ppm (s, 1H, COOH). MS (ESI, positive mode): $[M+Na]^+$ 597.05 (100%).

H_2N-β^{3}hLys-β^{3}hVal-β^{3}hLeu-β^{3}hGlu-β^{3}hVal-β^{3}hTrp-β^{3}hLys-β^{3}hVal-β^{3}hPhe-β^{3}hGlu-βAla-β^{3}hPra(TMR)-OH (6).

The HMBA resin (236 mg, 0.069 mmol) was derivatized with Fmoc-β^{3}hPra-OH (115 mg, 0.34 mmol) according to GP1. Solid-phase peptide synthesis was performed according to GP2 by sequential incorporation of Fmoc-βAla-OH, Fmoc-β^{3}hGlu(O*t*Bu)-OH, Fmoc-β^{3}hPhe-OH, Fmoc-β^{3}hVal-OH, Fmoc-β^{3}hLys(Boc)-OH, Fmoc-β^{3}hTrp-(Boc)-OH, Fmoc-β^{3}h-Val-OH, Fmoc-β^{3}hGlu(O*t*Bu)-OH, Fmoc-β^{3}hLeu-OH, Fmoc-β^{3}hVal-OH and Fmoc-β^{3}hLys(Boc)-OH. The resin was Fmoc-deprotected by treatment with 20% piperidine in DMF (1 mL, 4 x 10 min), washed with DMF and dried under h v. The side chain protecting groups were then removed by treatment with 2 mL of a solution of TFA/H_2O/TIS 95:2.5:2.5. After filtration, the resin was washed with DCM (1 mL, 3 x 1 min), with a solution of 10% DIPEA in DCM (1 mL, 3 x 10 min), and again with DCM (1 mL, 3 x 1 min). The labeling reaction and the cleavage from the resin were performed according to GP3. Purification by RP-HPLC (30-60% B in 20 min, 60-95% B in 5 min, C_{18}, 555 nm) yielded the title compound . Analytical RP-HPLC: t_R 13.6 min (30-60% B in 20 min, C_{18}, 555 nm). MS (ESI, positive mode): $[M+2H]^{2+}$ 1055.6 (15%), , $[M+3H]^{3+}$ 704.0 (100%), $[M+4H]^{4+}$ 528.3 (80%).

H_2N-β^{3}hLys-β^{3}hVal-β^{3}hLeu-β^{3}hGlu-β^{3}hVal-(d,l)-(6-Cl)β^{3}hTrp-β^{3}hLys-β^{3}hVal-β^{3}hPhe-β^{3}hGlu-βAla-β^{3}hPra(TMR)-OH (5).

The HMBA resin (103 mg, 0.03 mmol) was derivatized with Fmoc-β^{3}hPra-OH (50 mg, 0.15 mmol) according to GP1. Solid-phase peptide synthesis was performed according to GP2 by sequential incorporation of Fmoc-βAla-OH, Fmoc-β^{3}hGlu(O*t*Bu)-OH, Fmoc-β^{3}hPhe-OH, Fmoc-β^{3}hVal-OH, Fmoc-β^{3}hLys(Boc)-OH, Fmoc-6-Cl-(D,L)-β^{3}hTrp(Boc)-OH, Fmoc-β^{3}hVal-OH, Fmoc-β^{3}hGlu(O*t*Bu)-OH, Fmoc-β^{3}hLeu-OH, Fmoc-β^{3}hVal-OH and Fmoc-β^{3}hLys(Boc)-OH. The resin was Fmoc deprotection by treatment with 20% piperidine in DMF, filtered off, washed with DMF, and dried under high vacuum. The side chain protecting groups were then removed by treatment with 2 mL of a solution of TFA/H_2O/TIS 95:2.5:2.5. After filtration, the resin was washed with DCM (1 mL, 3 x 1 min), with a solution of 10% DIPEA in DCM (1 mL, 3 x 10 min), and again with DCM (1 mL, 3 x 1 min). The labeling reaction and the cleavage from

the resin were performed according to GP3. Purification by RP-HPLC (20-80% B in 20 min, 80-95% B in 5 min, C_{18}, 555 nm) yielded two diastereoisomers of the title compound. Analytical RP-HPLC: t_R 7.6 min (20-80% B in 20 min, C_{18}, 555 nm). MS (ESI, positive mode): $[M+3H]^{3+}$ 715.6 (30%), $[M+4H]^{4+}$ 536.9 (20%).and t_R 14.1 min (20-80% B in 20 min, C18, 555 nm). MS (ESI, positive mode): $[M+2H]^{2+}$ 1072.8 (15%); $[M+3H]^{3+}$ 715.5 (100%), $[M+4H]^{4+}$ 536.9 (98%).

H_2N-β^{3}hLys-β^{3}hVal-β^{3}hLeu-β^{3}hGlu-β^{3}hVal-(d,l)-β^{3}hTrp-β^{3}hLys-β^{3}hVal-β^{3}hPhe-β^{3}hGlu-OH (2).

The Wang resin (100 mg, 0.115 mmol) was modified with Fmoc-β^{3}hGlu(O*t*bu)-OH (253 mg, 0.575 mmol) using the method described in GP1 using MSNT (170 mg, 0.575 mmol) and MeIm (34 µL). The Fmoc protecting group was then removed by treatment with 20% piperidine in DMF (1 mL, 4 x 10 min). Solid phase synthesis was performed according to GP1 by sequential incorporation of Fmoc-β^{3}hPhe-OH, Fmoc-β^{3}hVal-OH, Fmoc-β^{3}hLys(Boc)-OH, Fmoc-(d,l)-β^{3}hTrp(Boc)-OH, Fmoc-β^{3}hVal-OH, Fmoc-β^{3}hGlu(O*t*bu)-OH, Fmoc-β^{3}hLeu-OH, Fmoc-β^{3}hVal-OH and Fmoc-β^{3}hLys-(Boc)-OH. The resin was fmoc deprotected by treatment with 20% piperidine in DMF, filtered off, washed with DMF, and dried under high vacuum. The resin was then treated with a solution of TFA/H_2O/TIS 95:2.5:2.5. The filtrate was evaporated under reduced pressure. Purification by RP-HPLC (20-80% B in 20 min, 80-95% B in 5 min, C_{18}, 220 nm) yielded two diastereoisomers of the title compound. Analytical RP-HPLC: first diastereoisomer t_R 8.8 min (20-80% B in 20 min, C_{18}, 220 nm), MS (ESI, positive mode): $[M+H]^+$ 1450.7 (10%), $[M+2H]^{2+}$ 726.2 (100%), $[M+3H]^{3+}$ 484.7 (80%). Second diastereoisomer t_R 14.3 min (20-80% B in 20 min, C_{18}, 220 nm). MS (ESI, positive mode) $[M+H]^+$ 1450.7 (10%), $[M+2H]^{2+}$ 726.2 (100%), $[M+3H]^{3+}$ 485.0 (60%).

H_2N-Lys-Lys-Trp-Lys-Met-Arg-Arg-Asn-Gln-Phe-Trp-Val-Lys-Val-Gln-Arg-Gly-$CONH_2$ (16).

(100 mg, 0.071 mmol) of Rink Amide AM resin was Fmoc-deprotected by treatment with 20% piperidine in DMF (1 mL, 4 x 10 min). Solid phase synthesis was performed according to GP1 by sequential incorporation of Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(*Trt*)-OH, Fmoc-Val-OH, Fmoc-Lys(Boc)-OH, Fmoc-Val-OH, Fmoc-Trp-(Boc)-OH, Fmoc-Phe-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg-(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Met-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Boc)-OH. A small amount of this resin (10 mg) containing the protected CPP was treated with

20% piperidine in DMF (1 mL, 4 x 10 min), filtered off, washed with DMF and dried under high vacuum. The resin was then treated with a solution of TFA/H_2O/TIS/EDT 95:2.5:1:2.5 and the filtrate was evaporated under reduced pressure. Purification by RP-HPLC (5-45% B in 20 min, 45-95% B in 5 min, C_{18}, 220 nm) yielded the title compound. Analytical RP-HPLC: t_R 11.9 min (5-45% B in 20 mn C_{18}, 220 nm). MS (ESI, positive mode), $[M+2H]^{2+}$ 1138.23 (20 %), $[M+3H]^{3}$ 758.99 (100 %).

H_2N-β^{3}hLys-β^{3}hVal-β^{3}hLeu-β^{3}hGlu-β^{3}hVal-(d,l)-β^{3}hCl-Trp-β^{3}hLys-β^{3}hVal-β^{3}hPhe-β^{3}hGlu-HN(CH_2CH_2O)$_2$$CH_2CO$-Lys-Lys-Trp-Lys-Met-Arg-Arg-Asn-Gln-Phe-Trp-Val-Lys-Val-Gln-Arg-Gly-$CONH_2$ (8).

The Rink Amide AM resin (90 mg, 0.071 mmol) described above, containing the protected CPP was Fmoc-deprotected by treatment with 20% piperidine in DMF (1 mL, 4 x 10 min). Solid phase synthesis was performed according to GP1 by sequential incorporation of Fmoc-8-amino-3,6-dioxaoctanoic acid, Fmoc-β^{3}hGlu(O*t*Bu)-OH, Fmoc-β^{3}hPhe-OH, Fmoc-β^{3}hVal-OH, Fmoc-β^{3}hLys(Boc)-OH, Fmoc-(d,l)-β^{3}hTrp(Boc)-OH, Fmoc-β^{3}hVal-OH, Fmoc-β^{3}hGlu(O*t*Bu)-OH, Fmoc-β^{3}hLeu-OH, Fmoc-β^{3}hVal-OH and Fmoc-β^{3}hLys(Boc)-OH. After removal of the last Fmoc protecting group, the resin was washed with DMF (2 mL, 4 x 1 min), DCM (2 mL, 4 x 1 min), filtered off and dried under h.v. The removal of the side chain protecting groups and the cleavage of the peptide from the resin was performed using a soln. of TFA/H_2O/TIS/EDT 95:2.5:1:2.5. Purification by RP-HPLC (20-80% B in 20 min, 80-95 % B in 5 min, C_{18}, 220 nm) yielded two diastereoisomers of the title compound. Anal. RP-HPLC: first diastereoisomer t_R 14.1 min (15-45% B in 20 min, C_{18}, 220 nm), MS (ESI, positive mode): $[M+3H]^{3+}$ 1284.1 (20%), $[M+4H]^{4+}$ 963.0 (40%), $[M+5H]^{5+}$ 770.5 (90%), $[M+6H]^{6+}$ 642.1 (100%), $[M+7H]^{7+}$ 550.3 (60%), second diastereoisomer t_R 7.5 min (30-65% B in 20 min, C_{18}, 220 nm). MS (ESI, positive mode): $[M+3H]^{3+}$ 1284.4 (60%), $[M+4H]^{4+}$ 963.2 (100%), $[M+5H]^{5+}$ 770.8 (70%), $[M+6H]^{6+}$ 642.7 (55%), $[M+7H]^{7+}$ 550.9 (30%), $[M+8H]^{8+}$ 481.9 (10%).

H_2N-Arg-Pro-Ala-Ser-Glu-Leu-Leu-Lys-Tyr-Leu-Thr-Thr-Pra(TMR)-OH (9).

The HMBA resin (103 mg, 0.03 mmol) was derivatized with Fmoc-Pra-OH (50 mg, 0.15 mmol) according to GP1. Solid-phase peptide synthesis was performed according to GP2 by sequential incorporation of Fmoc-Thr(OtBu)-OH, Fmoc-Thr(OtBu)-OH, Fmoc-Leu-OH, Fmoc-Tyr(OtBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Ser(O*t*Bu)-OH, Fmoc-Ala-OH, Fmoc-Pro-OH, Fmoc-

Arg(Pbf))-OH, The resin was Fmoc deprotection by treatment with 20% piperidine in DMF, filtered off, washed with DMF, and dried under high vacuum. The side chain protecting groups were then removed by treatment with 2 mL of a solution of TFA/ H_2O/TIS 95:2.5:2.5. After filtration, the resin was washed with DCM (1 mL, 3 x 1 min), with a solution of 10% DIPEA in DCM (1 mL, 3 x 10 min), and again with DCM (1 mL, 3 x 1 min). The labeling reaction and the cleavage from the resin were performed according to GP3. Purification by RP-HPLC (20-80% B in 20 min, 80-95% B in 5 min, C_{18}, 555 nm) yielded the title compound. Analytical RP-HPLC: t_R 7.3 min (20-80% B in 20 min, C_{18}, 555 nm). MS (ESI, positive mode): $[M+H]^{2+}$ 949.7 (100 %).

Affinity determination by confocal fluorescence fluctuation analysis using the 2D-FIDA-anisotropy method

The TMR labelled peptides were dissolved in PBS, pH 7.5, containing 0.005% Tween 20. For the competition titration experiments with the unlabeled peptide **5**, the assay buffer contained 5.5% DMSO as additive.

2D-FIDA measurements were performed using 384-well microtiter plates (Greiner) in a total assay volume of 70µL on the *PS02* instrument. The *PS02* instrument (developed jointly between Novartis and former Evotec Technologies, now Perkin Elmer, Germany) is based on an Olympus IX70 inverted microscope and equipped with two avalanche photodiode detectors. A HeNe laser (λ = 543 nm) was used as light source together with a linear polarization filter in the excitation path and a polarization beamsplitter in the fluorescence emission path. For TMR detection, a 560DRLP dichroic filter, an interference barrier filter with OD 5 (to block the excitation laser light from the optical detection path) and a 590DF60 bandpass filter were used in the optical path.

After instrument adjustment, TMR-labelled compounds were titrated against increasing $hDM2_{(2\text{-}188)}$ concentrations and fluorescence emission bursts were acquired for all samples for at least 10 x 10 s. All measurements were carried out at ambient temperature (constant at 23.5° C) in a low volume 384-well glass bottom microtiter plate. The molecular brightnesses q for each channel were obtained by data fitting, using the *FIDA Analyze* software package (Perkin Elmer, former Evotec Technologies): The confocal volume parameters (A0 and A1) and the channel-specific brightnesses of the adjustment solution were determined from the adjustment measurement. The G-factor was calculated according to Equation (1) prior to data fitting of the samples.

Finally, the anisotropies were calculated according to Equation (2) and averaged over ≥ 10 consecutive measurements.

$$G = \frac{q_1 * (1 - p_{true})}{q_2 * (1 + p_{true})} \quad (1)$$

$$r = \frac{q_1 - G * q_2}{q_1 + 2 * G * q_2} \quad (2)$$

r: anisotropy, q_1, q_2: molecular brightnesses in the parallel and perpendicular polarization channel, *G*: G-factor of the instrument, p_{true}: experimentally determined polarization (for TMR p_{true} = 0.034)

Equilibrium dissociation constants (K_d values) were obtained by performing a nonlinear least square regression fit of the fluctuation data sets, with the software package GraFit 5.0 and assuming a 1:1 binding model.

All competition titration experiments were performed on the PS02 instrument, using the 2D-FIDA anisotropy setup described above. TMR-labelled compounds at a concentration of 1 nM were titrated against increasing $hDM2_{(2\text{-}188)}$ concentrations in the presence of different concentrations of unlabeled competitor. Fluorescence fluctuation signals were acquired for all samples for at least 10x10 s. The concentration of unlabeled compound was kept constant at all measurement points in the individual titration curves. The resulting data were then fitted to the explicit algebraic description of binding equilibria in a mixture of 2 ligands competing for one receptor using the software package GraFit 5.0.

Cell Culture and Tumor Cell Viability

Cell culture: All cancer cell lines were obtained as frozen cell stocks from ATCC/ LGC Promochem and handled according to BSL-2 guidelines. RKO (ATCC, CRL-2577) is a poorly differentiated human colon carcinoma cell line with adherent growth properties and epithelial morphology. RKO cells were cultured in minimum essential medium (MEM) containing Earle's salts, 2.2 g L^{-1} sodium bicarbonate and 2 mM L-glutamine (Gibco, 31095), supplemented with 10% (*v/v*) fetal bovine serum (Gibco, 16000-044), 1 mM sodium pyruvate MEM (Gibco, 11360-039) and 1 x Antibiotic/Antimycotic solution (Sigma-Aldrich, A5955). SJSA-1 (ATCC, CRL-2098) is a human osteosarcoma cell line with adherent growth properties and fibroblast morphology. The cells

were cultured in RPMI 1640 medium, supplemented with 2.0 g L^{-1} $NaHCO_3$ and L-glutamine 10 % (*v/v*) fetal bovine serum (Gibco, 16000-044) and 1 x antibiotic/antimycotic solution (Sigma-Aldrich, A5955). All cell lines were cultured in a 37 ℃ incubator in an atmosphere containing 5 % CO_2 and a relative humidity of max. 95 %.

Subculturing of cancer cells: Typically, cells were subcultured when they reached ~80 % confluency. RKO cells were detached by incubation with non-enzymatic Cell Dissociation solution (Sigma-Aldrich, C5914). SJSA-1 cells were detached enzymatically using 1 x Trypsin-EDTA solution (0.25 % Trypsin with EDTA 4·Na, Gibco P/N 25200072). To harvest cells, complete growth medium was aspirated from the cell monolayer of the culture flask and the cells were washed twice with 10-15 mL sterile PBS def. to remove all traces of serum (contains trypsin inhibitor). 5 mL prewarmed Cell Dissociation solution or Trypsin-EDTA was added and the cells were observed under an inverted microscope until the cell layer was dispersed (usually within 3-5 minutes). To detach any remaining adherent cells, the solution was repeatedly pipetted over the surface of the culture flask. The cell suspension was transferred into a 50 mL polypropylene tube containing 25 mL complete growth medium for trypsin inactivation. The cells were harvested by centrifugation at 300 *g* (i.e. 1300 rpm on a Heraeus Megafuge 1.0R) for 5 min at RT. The supernatant was removed, and the cell pellet was resuspended in 10 mL complete growth medium. The cell number and viability was determined by Trypan Blue staining using a hemacytometer. For subculturing, cells were split in a ratio of 1:10 and 1:20 (*v/v*) in complete growth medium and seeded into new 75 cm^2 culture flasks (Costar, 3376).

Fibronectin coating of cell culture assay plates: Human fibronectin (hFN) was used to facilitate attachment of the cancer cells in 96-well assay plates. Lyophilized hFN (Chemicon; FC010) was dissolved at 37 ºC in sterile water to a concentration of 1 mg mL^{-1}, according to the manufacturer's instructions. For coating of 96-well plates the fibronectin solution was diluted to 20 µg mL^{-1} with sterile complete PBS. 100 µL diluted hFN was added to each well and allowed to adsorb for at least 1 h at 37℃ and 5 % CO_2. Residual PBS with unbound hFN was removed prior to plating of cells.

Determination of cell numbers and viability by Trypan-Blue staining: For routine determination of cell numbers and cell viability, a 10 µL aliquot of the cell suspension was removed and mixed with (usually an equal volume) Trypan blue solution (0.4 % w/v, Sigma-Aldrich P/N T8154). After incubation for 3-5 min, 10 µL of the stained cell

suspension was injected beneath the cover slip of a Neubauer hemacytometer. Unstained (viable) and stained (non-viable) cells were counted from at least four of the central large squares of the hemacytometer using a binocular microscope. The number of cells per mL of cell suspension was calculated according to

$$\left[cells \cdot ml^{-1}\right] = {}^{N_{viable}}\!/_{square} \cdot 10^4 \cdot DilutionFactor$$

The percentage of viable cells was calculated as:

$$\%\,viability \;\; = {}^{N_{viable}}\!/_{(N_{viable} + N_{non-viable})} \cdot 100$$

The viability of the harvested cells was usually >98 %.

Tumor cell viability assay: The effect of β-peptidic Hdm2 inhibitors on RKO and SJSA1 tumor cell viability was determined using a standard enzymatic viability assay. Briefly, the MTT Cell Proliferation Assay is a colorimetric assay that measures metabolic activity of cells and conversely, the reduction in cell metabolism due to apoptotic or necrotic cell death. In metabolically active cells, the yellow tetrazole substrate MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by mitochondrial reductases to the purple formazan. The intracellular formazan reaction product can be solubilized in detergents or organic solvents and quantified by spectrophotometric detection at 570 nm. As the reduction takes place only when mitochondrial reductase enzymes are active, the amount of conversion product can be directly related to the number of viable cells. The MTT Cell Proliferation Assay Kit from LGC Promochem/ATCC (Catalog Number 30-1010K) is a ready to use kit containing MTT reagent and detergent solution. The manufacturer´s formulation of the reagents is not specified in detail.

Seeding of cells into assay plates: Cells were plated in sterile 96-well flat bottom plates (Costar, 3595), which were previously coated with human fibronectin. In the first experimental series cells were seeded at a cell density of 1.2 x 10^4 cells (RKO) or 0.8 x 10^4 cells (SJSA-1) in 100 µL complete growth medium per well. Prior to compound addition, the cells were grown at 37° C, 5 % CO_2 for 3 days. In the second viability assay series cells were seeded at a cell density of 1.8 · 10^4 cells (RKO) and

1.2×10^4 cells (RKO) in 100 µL complete growth medium per well. Prior to compound addition, the cells were grown at 37° C, 5 % CO_2 for 24 h.

Compound dilution and administration: All compounds were dissolved to a stock concentration of 5 mM in cell culture grade DMSO (Sigma, D2650), diluted in each cell line's individual complete growth medium and added to the cells in an assay volume of 50 µL. The final compound concentrations were 100, 50, 20 and 5 µM, corresponding to final DMSO concentrations of 2, 1, 0.4 or 0.1 % (*v/v*), respectively. Control samples were treated with DMSO only, or left untreated. The cells were incubated with the compounds over night at 37° C , 5 % CO_2. Each condition was tested at least in triplicates.

MTT assay protocol: After incubation with the compound for 20 h (experimental series I) or 17 h (experimental series II), the cell viability was determined using the MTT-assay. To each well, 5 µL of the MTT reagent was added and incubated with the cells at 37° C, 5 % CO_2 until a homogenous purple precipitate became visible within the cytoplasm (1.5 h for RKO cells, 2 h for SJSA-1 cells). To lyse the cells and solubilise the precipitate, 50 µL of detergent solution was added to each well, followed by an incubation for 3-4 hours at room temperature (protected from light and with shaking of the plates every 30 min), until the precipitate was completely dissolved. Absorbance at 570 nm was measured on a Spectramax Gemini spectrophotometer (Tecan) and analyzed with the SoftMax Pro software. The viability of cells was calculated relative to untreated, vital cells as

$$\%\,viability = OD_{570\,nm\_sample\_well} \Big/ OD_{570\,nm\_untreated\_well} \cdot 100$$

Microscopy of cells after compound treatment: Previous to MTT addition, the cell morphology was assessed by bright field microscopy on a Zeiss Axiovert 200 microscope. A detailed description of this commercial microscope can be found, for instance, via the link http://www.zeiss.com/micro. For the cellular viability assay, a long distance 40× objective with 0.6 numerical apperture (NA) was used. The image mode was set to bright field, and the typical exposure time of the CCD camera was 200 ms.

Mouse plasma stability

Plasma stability analysis: Concentrated stock solutions of the peptides in water/methanol 8:2 were prepared, UV/Vis spectra measured and the concentration calculated on the basis of the molar extinction coefficient of the attached dye (TMR, ε = 78 000 cm^{-1} mol^{-1} l). Mouse plasma, strain BALB/C, with Li-Heparin, was obtained from Harlan Sera-Lab Ltd., Loughborough, UK. 600 µL plasma per series were spiked with small amounts of concentrated water/MeOH 8:2 stock solutions of the peptides to a final concentration of 10 µM and incubated at 37°C. Time point 0 h was taken immediately after spiking and mixing.

For each time point, the plasma was shortly mixed, 40 µL plasma were removed, vortexed with 120 µL acetonitrile for 20 sec to precipitate the proteins and centrifuged at 12000 rpm for 2 min. The clear supernatant was completely transferred to small microcentrifuge vials and evaporated in a vacuum centrifuge. The residue was dissolved in water/acetonitrile 8:2 (50 µL) by ultrasonic treatment, centrifuged for 1 min and the clear solution was completely transferred to autosampler vials. 15-25 µL aliquots were analyzed by HPLC.

HPLC was performed on a Dionex System with P-680 pump, ASI-100 autosampler, PDA-100 DAD detector, Jasco FP 2020Plus fluorescence detector and Chromeleon data system. Column: Zorbax SB-C18 3.5µm, 4.6x150mm with 4 x 4 mm Merck Lichrospher pre-column. Gradient: 11 min 5-90% B, 5 min 90% B, 3 min 90-5% B, 3 min 5% B, A: water 0.1% TFA, B: acetonitrile 0.1% TFA. Detection at 215, 260, 280, 333 or 555 nm (TMR) and fluorescence λ_{ex} 550 nm, λ_{em} 585 nm, gain 10.

The compound stability to degradation was monitored by detecting the HPLC chromatographic peak areas of the compound's fluorescence emission and the respective maximum of the absorbance of the attached dye in relation to either the total peak area of each analysis or the corresponding compound peak at time 0 h. Degradation products appeared as earlier eluting fluorescent and absorbing peaks.