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

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Protein–protein interactions are at the centre of most cellular processes, such as signal transduction[1] or programmed cell death.[2] Therefore, proteins mediating key components in disease related pathways are promising targets for drug discovery. Targeting protein–protein interactions is, however, extremely challenging.[3,4] Alpha helices are the most common secondary structure elements involved in protein–protein interactions besides linear stretches and turns. The key recognition motifs of alpha helices often involve hydrophobic side chains of amino acids located on one face of the helix at position *i*, *i*+3 and *i*+7 (Figure 1). Recently, considerable progress has been made to design foldamers—that is, synthetic scaffolds that can mimic the interaction face of a helix thereby disrupting the binding of a ligand to its receptor.[5] Oligomers of β-amino acids (so called β-peptides) are one class of such new peptidomimetics.[6] Depending on the substitution pattern, β-peptides have the ability to fold into a variety of stable secondary structures such as helices,[7–10] sheets[11] or turns.[12] Indeed, the group of Schepartz et al. designed and synthesized a β-peptidic mimic (**1**) of the tumour suppressor protein p53 binding domain to $hDM2_{(2-188)}$ with a K_d of 368±76 nM (Figure 2 A).[13,14] Furthermore, their stability towards proteolytic degradation[15] suggests that β-peptides could be used as scaffolds to mimic natural ligands.

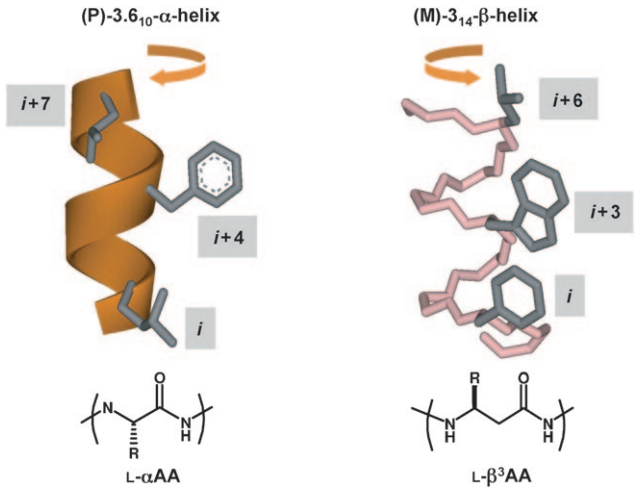

Figure 1. Schematic representation of a natural α-peptidic helix (left) and the β-peptidic (M)-3_{14}-helix (right). The two helices differ in their handedness and their overall dipole moment. However, residues *i*, *i*+4 and *i*+7 on the natural α-helix can be readily mimicked by residues *i*, *i*+3 and *i*+6 of the (M)-3_{14} helix.

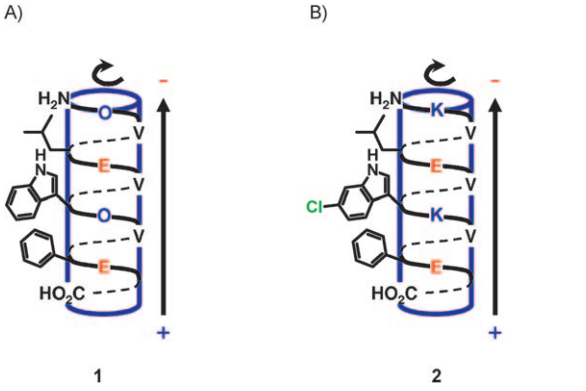

Figure 2. Helix representation of the β-peptidic ligand **1** published by Kritzer et al.[13] and the newly designed β^3-h(6-Cl)Trp containing hDM2 inhibitor. In addition to the incorporation of β^3-h(6-Cl)Trp in the new inhibitor, also ornithines were are replaced by lysines.

Herein, we report the design, synthesis and characterisation of a new β-peptidic p53 mimic with a tenfold improved binding affinity (K_d) for $hDM2_{(2-188)}$ as compared to the previously described β-peptidic ligand *β53-1* **1**. The improvement in hDM2 binding affinity was achieved by introducing a β^{3}h(6-Cl)Trp, a new β^3-homoamino acid with a nonnatural halogenated indole side chain, into the (M)-3_{14}-helical scaffold. Similar to the result found by others using alpha peptides,[16,17] the Cl atom on the indole ring of β^{3}h(6-Cl)Trp is supposed to fill a deep pocket on the hDM2 binding interface and hence increases the binding affinity of the p53 mimetic to hDM2. Furthermore, when tested in a cellular assay, the penetratin-conjugated form of the new p53 ligand led to a significant reduction in tumour cell viability and furthermore exhibited exquisite proteolytic stability. Our results further suggest that the combination of a β-peptidic helix-foldamer with the appropriate nonnatural amino acid side chains may provide a generalizable route to biologically relevant β-peptidic ligands. Furthermore,

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our ligand design strategy incorporates a propargylglycine for fluorescence labelling and a spacer unit. This generic labelling strategy allows for a fast and convenient determination of direct binding affinities, for example by using confocal fluorescence spectroscopy methods at single molecule resolution. In contrast to the often time-consuming iterative development of classical peptidomimetics, the combination of a well-known folding pattern with affinity enhancing side chains and a generic fluorescence labelling strategy for integrated quantitative analysis might provide an efficient strategy for tool compound and inhibitor identification.

Design and synthesis of a high-affinity β-peptidic p53 helix mimetic

Extensive structure activity relationship and protein mutagenesis studies have shown that the p53/hDM2 interaction is dominated by three hydrophobic residues (Phe19, Trp23, Leu26) aligned along one face of an amphipathic α-helix in p53 at position *i*, *i*+4 and *i*+7, filling hydrophobic pockets on the surface of hDM2.[18] The three side chains make direct contact with a cleft on the hDM2 surface and, despite the nice fit of the indole ring of Trp23 into a deep hydrophobic pocket in hDM2, empty space corresponding to a methylene group or Cl atom remains unoccupied.

The (M)-3_{14}-helix made of (L)-β^3-homoaminoacids is probably the best studied and characterized β-peptidic secondary structure (Figure 1).[19] Starting from a previously reported stable (M)-3_{14}-helix β-peptidic mimic of p53 (**1**, Figure 2A) by Kritzer et al,[13] we replaced the β^3hTrp by a β^3h(6-Cl)Trp-OH (**2**, Figure 2B, Figure 3). For this purpose, the new and suitably protected 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**, Scheme S1 and synthetic procedures in the Supporting Information). The new β^3h(6-Cl)Trp-OH-containing helix mimetic (**5**) along with parent compound **6** was synthesized on a HMBA TentaGel resin using standard FMOC/HATU chemistry (Figure 3, synthetic procedures in the Supporting Information). For a reliable comparison of direct binding affinities of the β-peptidic ligands to $hDM2_{(2-188)}$ a C-terminal fluorescence

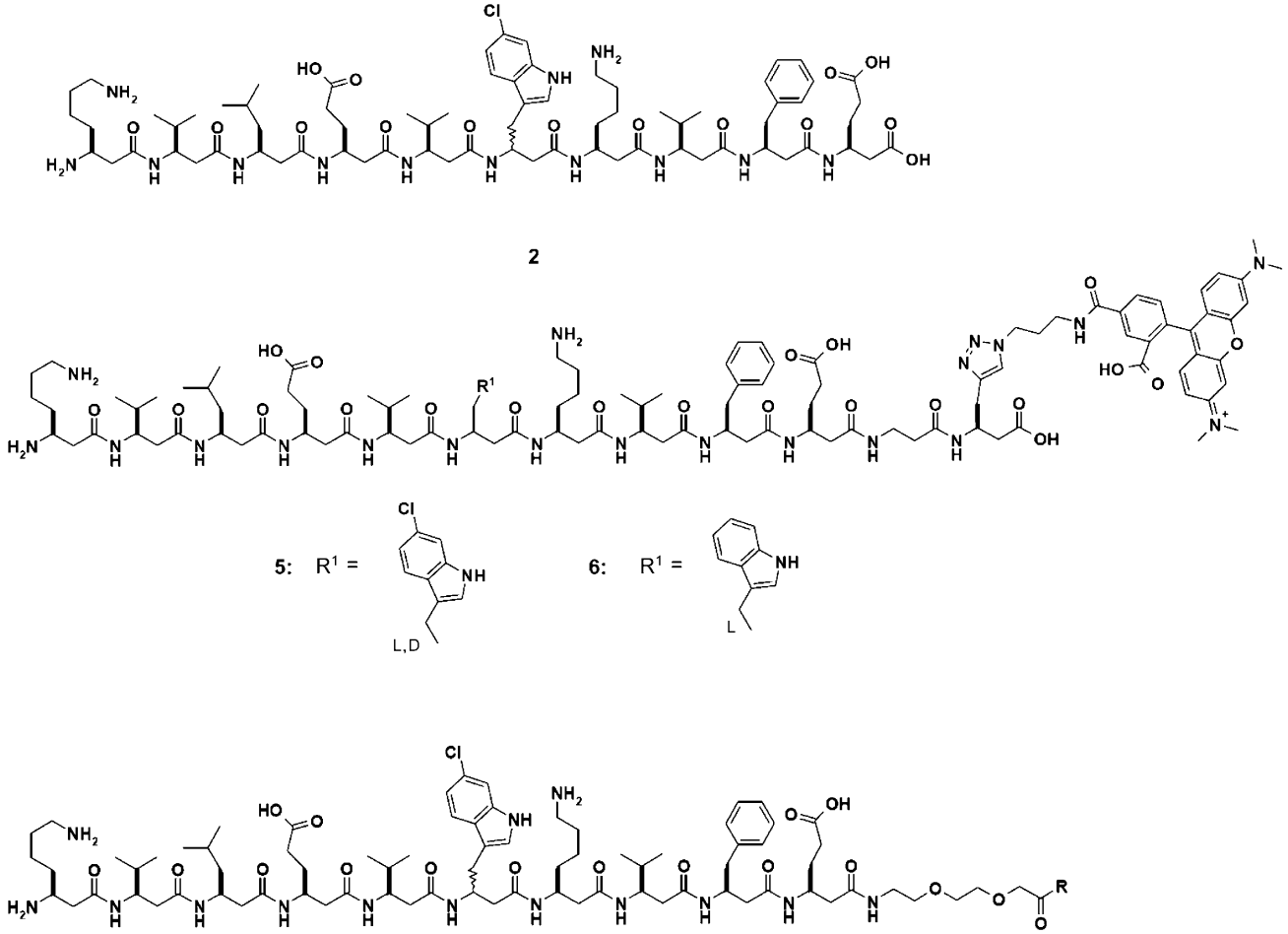

Figure 3. Primary sequences of the newly synthesized hDM2 inhibitors **2**, **5**, **6** and **8**. For the β^3-h(6-Cl)Trp-containing peptides, two diastereoisomers were obtained that were separated by HPLC purification and tested separately in the subsequent assays.

labelling site comprising a β^3-homopropargylglycine and a β-alanine were included in both helix mimetics. On-resin "click"-reaction, the highly chemoselective Cu^I-catalysed [3+2]cycloaddition reaction[20,21] with azide modified tetramethylrhodamine (TMR), azido-TMR **7** (synthesis in the Supporting Information), yielded labelled peptides **5** (as two separated isomers, denoted **5a** and **5b** subsequently) and **6** after cleavage and HPLC purification. For competition titrations, the new helix mimetic **2**, which lacked the labelling site and the TMR label, was synthesized on a Wang resin, by using the same coupling and deprotection conditions as described for compounds **5** and **6**. Furthermore, in order to test the efficiency of our new helix-mimetic we generated a cell penetrating peptide (CPP)-conjugated variant **8**. Construct **8** contains the penetratin sequence,[22,23] an Antennapedia homeodomain derived CPP, followed by an 8-amino-3,6-dioxaoctanoic acid spacer unit and the β-peptidic hDM2 recognition element on the C terminus (Figure 3, synthetic procedures in the Supporting Information).

To measure direct binding affinities (K_ds) of peptides **5a,b** and **6** to $hDM2_{(2-188)}$ we developed a 2D-FIDA-anisotropy[24] assay based on fluorescence fluctuation analysis at single molecule resolution (for details see the Supporting Information). 2D-FIDA-anisotropy is the confocal microscopy equivalent of the widely used fluorescence anisotropy method for detection of molecular volume changes in biological binding assays. Based on the analysis of the frequency distribution of recorded single photons in two polarization channels, the essential readout parameters of 2D-FIDA-anisotropy are molecular concentration and brightness (fluorescence intensity per molecule) of different fluorescent species. With a femtoliter-sized detection volume this method is ideally suited for the analysis of minimal amounts of fluorescently labelled compounds synthesized on solid-phase resin beads. Furthermore it avoids anisotropy artefacts that arise from adsorption of fluorescent material on the walls of the sample compartment.

The titration data, obtained with 1 nM labelled compound **5a**, **5b** or **6** as a function of the total concentration of $hDM2_{(2-188)}$ protein, were fitted to a 1:1 binding model (Figure 4A). The nonlinear curve fitting resulted in a dissociation constant of 102 ± 7 nM for the active isomer of peptide **5**, containing the β^3h(6-Cl)Trp. The parent β^3-peptide **6** turned out to be tenfold less potent, with a K_d of 1.44 ± 0.14 μM. The second isomer of peptide **5**, on the other hand, did not result in any measurable K_d under these experimental conditions.

To ensure that the high affinity of TMR-β^3h(6-Cl)Trp-p53 mimetic **5** for hDM2 was not induced by the hydrophobicity of the fluorescence label, and to test the hDM2 binding affinity of the penetratin-conjugated construct **8**, equilibrium competition titrations of the labelled ligands **5** and **6** versus the unlabelled ligands **2** and **8** were performed. The unlabelled competitors **2** or **8** at a concentration of 1 μM and 0.1 μM were premixed with 1 nM of labelled **5** and **6**, respectively, and the competition titration curves were recorded as a function of total $hDM2_{(2-188)}$ concentration (Figure 4B, C). Nonlinear curve fitting of the experimental data using the explicit algebraic description of binding equilibria in a mixture of two ligands competing for one receptor[25] yielded a dissociation constant of

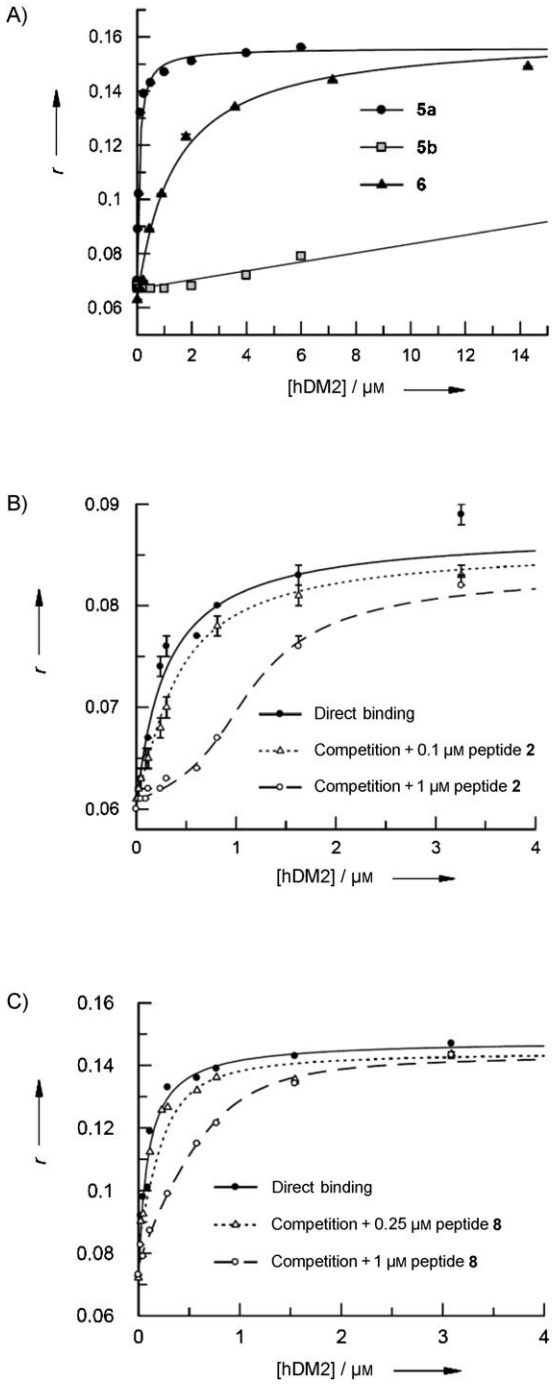

Figure 4. Direct binding and competition data for peptides **5**, **6**, **2** and **8**. All titrations were performed in 7 μL mixtures. 2D-FIDA anisotropy, the confocal fluorescence fluctuation analysis method equivalent to fluorescence anisotropy in ensemble averaging fluorescence detection was used for monitoring of binding followed by curve fitting applying the quadratic and cubic algebraic equations describing binding equilibria of a labelled ligand without or with competitor as function of the total amount of $hDM2_{(2-188)}$. A) direct binding of the two diastereoisomers of peptide **5** ($K_d = 102 \pm 7$ nM, active isomer) and the parent peptide **6** ($K_d = 1.44 \pm 0.14$ μM) to $hDM2_{(2-188)}$. B) competition titrations for the active isomer of peptide **5** in presence of 0.1 and 1 μM of the active isomer of competitor **2**. C) competition titration of active isomer of peptide **5** in presence of 0.25 and 1 μM of CPP-conjugated competitor **8**.

53 ± 10 nM for the active isomer of the β[3]h(6-Cl)Trp-helix mimetic **2** binding to $hDM2_{(2-188)}$. Due to the reduced solubility of unlabelled and untagged **2**, the competition titration assay was performed at 5.5% DMSO. This reduced the binding affinity of the labelled derivative **5** to a K_d of 340 ± 60 nM compared to the 102 ± 7 nm in native buffer. The actual affinity of **2** in native buffer might therefore even be in the single digit nM range. For the active isomer of the CPP-conjugated compound **8**, the curve fitting of the competition titration data resulted in a K_d of 156 ± 25 nM (Figure 3C).

In order to assess the cellular efficacy of the CPP-conjugated β-peptidic hDM2 inhibitor **8**, we further tested its ability to decrease cancer cell viability in vitro. Two different cell lines, a colon cancer cell line (RKO) and an osteosarcoma cell line (SJSA-1) were treated with both isomers of β[3]-peptide **8** and control compounds, including penetratin alone and non-CPP-conjugated inhibitor **2**. The metabolic activity (cell viability) was measured using a standard enzymatic assay (MTT viability assay).

Treatment with the active isomer of β[3]-peptide **8** reproducibly decreased the viability of both, RKO and SJSA-1 cells as compared to the controls with IC_{50} values in this assay being (53 ± 1) μM for RKO cells and (34 ± 3) μM for SJSA-1 cells (Figure 5A, B). The measured de-

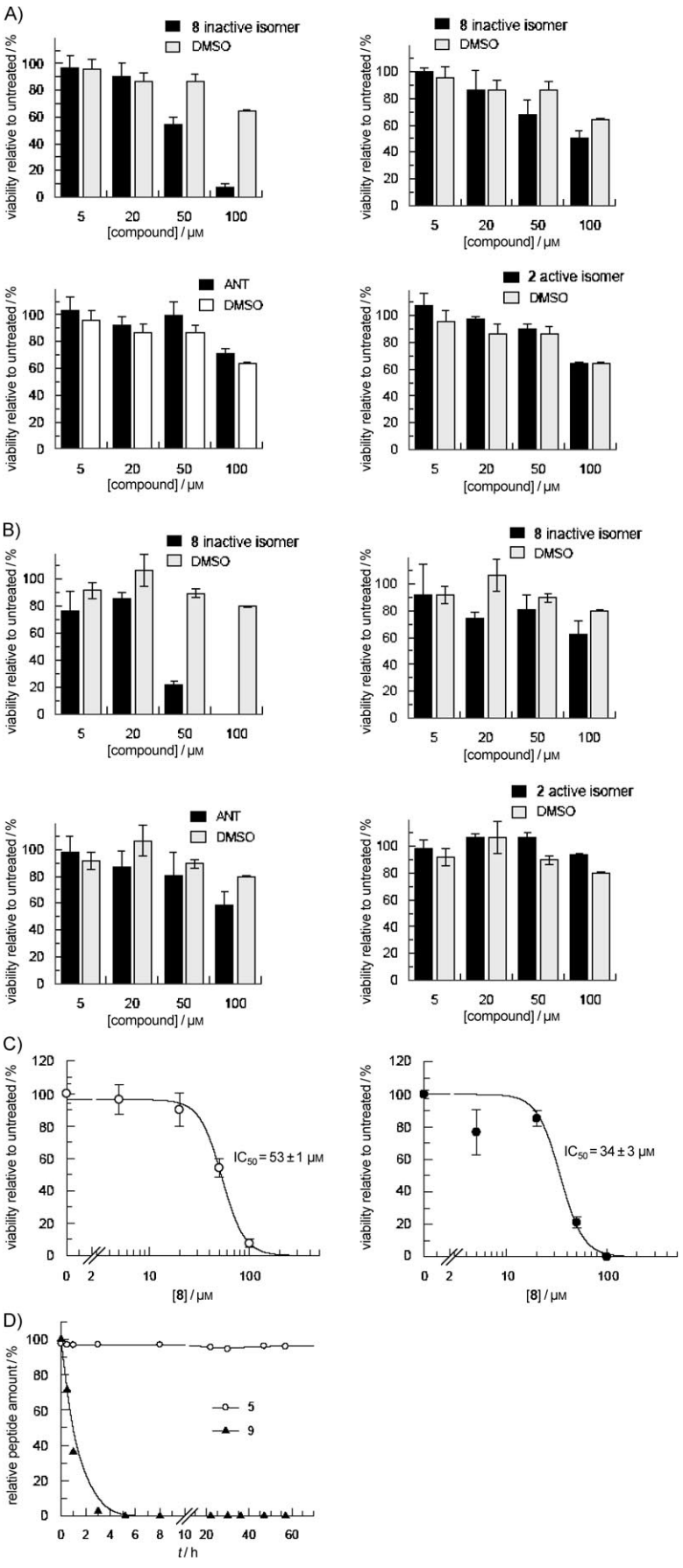

Figure 5. Cellular activity and plasma stability. Viability of A) RKO and B) SJSA1 cells after treatment with CPP conjugated β[3]-peptide **8** and the nonconjugated β[3]-peptide **2**. *ANT* indicates treatment with a peptide containing only the Antennapedia derived penetratin sequence of peptide **8**. C) IC_{50} determination of CPP-conjugated β[3]-peptide **8** from the tumour cell viability assay with RKO cells (left) and SJSA1 cells (right). D) Stability of 10 μM peptide solutions of β[3]-peptide **5** (active isomer) and α-peptide **9** in heparin mouse plasma over a period of 100 h at 37 °C.

crease in metabolic activity is in accordance with morphologic changes assessed by bright field microscopy (data not shown) suggesting that cell death was responsible for the measured reduction in cell metabolism. As the viability of the cells was unaffected by treatment with the CPP alone, this supports an hDM2 dependent effect. Finally, the inactivity of non-CPP conjugated β^3-peptide **2** suggests that the CPP is crucial for the cellular activity of this compound.

After the promising cellular results, we investigated the stability of the TMR-labelled β-peptide **5** in mouse plasma at 37 °C in comparison to an unrelated TMR-labelled α-peptide **9**, that contained the random sequence RPASELLKWLT and was synthesized with an identical labelling site and spacer (Figure 5C, Supporting Information). Mouse plasma was spiked with the peptides to a final concentration of 10 μM, incubated at 37 °C up to nine days in duplicates and aliquots were analyzed by HPLC in time intervals. Whereas the α-peptide **9** was proteolytically degraded within five to eight hours, the β-peptidic hDM2 inhibitor **5** remained nearly completely intact within the monitored time period of about nine days. Only about 5% of an earlier eluting fluorescent peak was detectable after 100 h.

In summary we have designed and characterized a new high-affinity p53-helix mimetic based on a β-peptidic (M)-3_{14}-helix. The design strategy utilizes the well-characterized property of β^3-peptides to fold into stable helical structures. The exchange of a tryptophan in the native recognition motif to a 6-chloro-tryptophan produced an hDM2 inhibitor with a low nanomolar dissociation constant. Furthermore, when attached to a cell-penetrating peptide, the new peptidomimetic exhibited biological activity against two common tumour-cell lines. Finally, plasma stability studies confirmed the superior proteolytic stability of β-peptidic ligands, as compared to α-peptides. In a broader sense, our results demonstrate the potential of β-peptidic secondary structure mimetics for cellular and prospective in-vivo applications.

Abbreviations

K_d: dissociation constant, HMBA: 4-hydroxymethylbenzoic acid; Fmoc: 9-fluorenylmethoxycarbonyl; HATU: *O*-(7-azabenzotriazol-1-yl) *N*,*N*,*N*,*N*-tetramethyluronium hexafluorophosphate; TMR: tetramethylrhodamine, CPP: cell-penetrating peptide.

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Keywords: antitumor agents · beta-peptides · hDM2 · inhibitors · p53 · protein–protein interactions

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