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Citation for published version:

Bautista, AD, Appelbaum, JS, Craig, CJ, Michel, J & Schepartz, A 2010, 'Bridged beta(3)-Peptide Inhibitors of p53-hDM2 Complexation: Correlation between Affinity and Cell Permeability' *Journal of the American Chemical Society*, vol 132, no. 9, pp. 2904-+. , 10.1021/ja910715u

Digital Object Identifier (DOI):

[10.1021/ja910715u](https://doi.org/10.1021/ja910715u)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of the American Chemical Society

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Published in final edited form as:

J Am Chem Soc. 2010 March 10; 132(9): 2904–2906. doi:10.1021/ja910715u.

Bridged β^3 -Peptide Inhibitors of p53-hDM2 Complexation— Correlation Between Affinity and Cell Permeability

Arjel D. Bautista[#], Jacob S. Appelbaum[§], Cody J. Craig[#], Julien Michel[#], and Alanna Schepartz^{#,¶}

Alanna Schepartz: alanna.schepartz@yale.edu

[#]Department of Chemistry, Yale University, New Haven, Connecticut 06520-8107

[§]Department of Cell Biology, and Molecular, Yale University, New Haven, Connecticut 06520-8107

[¶]Department of Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520-8107

Abstract

β -peptides possess several features that are desirable in peptidomimetics; they are easily synthesized, fold into stable secondary structures in physiologic buffers, and resist proteolysis. They can also bind to a diverse array of proteins to inhibit their interactions with α -helical ligands. β -peptides are not usually cell permeable, however, and this feature limits their utility as research tools and potential therapeutics. Appending an Arg₈ sequence to a β -peptide improves uptake but adds considerable mass. We reported that embedding a small cationic patch within a PPII, α - or β -peptide helix improves uptake without the addition of significant mass. In another mass-neutral strategy, Verdine, Walensky, and others have reported that insertion of a hydrocarbon bridge between the *i* and *i*+4 positions of an α -helix also increases cell uptake. Here we describe a series of β -peptides containing diether and hydrocarbon bridges and compare them on the basis of cell uptake and localization, affinities for hDM2, and 14-helix structure. Our results highlight the relative merits of cationic patch and hydrophobic bridge strategies for improving β -peptide uptake and identify a surprising correlation between uptake efficiency and hDM2 affinity.

β -peptides^{1–4} possess several features that are desirable in peptidomimetics;^{5,6} they are easily synthesized, fold into helices^{1–3,7} in physiologic buffers,⁸ and resist proteolysis.⁹ They also bind *in vitro* to proteins such as hDM2,^{10–14} hDMX,¹⁰ gp41,^{15,16} and others,^{17–19} and inhibit their interactions with α -helical ligands. β -peptides are not usually cell permeable, however, and this feature limits their utility as research tools and potential therapeutics. Appending an Arg₈ sequence to a β -peptide can improve uptake^{20,21} but adds considerable mass. We reported that embedding a small cationic patch within a PPII,²² α -²³ or β -peptide¹¹ helix improves uptake without the addition of significant mass.^{24,25} Similarly, Verdine, Walensky, and others^{26–33} reported that insertion of a hydrocarbon bridge (a “staple”) between the *i* and *i*+4 positions of an α -helix³⁴ increases uptake.^{26,29,32,34–38} Here we describe a variety of β -peptides containing diether- and hydrocarbon bridges and compare them on the basis of cell uptake and localization, affinity for hDM2, and 14-helix structure. Our results highlight the relative merits of cationic patch and hydrophobic bridge strategies for improving β -peptide uptake and identify an unprecedented correlation between uptake efficiency and hDM2 affinity *in vitro*.

Correspondence to: Alanna Schepartz, alanna.schepartz@yale.edu.

Supporting Information **Available:** β -peptide synthesis, binding and cell uptake assays, and confocal microscopy images. This material is available free of charge on the Internet at <http://pubs.acs.org>.

Our studies began with an analysis of available x-ray^{39,40} and NMR structures^{13,41} of β -peptide 14-helices to identify those position pairs that would best tolerate an ether^{42,43} or hydrocarbon³⁴ bridge. This analysis, supported by recent work of Perlmutter⁴² and Seebach⁴⁴ suggested that a 21-atom bridge could be accommodated between most *i* and *i*+3 positions of a 14-helix. To test this prediction, we synthesized an analog of β -peptide **2**⁷ containing (O-allyl)- β^3 -L-Ser at positions 3 and 6 (**2(3-6)**, Figure 1), and subjected it to on-resin ring-closing metathesis using bis(tricyclohexylphosphine)benzylidene ruthenium (IV) dichloride³⁴ to generate **2(3-6)s**.⁴⁵ The circular dichroism (CD) spectra of **2**, **2(3-6)** and **2(3-6)s** were identical (Figure S1), indicating that this 21-atom diether bridge is accommodated between positions 3 and 6. Introduction of the diether bridge did not significantly increase or decrease the extent of 14-helix structure as judged by CD.

In order to evaluate the relative uptake of bridged β -peptides in the context of a functional molecule of diverse sequence, we synthesized a series of variants of β **53-12**,¹⁰ an inhibitor of p53-hDM2 complexation (Figure 1). These variants contained either (O-allyl)- β^3 -L-Ser (to generate a diether bridge) or (*S*)-3-aminooct-7-enoic acid (to generate a hydrocarbon bridge) at *i* and *i*+3 positions 2 and 5 (**25.O-s** and **25.C-s**, respectively) or 4 and 7 (**47.O-s** and **47.C-s**, respectively). According to the CD spectra (Figure 2), all bridged β -peptides assumed a 14-helical structure and were modestly more helical than unbridged analogs (Figure S2).

As a prelude to evaluating cell uptake and localization, we employed a direct fluorescence polarization assay to compare hydrocarbon and diether bridged β -peptides on the basis of affinity for hDM2₁₋₁₈₈ (Figure 2B). β -peptides containing a diether or hydrocarbon bridge between positions 4 and 7 bound hDM2₁₋₁₈₈ 2-fold better ($K_d = 53.9 \pm 22.7$ and 94.1 ± 18.4 nM, respectively) than the corresponding unbridged analogs ($K_d = 114 \pm 28$ and 253 ± 75 nM, respectively), in line with analogous comparisons in an α -peptide context.³⁵ By contrast, β -peptides containing a diether or hydrocarbon bridge between positions 2 and 5 bound hDM2₁₋₁₈₈ between 4 and 8-fold worse ($K_d = 548 \pm 58$ and 546 ± 96 nM, respectively) than unbridged analogs ($K_d = 139 \pm 13$ and 68.1 ± 7.8 nM, respectively). *In silico* analysis suggests that the lower hDM2₁₋₁₈₈ affinity of β -peptides **25.C-s** and **25.O-s** results from steric hindrance between the hydrocarbon bridge and the hDM2 surface that is absent in the complex with peptides **47.C-s** and **47.O-s** (Figure 3, compare A and B).

We next set out to monitor the mammalian cell uptake and sub-cellular localization of diether- and hydrocarbon bridged β -peptides based on β **53-12**. Uptake was monitored using flow cytometry, whereas sub-cellular localization was assessed using confocal microscopy (Figure 4). β -peptides containing diether or hydrocarbon bridges between positions 4 and 7 were taken up significantly more efficiently (MCF = 8.21 ± 0.45 and 8.63 ± 0.77 , respectively) than unbridged analogs (MCF = 3.23 ± 0.31 and 2.63 ± 0.32 , respectively), irrespective of bridge structure. By contrast, β -peptides containing diether or hydrocarbon bridges between positions 2 and 5 were taken up poorly, irrespective of bridge structure, and behaved much like the unbridged analogs. In all cases, as judged by flow cytometry, the greatest uptake was observed with β -peptide β **53-12SB3**, which contains a cationic patch on one 14-helix face but no bridge of any kind (Figure 4AB).

The localization of bridged β -peptides upon cell uptake was explored in more detail using confocal microscopy. HeLa cells were treated with fluorescently labeled β -peptide (green) as well as Alexa Fluor® 647 labeled transferrin and Hoechst 33342 to visualize recycling endosomes^{46,47} (red) and nuclei (blue). β -peptides containing a diether or hydrocarbon bridge between positions 4 and 7 are distributed widely among Tf+ and Tf- endosomes, as well as nuclear and cytosolic compartments, whereas those containing the analogous bridge between positions 2 and 5 are not (Figure 3). Indeed, β -peptides containing a diether or hydrocarbon bridge between positions 2 and 5 are taken up more poorly than the unbridged analog (Figure

S4). These results highlight an intriguing correlation between hDM2 affinity and cell uptake; it is possible that the structural features that lower hDM2 affinity (Figure S3) also lower uptake efficiency. Indeed, it appears that for these β -peptides, an increase in 14-helix secondary structure does not necessarily confer increased cell uptake.²⁶

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

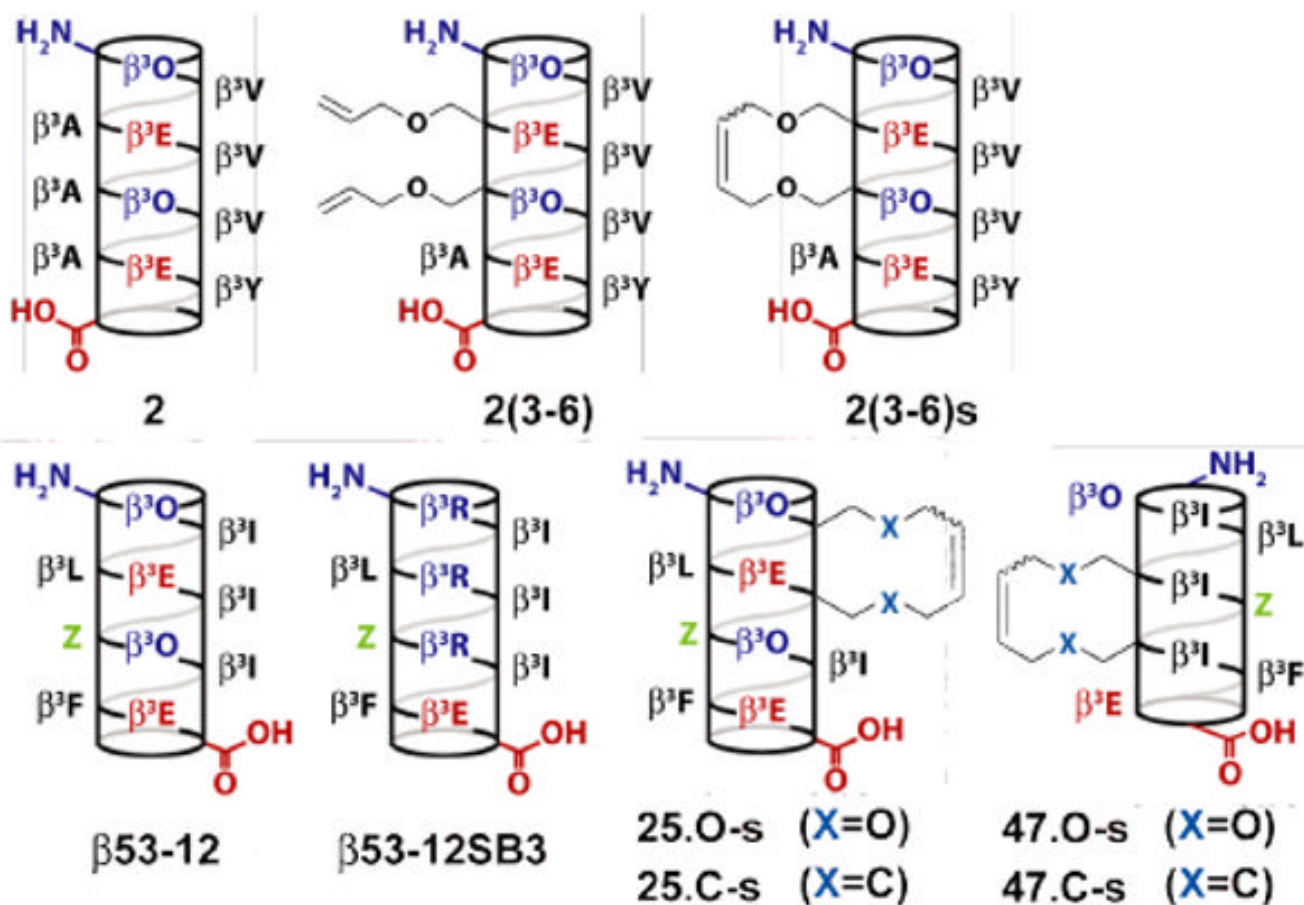
Acknowledgments

This work was supported by the NIH (GM 74756), the National Foundation for Cancer Research, and a Marie Curie International Outgoing Fellowship within the 7th European Community Framework Programme (J.M.). A.D.B. is grateful to Bristol-Myers Squibb for a graduate research fellowship.

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**Figure 1.**

Helical net representation of β -peptides studied herein. β^3 -homoamino acids are identified by the single-letter code used for the corresponding α -amino acid. Orn represents ornithine. Z represents 3-(S)-3-amino-4-(2-trifluoromethylphenyl)-butyric acid.

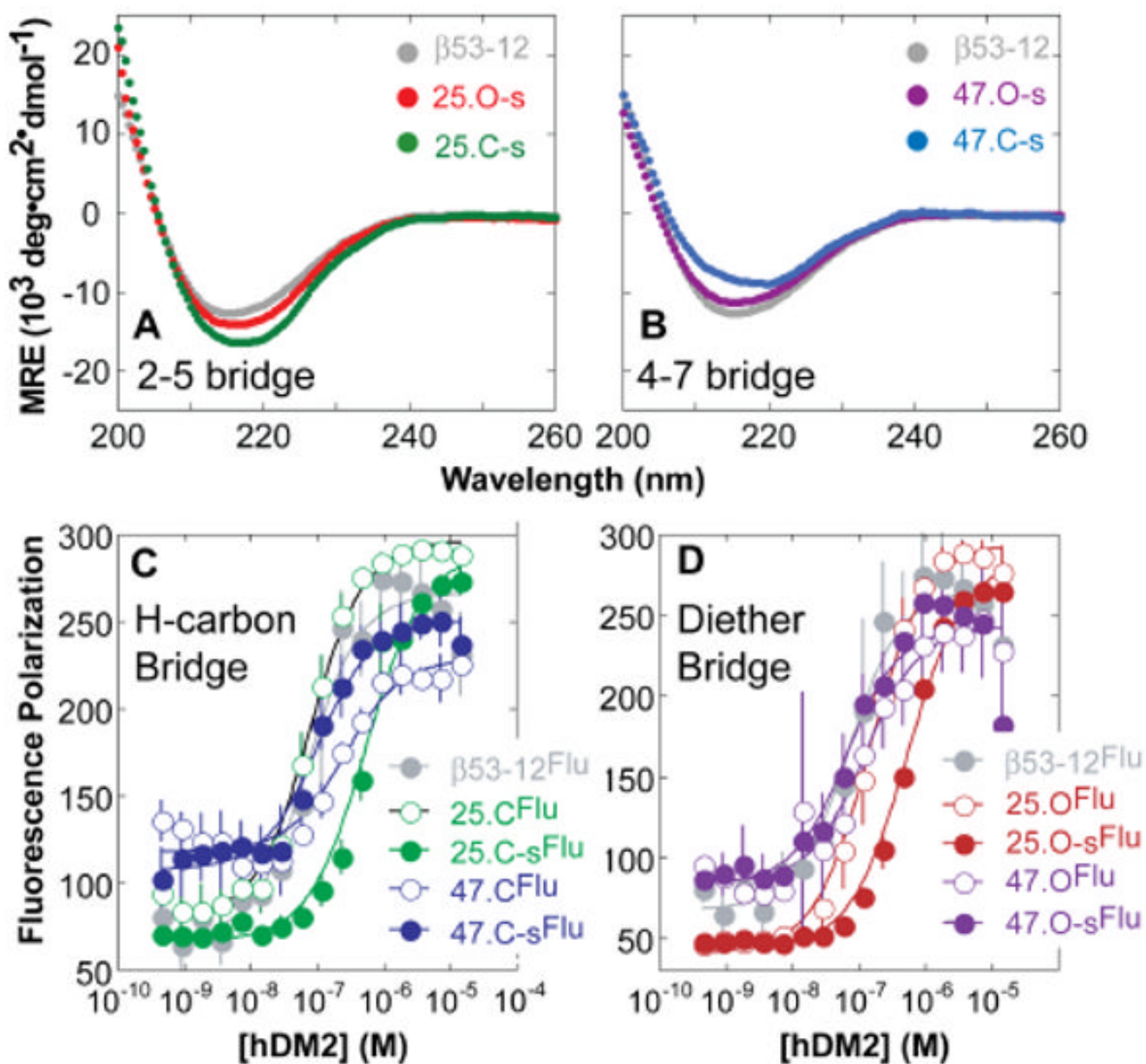


Figure 2. CD analysis of β -peptides containing hydrocarbon or diether bridges between residues (A) 2 and 5 or (B) 4 and 7. Fluorescence polarization (FP) analysis of hDM2 binding by β -peptides containing (C) hydrocarbon or (D) diether bridges.

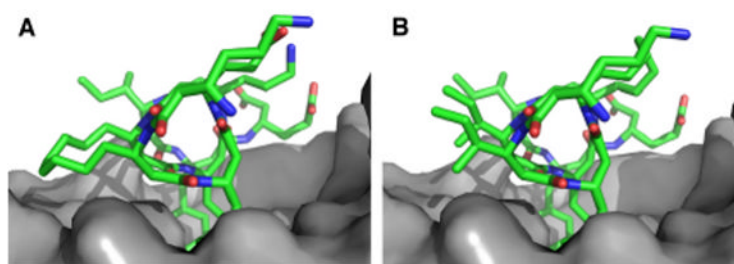
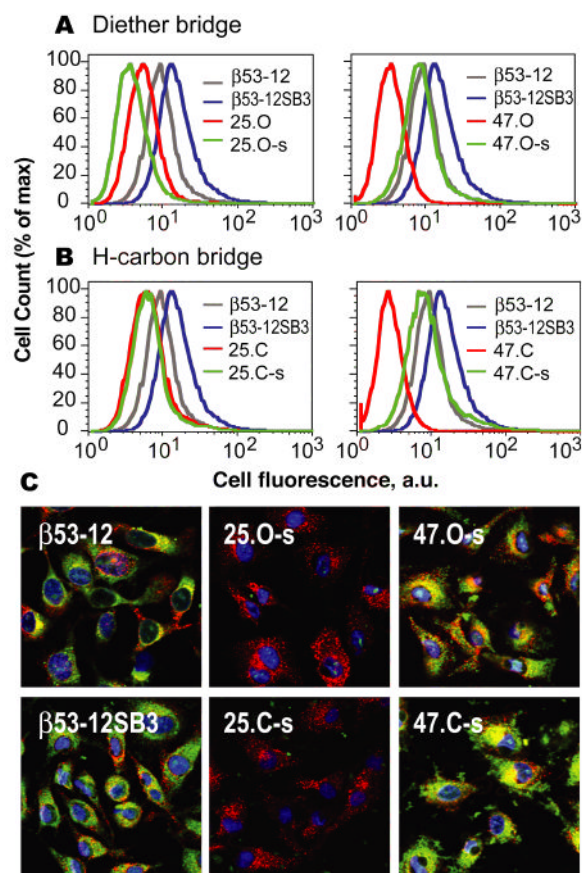


Figure 3.
Computational model of hDM2 (grey) in complex with (A) **25.C-s** or (B) **47.C-s**.⁴⁵

**Figure 4.**

HeLa cell uptake and localization of Flu-labeled β -peptides. (A,B) HeLa cells were incubated with 2 μ M β -peptide for 4 h, treated with 0.25% trypsin for 10 min, washed with cold DMEM and PBS, and analyzed using flow cytometry. (C) Confocal microscopy of HeLa cells treated with 20 μ M of the indicated β -peptide (green), 5 $\text{mg}\cdot\text{mL}^{-1}$ Alexa Fluor 647-transferrin (red) and 150 nM Hoescht 33342 (blue).