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## Bridged $\beta^3$ -Peptide Inhibitors of p53-hDM2 Complexation— Correlation Between Affinity and Cell Permeability

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### Abstract

$\beta$ -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  $\alpha$ -helical ligands.  $\beta$ -peptides are not usually cell permeable, however, and this feature limits their utility as research tools and potential therapeutics. Appending an Arg<sub>8</sub> sequence to a  $\beta$ -peptide improves uptake but adds considerable mass. We reported that embedding a small cationic patch within a PPII,  $\alpha$ - or  $\beta$ -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  $\alpha$ -helix also increases cell uptake. Here we describe a series of  $\beta$ -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  $\beta$ -peptide uptake and identify a surprising correlation between uptake efficiency and hDM2 affinity.

$\beta$ -peptides<sup>1-4</sup> possess several features that are desirable in peptidomimetics;<sup>5,6</sup> they are easily synthesized, fold into helices<sup>1-3,7</sup> in physiologic buffers,<sup>8</sup> and resist proteolysis.<sup>9</sup> They also bind *in vitro* to proteins such as hDM2,<sup>10-14</sup> hDMX,<sup>10</sup> gp41,<sup>15,16</sup> and others,<sup>17-19</sup> and inhibit their interactions with  $\alpha$ -helical ligands.  $\beta$ -peptides are not usually cell permeable, however, and this feature limits their utility as research tools and potential therapeutics. Appending an Arg<sub>8</sub> sequence to a  $\beta$ -peptide can improve uptake<sup>20,21</sup> but adds considerable mass. We reported that embedding a small cationic patch within a PPII,<sup>22</sup>  $\alpha$ -<sup>23</sup> or  $\beta$ -peptide<sup>11</sup> helix improves uptake without the addition of significant mass.<sup>24,25</sup> Similarly, Verdine, Walensky, and others<sup>26-33</sup> reported that insertion of a hydrocarbon bridge (a “staple”) between the *i* and *i+4* positions of an  $\alpha$ -helix<sup>34</sup> increases uptake.<sup>26,29,32,34-38</sup> Here we describe a variety of  $\beta$ -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  $\beta$ -peptide uptake and identify an unprecedented correlation between uptake efficiency and hDM2 affinity *in vitro*.

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Supporting Information **Available:**  $\beta$ -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<sup>39,40</sup> and NMR structures<sup>13,41</sup> of  $\beta$ -peptide 14-helices to identify those position pairs that would best tolerate an ether<sup>42,43</sup> or hydrocarbon<sup>34</sup> bridge. This analysis, supported by recent work of Perlmutter<sup>42</sup> and Seebach<sup>44</sup> 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  $\beta$ -peptide **2**<sup>7</sup> containing (O-allyl)- $\beta^3$ -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) dichloride<sup>34</sup> to generate **2(3-6)s**.<sup>45</sup> 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  $\beta$ -peptides in the context of a functional molecule of diverse sequence, we synthesized a series of variants of  $\beta$ **53-12**,<sup>10</sup> an inhibitor of p53-hDM2 complexation (Figure 1). These variants contained either (O-allyl)- $\beta^3$ -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  $\beta$ -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  $\beta$ -peptides on the basis of affinity for hDM2<sub>1-188</sub> (Figure 2B).  $\beta$ -peptides containing a diether or hydrocarbon bridge between positions 4 and 7 bound hDM2<sub>1-188</sub> 2-fold better ( $K_d = 53.9 \pm 22.7$  and  $94.1 \pm 18.4$  nM, respectively) than the corresponding unbridged analogs ( $K_d = 114 \pm 28$  and  $253 \pm 75$  nM, respectively), in line with analogous comparisons in an  $\alpha$ -peptide context.<sup>35</sup> By contrast,  $\beta$ -peptides containing a diether or hydrocarbon bridge between positions 2 and 5 bound hDM2<sub>1-188</sub> between 4 and 8-fold worse ( $K_d = 548 \pm 58$  and  $546 \pm 96$  nM, respectively) than unbridged analogs ( $K_d = 139 \pm 13$  and  $68.1 \pm 7.8$  nM, respectively). *In silico* analysis suggests that the lower hDM2<sub>1-188</sub> affinity of  $\beta$ -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  $\beta$ -peptides based on  $\beta$ 53-12. Uptake was monitored using flow cytometry, whereas sub-cellular localization was assessed using confocal microscopy (Figure 4).  $\beta$ -peptides containing diether or hydrocarbon bridges between positions 4 and 7 were taken up significantly more efficiently (MCF =  $8.21 \pm 0.45$  and  $8.63 \pm 0.77$ , respectively) than unbridged analogs (MCF =  $3.23 \pm 0.31$  and  $2.63 \pm 0.32$ , respectively), irrespective of bridge structure. By contrast,  $\beta$ -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  $\beta$ -peptide  $\beta$ 53-12SB3, which contains a cationic patch on one 14-helix face but no bridge of any kind (Figure 4AB).

The localization of bridged  $\beta$ -peptides upon cell uptake was explored in more detail using confocal microscopy. HeLa cells were treated with fluorescently labeled  $\beta$ -peptide (green) as well as Alexa Fluor® 647 labeled transferrin and Hoescht 33342 to visualize recycling endosomes<sup>46,47</sup> (red) and nuclei (blue).  $\beta$ -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,  $\beta$ -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  $\beta$ -peptides, an increase in 14-helix secondary structure does not necessarily confer increased cell uptake.<sup>26</sup>

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

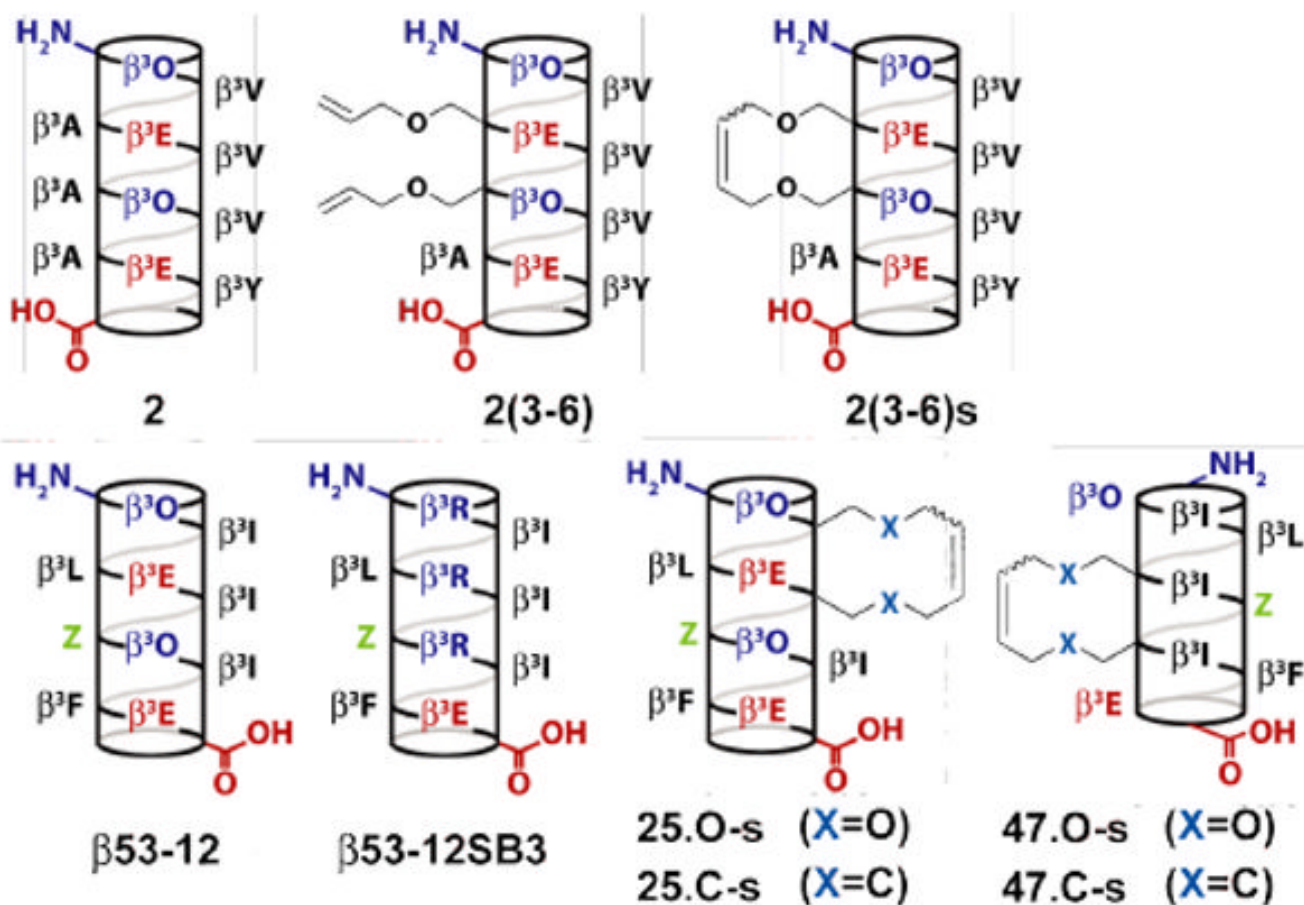
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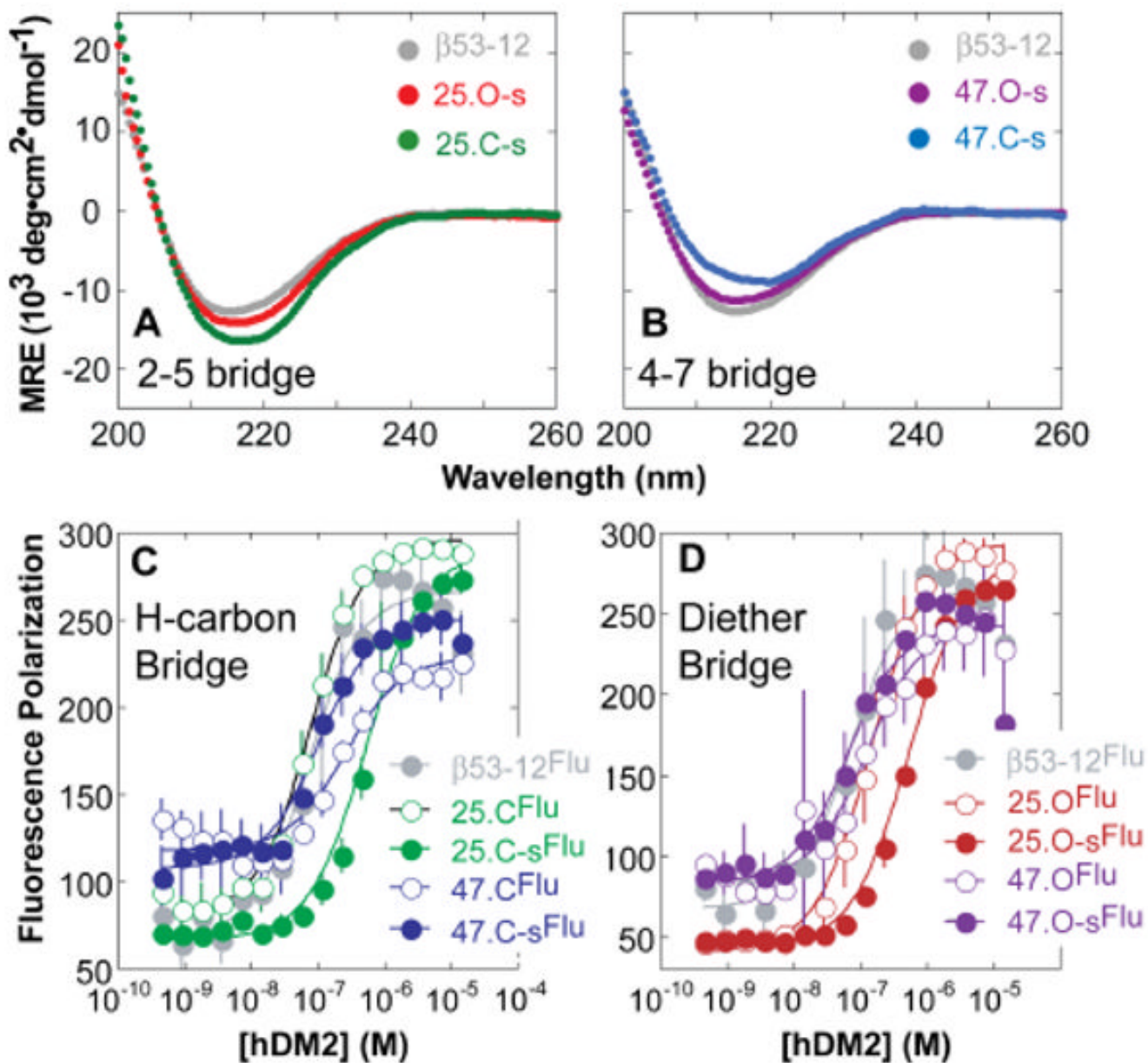
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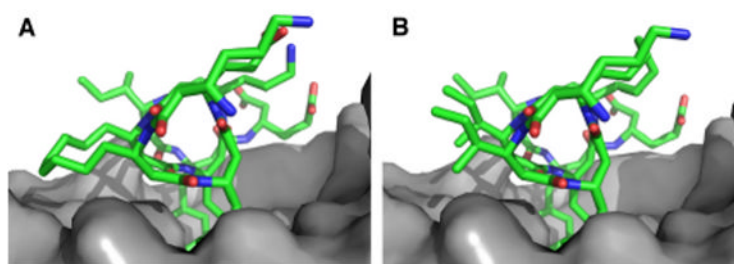
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**Figure 1.** Helical net representation of  $\beta$ -peptides studied herein.  $\beta^3$ -homoamino acids are identified by the single-letter code used for the corresponding  $\alpha$ -amino acid. Orn represents ornithine. Z represents 3-(S)-3-amino-4-(2-trifluoromethylphenyl)-butyric acid.

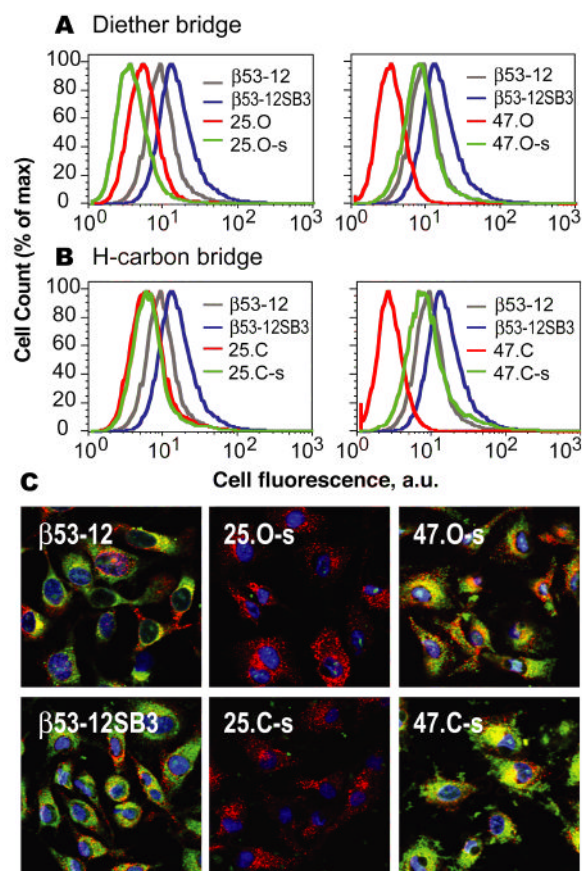


**Figure 2.** CD analysis of  $\beta$ -peptides containing hydrocarbon or diether bridges between residues (A) 2 and 5 or (B) 4 and 7. Fluorescence polarization (FP) analysis of hDM2 binding by  $\beta$ -peptides containing (C) hydrocarbon or (D) diether bridges.



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





**Figure 4.**

HeLa cell uptake and localization of Flu-labeled  $\beta$ -peptides. (A,B) HeLa cells were incubated with 2  $\mu$ M  $\beta$ -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  $\mu$ M of the indicated  $\beta$ -peptide (green), 5  $\text{mg}\cdot\text{mL}^{-1}$  Alexa Fluor 647-transferrin (red) and 150 nM Hoescht 33342 (blue).