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In Silico Improvement of β^3-Peptide Inhibitors of p53•hDM2 and p53•hDMX

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There is great interest in molecules that inhibit the interactions between p53 and its negative regulators hDM2 and hDMX, as such molecules have validated potential against cancers that overexpress one or both of these oncoproteins.[1,2] We reported that substituted β^3-peptides can inhibit these interactions[3,4] and, more recently, that minimally cationic β^3-peptides are sufficiently cell permeable to upregulate p53-dependent genes in live cells.[5,6] These observations, coupled with the established intracellular stability of β-peptides[7−9] and the recently reported structures of hDM2[10] and hDMX,[11] motivated us to exploit computational methods to identify β-peptides with improved potency and/or selectivity. This exercise successfully identified a new β^3-peptide, **β53-16**, that possess the desirable attributes of high affinity for hDM2 and hDMX and points to the 3,4-dichlorophenyl moiety as a novel determinant of hDMX affinity.

Our computational modeling began with the application of Visual Molecular Dynamics (VMD)[12] to generate a model of previously reported **β53-8** bound to the p53 binding site on hDM2 (Figure 1A). In this model, **β53-8** is bound as a 14-helix that is slightly unwound at the C-terminus, mimicking its conformation in solution.[13] The three hDM2 hydrophobic pockets occupied in the native structure by the p53 side chains of Leu_{26}, Trp_{23}, and Phe_{19}[10] are occupied in the modeled complex by the corresponding β^3-amino acid side chains at positions 3, 6, and 9. An analogous model of **β53-8** bound to hDMX was also prepared (Figure 1B).[11]

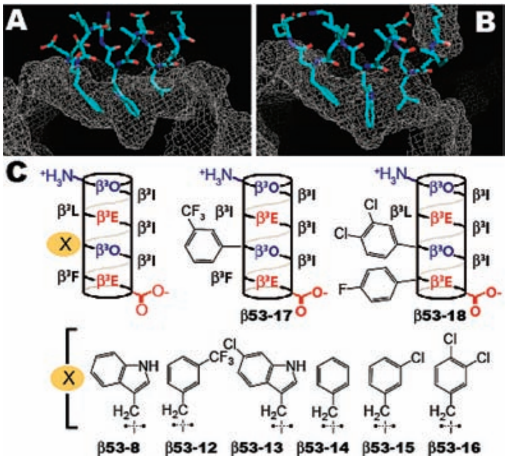
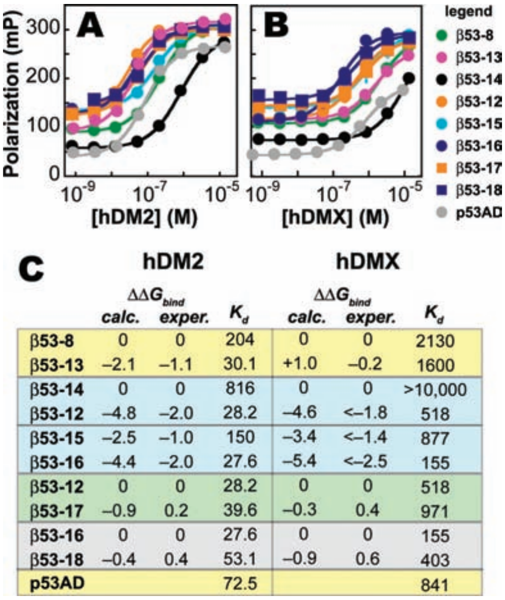


Figure 1. Computationally generated models of **β53-8** (blue) in complex with (a) hDM2 and (b) hDMX illustrating differences in binding site topologies. (c) Helical net representations of β^3-peptides studied herein.

We then applied a hierarchical computational strategy to search for alternative side chains that would improve packing at one or both interfaces. With the *de novo* design program BOMB[14] we screened over 10 000 **β53-8** analogues containing substituted aromatic and nonaromatic heterocycles and short hydrocarbon side chains in place of Leu_{26}, Trp_{23}, and Phe_{19}.[10] Approximately 50 candidates were identified by scoring and visualization for further evaluation with MCPRO.[15] Binding free energies were predicted *via* Monte Carlo Free Energy Perturbation (MC/FEP) calculations using the OPLS-AA force field[16] for the protein−ligand complex and the TIP4P model for water.[17] In these simulations, the protein backbones remained fixed; the affinities of the eight most interesting and synthetically accessible compounds (Figure 1C) were subsequently reevaluated in a second round of MC/FEP calculations that permitted backbone motions.[18]

The models were first validated by evaluating whether they would predict the large increase in hDM2 affinity realized when the tryptophan side chain at position 6 is replaced by 6-chlorotryptophan ($^{6\text{-Cl}}$W) (compare **β53-8** and **β53-13**, Figure 1C).[5] The calculations predict that substitution of $^{6\text{-Cl}}$W at position 6 should significantly improve binding to hDM2 ($\Delta\Delta G = -2.1$ kcal•mol^{-1}) but not hDMX ($\Delta\Delta G = +1.0$ kcal•mol^{-1}, Figure 2C). These predictions are fully aligned with the experimental results: the stability of the hDM2•**β53-13** complex is significantly higher ($K_d = 30.1$ nM, $\Delta G = -10.25$ kcal•mol^{-1}) than that of the hDM2•**β53-8** complex ($K_d = 204$ nM, $\Delta G = -9.12$ kcal•mol^{-1}), whereas the stabilities of the analogous hDMX complexes are comparable ($K_d = 1.6$ and 2.1 μM for **β53-13** and **β53-8**, respectively). The improvement in hDM2 but not hDMX affinity upon substitution of $^{6\text{-Cl}}$W is largely consistent with results observed in the context of previously reported ligands.[20−23]



	hDM2			hDMX		
	$\Delta\Delta G_{bind}$ calc.	$\Delta\Delta G_{bind}$ exper.	K_d	$\Delta\Delta G_{bind}$ calc.	$\Delta\Delta G_{bind}$ exper.	K_d
β53-8	0	0	204	0	0	2130
β53-13	–2.1	–1.1	30.1	+1.0	–0.2	1600
β53-14	0	0	816	0	0	>10,000
β53-12	–4.8	–2.0	28.2	–4.6	<–1.8	518
β53-15	–2.5	–1.0	150	–3.4	<–1.4	877
β53-16	–4.4	–2.0	27.6	–5.4	<–2.5	155
β53-12	0	0	28.2	0	0	518
β53-17	–0.9	0.2	39.6	–0.3	0.4	971
β53-16	0	0	27.6	0	0	155
β53-18	–0.4	0.4	53.1	–0.9	0.6	403
p53AD			72.5			841

Figure 2. Direct fluorescence polarization analysis of the affinity of each β^3-peptide shown for (A) hDM2 and (B) hDMX. (C) Comparison of calculated and experimental binding free energies expressed in terms of $\Delta\Delta G_{\text{bind}}$ relative to the standard shown (kcal•mol^{-1}); K_d values in nM units.

10.1021/ja901478e CCC: $40.75

The models were further validated by their ability to predict the large increase in hDM2 and hDMX affinity observed for β^3-peptides containing a central *meta*-trifluoromethyl phenyl substituent (^{CF3}F) when compared with an unsubstituted phenyl ring (compare ***β*****53-12** with ***β*****53-14**, Figure 1C). The calculations predict that the ^{CF3}F side chain should favor binding to both hDM2 and hDMX ($\Delta\Delta G = -4.8$ and -4.6 kcal·mol^{-1}, respectively). This increase was also realized experimentally, albeit in an attenuated way: the stability of the hDM2•***β*****53-12** complex is significantly higher ($K_d = 28.2$ nM, $\Delta G = -10.29$ kcal·mol^{-1}) than that of the hDM2•***β*****53-14** complex ($K_d = 816$ nM, $\Delta G = -8.3$ kcal·mol^{-1}); analogous differences are seen for the hDMX complexes (Figure 2).[24]

Next we examined whether the affinity of ***β*****53-12** could be increased further by substituting the leucine side chain at position 3 with one of eight cyclic and acyclic hydrocarbon alternatives. Although few promising candidates emerged from the BOMB and MC/FEP analyses, we did investigate ***β*****53-17**, in which the Leu side chain is replaced by Ile. This substitution was predicted to slightly favor the binding of both hDM2 and hDMX ($\Delta\Delta G_{\text{bind}} = -0.9$ and -0.3 kcal·mol^{-1}, respectively). However, no increase in affinity was observed, and these molecules were not studied further. We note that ***β*****53-17** is significantly less 14-helical than ***β*****53-12** as judged by circular dichroism analysis (Figure SI-1). As the computational model does not account for changes in β-peptide secondary structure, it is possible that the observed change in secondary structure accounts for the poor agreement between prediction and experiment in this case. The predictions may also be affected by uncertainty in the structures of unliganded hDM2 and hDMX as the 23 N-terminal residues of both proteins are only partially resolved due to their flexibility.[25,26]

Based on these observations, we returned attention to the central side chain of the hDM2/hDMX epitope and evaluated the relative hDM2 and hDMX affinities of hundreds of ***β*****53-12** analogues containing substituted phenylalanine analogues at position 6. This analysis suggested that β-peptides containing either *meta*- or *para*-chlorophenylalanine at this position would show improved affinity for both hDM2 and hDMX when compared with ***β*****53-14** (-3.5 kcal·mol^{-1} < $\Delta\Delta G_{\text{bind}}$ < -2.5 kcal·mol^{-1}). Indeed, the stability of the hDM2•***β*****53-15** complex (*meta*-chloro substituent, Figure 1C) is significantly higher ($K_d = 150$ nM, $\Delta G = -9.3$ kcal·mol^{-1}) than that of hDM2•***β*****53-14**; analogous differences are observed for the hDMX complexes (Figure 2). However, as predicted, the stabilities of the ***β*****53-15** complexes were not greater than those of the ***β*****53-12** complexes. Therefore, since the gains in affinity for the *para*-chlorophenylalanine were predicted to be similar to those of ***β*****53-15**, this additional analogue was not tested experimentally.

Finally we examined the effect of a *meta*,*para*-dichlorophenylalanine side chain at the central position of the recognition epitope (***β*****53-16**, Figure 1), whose inclusion was predicted to significantly improve affinity for both hDM2 and hDMX compared to ***β*****53-14** ($\Delta\Delta G_{\text{bind}} = -4.4$ and -5.4 kcal·mol^{-1}, respectively). Indeed, the stabilities of both hDM2•***β*****53-16** and hDMX•***β*****53-16** are significantly higher than those of the corresponding ***β*****53-14** complexes ($K_d = 27.6$ and 155 nM, respectively for ***β*****53-16**, Figure 2). They also equal or exceed the stabilities of the corresponding complexes with ***β*****53-12**. Competition fluorescence polarization experiments confirm that ***β*****53-16** competes with p53AD for binding to hDM2 and hDMX and shows improved inhibitory potency toward hDMX (Figure SI-2).

Thus, ***β*****53-16** offers significantly improved affinity for hDMX without loss of affinity for hDM2. Analysis of the MC/FEP simulations suggests more favorable interaction of the dichlorophenyl group with residues 50−54 in hDMX than with equivalent residues 54−58 in hDM2. We subsequently examined whether the affinity of ***β*****53-16** could be improved further upon replacement of the adjacent phenylalanine side chain with 1 of 12 substituted analogues. This scan failed to identify promising substitutions as the phenylalanine side chain appears to bind tightly to the hydrophobic pocket of both proteins; however minor gains in affinity for both hDM2 and hDMX were predicted for a *para*-fluorophenylalanine substitution ($\Delta\Delta G_{\text{bind}} = -0.4$ and -0.9 kcal·mol^{-1}, respectively). No increase in affinity was observed experimentally with this peptide, ***β*****53-18**, so further modification at this position was not pursued (Figure 1, Table 1). ***β*****53-16** represents the highest affinity β^3-peptide for hDMX reported to date, with a significantly higher affinity than the prototypic hDM2 ligand, Nutlin-3. Thus, ***β*****53-16** embodies the pan-specificity of well-known peptidic hDM2/hDMX inhibitors[27,28] without the limitations of protease sensitivity or poor uptake.

Acknowledgment. This work was supported by the NIH (GM 74756 and GM 32136) and the National Foundation for Cancer Research.

Supporting Information Available: Details of computational protocols, CD and binding assays, and the complete citation for ref 20. This material is available free of charge via the Internet at http://pubs.acs.org

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JA901478E