Protein–Protein Interactions

Terphenyl-Based Helical Mimetics That Disrupt the p53/HDM2 Interaction**

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The p53 protein plays a key role in the apoptosis pathway.[1] Increased expression of wild-type p53 in stressed cells leads to cell-cycle arrest or apoptosis,[2] whereas in normal cells p53 is present at very low levels owing to regulation by human double minute 2 (HDM2), which promotes the degradation of p53 through an ubiquitin-dependent proteasome pathway.[3] The p53 protein is found in a mutated or inactive state in over 50% of cancerous tumors.[4] Moreover, overexpression of HDM2 has been implicated in the development of cancerous tumors, such as human osteogenic sarcomas and soft-tissue sarcomas,[5] as the overexpressed HDM2 abrogates the ability of p53 to induce cell-cycle arrest and apoptosis.[2] For these reasons, disruption of the p53/HDM2 interaction by using small-molecule agents has become an important goal for anticancer-drug development.[6]

HDM2 regulates p53 by complex formation that involves amino acid residues 18–102 of HDM2 and a helical region of p53 (amino acids 16–28).[7] Crystallographic analysis of the HDM2/p53 complex has revealed that three hydrophobic residues (F19, W23, L26) along one face of the p53 helical peptide are essential for binding (Figure 1a).[8] Several groups

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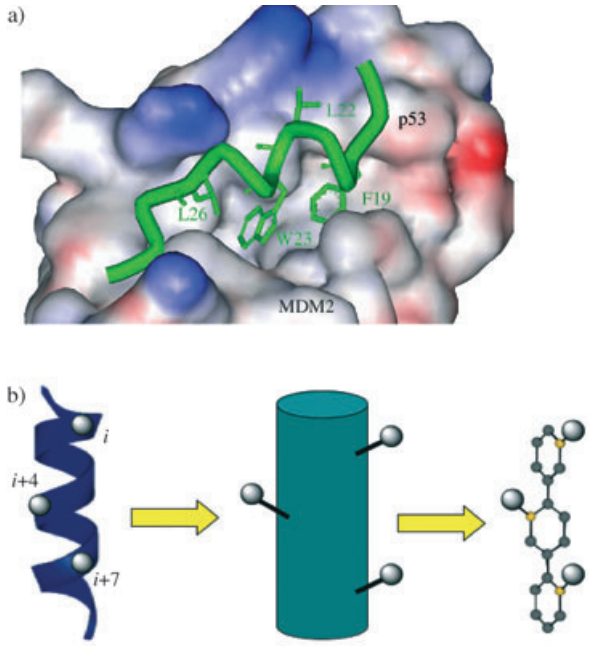


Figure 1. a) X-ray crystal structure of the HDM2/p53 complex. The key side chains of F19, W23, and L26 are shown in stick representation. b) α-Helical mimicry based on a terphenyl scaffold.

have reported small-molecule agents that disrupt p53/HDM2 dimerization. For example, Vassilev et al. identified *cis*-imidazoline analogues as low-molecular-weight antagonists of HDM2 in a high-throughput screening.[9] Most recently, peptidomimetics of the helical region of p53 based on a β hairpin[10] and 14-helix scaffolds[11] that disrupt the p53/HDM2 complexation were developed by the groups of Robinson and Schepartz, respectively. These studies serve as the proof-of-principle that the protein–protein interface of the HDM2/p53 complex provides a sound target for small-molecule agents.

Previously, we showed that terphenyl derivatives can mimic one face of a α-helical peptide (Figure 1b).[12] By substituting the appropriate alkyl or aryl substituents on the three *ortho*- positions of the terphenyl scaffold, the side chains are projected in an analogous way to the i, $i+4$, and $i+7$ residues of an α helix. Herein we report the use of this strategy in which a group of terphenyl-based antagonists mimic the α-helical region of the p53 peptide and disrupt the HDM2/p53 complexation.

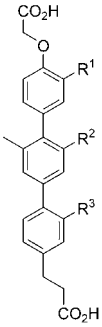
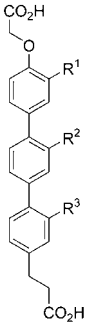
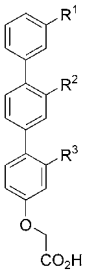
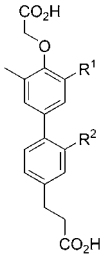
We prepared an extensive library of terphenyl inhibitors through a synthesis involving sequential Suzuki couplings of the appropriate *ortho*-substituted methoxyphenylboronate and phenyltriflate. A previously reported fluorescence polarization (FP) competition assay with a fluorescein-labeled p53 peptide, which contains residues 15–31 of p53 with a cysteine residue appended to the C-terminus (SQETFSDLWKLLPENNVC), was used to assess the binding affinities of these terphenyl derivatives to HDM2.[11] Displacement of this probe through competitive binding of the terphenyl into the hydrophobic cleft of HDM2 leads to a decrease in its fluorescence polarization. Regression analysis was conducted to determine the K_i values by using the previously reported method.[13] To test the validity of this assay, we used non-labeled p53 peptide as the competitive inhibitor to bind HDM2, giving a K_i of 3.51 ± 0.11 μM, which closely matches the K_d value (3.02 ± 0.33 μM) obtained from saturation titration experiments.

Good in vitro inhibition potencies in disrupting the p53/HDM2 heterodimerization were observed for certain compounds within the terphenyl series (Table 1). The terphenyl **14** with an isobutyl, 2-naphthylmethylene, isobutyl side-chain sequence showed a K_i value of 0.182 ± 0.020 μM for displacing p53 binding to HDM2. Terphenyls **16**, **20**, **21** bear only some of the key side chains of **14**, and their affinities for HDM2 are significantly lower. These results confirmed the importance of all three key side chains. The role of these side chains is further emphasized by the weak binding of control compounds (**9** and **17**), which indicated that there is no nonspecific hydrophobic interaction between the terphenyl backbone and the protein surface. Comparison of **1** ($K_i = 3.83$ μM) and **4** ($K_i = 297$ μM), which have reverse side-chain sequences, indicated that the orientation of the terphenyl backbone is critical in the binding. The terphenyl compounds with 2′,6′-dimethyl substituents showed improved affinities, as seen in the comparison of **1** and **10**, which may be due to the increased rigidity of the terphenyl backbone and the lowered entropic penalty on binding.

The displacement of the p53 peptide from the surface of HDM2 in the fluorescence experiment strongly suggested that the potent terphenyl compounds bind to the same cleft on the HMD2 surface. This location of the binding domain was further confirmed by computational and NMR spectroscopy experiments. The binding surface of p53 is dominated by a triad of p53 amino acids (F19, W23, and L26) that interacts with a hydrophobic cleft on the HDM2 surface (Figure 1a).[8] In this classification, the F19-pocket is defined by R65, Y67, E69, H73, I74, V75, M62, and V93 residues of HDM2, the W23-pocket comprises S92, V93, L54, G58, Y60, V93, and F91, the L26-pocket is composed of Y100, T101, and V53. To study the binding mode of the terphenyl derivatives, we conducted computational simulations with the Autodock program.[14] The top-ranked docking results for terphenyl **14** (shown in Figure 2) suggested that the terphenyl compounds target the same surface area where p53 binds, inserting their side chains into the F19, W23, and L26-pockets.

We mapped the binding surface of the terphenyl compounds on HDM2 using ^{15}N HSQC NMR experiments. The chemical shift perturbation of the HDM2 amide backbone was monitored upon addition of six different terphenyl compounds: **1**, **3**, **6**, **11**, **12**, and **14** (results shown in Table 2). All six compounds consistently induced chemical-shift changes at residues V28, F55, G58, V93, and/or K94, which all lie in the p53-binding pocket (with an exception of V28, whose shift is possibly due to the global conformational change of the protein). The chemical-shift changes at these positions were also observed in the binding of the p53 peptide and HDM2,[15] demonstrating that these terphenyl derivatives recognize the protein surface in a similar manner to the p53 peptide. Further analysis of **3**, **12**, and **14** demonstrated that bulkier side chains at the R^2 position resulted in more-significant chemical shifts at the F55, L57, V93, and K94

Table 1: Results of the fluorescence polarization assays.

Structure	Ligand	R^1	R^2	R^3	$K_i \pm$ S.D. [μM][a]
	**1**	Bn	Me	Me	3.83 ± 0.70
	2	Me	Me	*i*Bu	35.3 ± 4.4
	3	*i*Bu	Me	*i*Bu	2.97 ± 0.15
	4	Me	Me	Bn	297 ± 92
	5	*i*Bu	Me	Bn	2.83 ± 0.63
	6	Bn	Me	Bn	2.17 ± 0.14
	7	Bn	Me	*i*Pr	13.1 ± 3.8
	8	Bn	Me	*i*Bu	6.25 ± 1.50
	**9**	H	H	H	> 1000
	10	Bn	Me	Me	12.6 ± 2.1
	11	Bn	Bn	*i*Bu	0.978 ± 0.171
	12	*i*Bu	Bn	*i*Bu	3.50 ± 1.00
	13	*i*Bu	CH_2(1-Naph)	*i*Bu	25.7 ± 11.9
	14	*i*Bu	CH_2(2-Naph)	*i*Bu	0.182 ± 0.020
	15	CH_2(1-Naph)	*i*Bu	*i*Bu	11.6 ± 2.1
	16	*i*Bu	CH_2(1-Naph)	H	82.0 ± 8.6
	**17**	H	H	H	> 1000
	18	*i*Pr	Me	Me	> 1000
	19	*i*Pr	*i*Bu	*i*Bu	1.89 ± 0.30
	**20**	Bn	Me		70.8 ± 9.8
	21	Me	Me		479 ± 144

[a] The K_i and standard deviation (S.D.) values were obtained from three independent titrations.

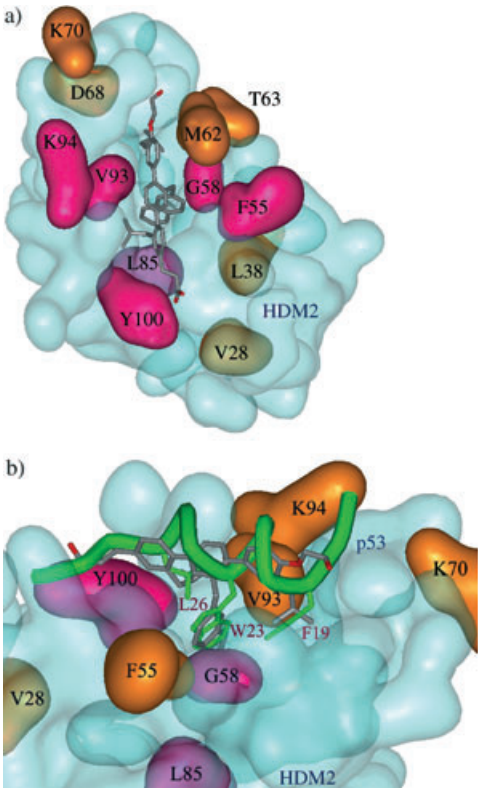


Figure 2. Results of the ^{15}N HSQC NMR spectroscopy and the molecular-docking experiments of terphenyl **14** binding to HDM2. a) The residues that showed significant and moderate chemical-shift changes upon addition of the terphenyl compounds are shown in magenta and orange, respectively. b) Overlay of the top-ranked docking result of **14** and the p53 peptide (green). The key hydrophobic side chains of F19, W23, and L26 are shown in stick representations.

positions. Terphenyl **14**, which shows the strongest binding ($K_i = 0.182 \pm 0.020$ μM), induced significant peak shifts for all these residues. In addition, a residue deep inside the W23 pocket, L85, is affected by **14**, suggesting that the 2-naphthylmethylene side chain inserts into the W23 pocket, thus resulting in a stronger binding (Figure 2b). Terphenyl **11**, which has a benzyl side chain at the R^1 position, clearly affected the residues M62 and H73 in the F19 pocket on HDM2, whereas **12** (*i*Bu at R^1) does not, which indicates that **11** inserts the benzyl side chain into the F19 pocket as p53 does. Taking all these results together, we concluded that the terphenyl derivatives are successful p53 mimetics and that these compounds present side chains into the F19, W23, and L26 binding pockets of HDM2.

A critical issue in the design of small-molecule α-helix mimetics is the selectivity of these compounds among different helix-binding proteins.[16] Nature frequently uses general secondary-structure modules, such as α helices, to recognize different protein targets and achieves high specificity through spatial and charge complementarity.[17] Previous studies have shown that the p53 peptide selectively binds to HDM2 over other oncogenic proteins, such as Bcl-x_L and Bcl-2, which both complex with the α-helical Bak BH3 domain.[18] In a similar manner to the p53/HDM2 interaction, the Bak peptide also projects key side chains at the *i*, *i* + 4, and *i* + 7 positions (V74, L78, and I81) into hydrophobic clefts on the targeted protein surfaces.[19] Comparison of terphenyl isomers **13** and **14,** which bear 1- and 2-naphthylmethylene side chains, respectively, on the middle phenyl rings, showed that terphenyl derivatives can selectively bind to different helix-binding proteins (Table 3).

Table 2: Summary of the amino acid residues of HDM2 that showed chemical-shift changes upon addition of terphenyl inhibitors.

Ligand	Significantly shifted residues[a]	Moderately shifted residues[a]
1	S22, V28, F55, L57, G58, I61, K94, K98, Y104	T26, L35, E52, V53, D68
3	G12, T15, S22, V28, G58	R29, L35, F55, I74, S92, K94
6	V8, G12, S22, V28, K51, V53, Y56, M62, V93	L33, L35, E52, F55, T63, K70, H73, L82, L85, F91, S92, H96
11	G12, T15, V28, K51, G58, H73, F91, S92, V93	S22, K45, I61, M62, I74, V75, K94, K98, I99, L107
12	V8, T15, S22, V28, F55, L57, I74, F91, E95	R29, Y60, Y67, K70, S92
14	V8, G12, T15, S22, V28, F55, G58, K70, L85, V93, K94, Y100	L38, M62, T63, D68

[a] See Supporting Information.

Table 3: Comparison of terphenyl derivatives **13** and **14** in inhibition of different protein–protein complexes.

K_i [μM]	HDM2/p53	Bcl-x_L/Bak	Bcl-2/Bak
13	25.7	0.114	0.121
14	0.182	2.50	15.0

Terphenyl **14** binds to HDM2 over 100-fold more strongly than **13** and has a 14:82 fold selectivity over Bcl-x_L/Bcl-2. This is consistent with the deeper pocket in HDM2 for W23 at the $i+4$ position compared to the L78-pocket of Bcl-x_L or Bcl-2. These results confirm the generality of the terphenyl scaffold as a mimic of the side-chain-induced selectivity of α helices and provides a useful tool for the rational design of protein-binding agents. Evaluation of the inhibitory effects of terphenyl derivatives in whole cells is currently underway.

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