Protein Structures

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Apamin as a Template for Structure-Based Rational Design of Potent Peptide Activators of p53**

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The activity and stability of the tumor suppressor protein p53 is negatively regulated by the oncogenic proteins MDM2 and MDMX—the cellular process is initiated by MDM2/MDMX binding to the N-terminal transactivation domain of p53.[1] MDM2, an E3 ubiquitin ligase, primarily controls p53 stability by targeting the tumor suppressor protein for ubiquitin-mediated constitutive degradation, whereas MDMX mainly functions as an effective transcriptional antagonist of p53 that blocks its ability to regulate responsive genes expression. Antagonists that block the p53-binding pocket of MDM2/MDMX kill tumor cells both in vitro and in vivo by re-activating the p53 pathway, resulting in cell cycle arrest, senescence, or apoptosis.[2] MDM2 and MDMX act synergistically in tumor cells and have become highly attractive molecular targets for anticancer drug development. Using phage display, we identified a 12-mer peptide, termed PMI (p53-MDM2/MDMX inhibitor), with affinity for both MDM2 and MDMX in the low nM range.[3] Herein we report that grafting four residues of PMI critical for MDM2/MDMX binding to apamin, a highly specific blocker of Ca^{2+}-activated K^+ channels of small conductance,[4] converted the 18 amino acid residue bee-venom neurotoxin into several potent peptide inhibitors of the p53–MDM2/MDMX interactions with different specificities. The rational design of these apamin-derived p53 activators, termed stingins, was structurally validated by X-ray crystallography.

The N-terminal transactivation domain of p53 encompasses the sequence $T^{18}F^{19}S^{20}D^{21}L^{22}W^{23}K^{24}L^{25}L^{26}$ minimally required for effective MDM2/MDMX binding.[5] Residues Phe19, Trp23 and Leu26 of p53, constituting an amphipathic α-helix, dock their side chains inside a hydrophobic cavity of MDM2/MDMX, and are energetically the most critical residues for MDM2/MDMX recognition.[6] We and others have also found that Tyr22 is superior to Leu22 in contributing to MDM2/MDMX binding.[3,7] Apamin consists of an N-terminal loop and a C-terminal α-helix globally stabilized by two disulfide bridges (Cys1–Cys11 and Cys3–Cys15),[8] providing an attractive structural template for de novo design of new functionalities.[9] To convert apamin into an inhibitor that emulates the activity of $^{18–26}$p53, we grafted Phe19, Tyr22, Trp23, and Leu26 to the topologically equivalent positions of apamin in its α-helical region, generating, by additional C-terminal truncation, stingins 1–5, ranging in length from 16 to 18 amino acid residues (Table 1). Two sets of residues in apamin were structurally permissible to the grafting: Ala9-Ala12-Arg13-Gln16 and Leu10-Arg13-Arg14-Gln17. Replacement of the former resulted in stingins 1–3, whereas substitutions of the latter yielded stingins 4 and 5. Notably, as residues Arg13, Arg14, and Gln17 are functional determinants of apamin,[8] conversion into stingins is conveniently expected to decimate its neurotoxin activity. All five stingin peptides along with apamin were chemically synthesized, spontaneously and quantitatively folded within 2 h under thiol–disulfide shuffling conditions, and purified to homogeneity by reversed-phase HPLC. All stingin peptides are highly soluble in aqueous solution. As is the case with native apamin, the C termini of synthetic apamin and stingins are all amidated.

Table 1: Amino acid sequences of apamin, PMI, and stingins and their dissociation equilibrium constants for p53-binding domains of MDM2 and MDMX.[a]

Name	Sequence	K_d [nM]	
		MDM2	MDMX
apamin	CNCKAPETALCARRCQQH	N.B.	N.B.
PMI	TS**F**AE**YW**NL**L**SP	3.2 ± 1.1	8.5 ± 1.7
stingin 1	CNCKAPET**F**LC**YW**RC**L**QH	25.1 ± 5.1	11.4 ± 2.3
stingin 2	CNCKAPET**F**LC**YW**RC**L**Q	35.2 ± 3.7	18.0 ± 2.3
stingin 3	CNCKAPET**F**LC**YW**RC**L**	57.5 ± 7.2	16.0 ± 4.5
stingin 4	CNCKAPETA**F**CA**YW**CQ**L**H	83.2 ± 8.4	252 ± 23
stingin 5	CNCKAPETA**F**CA**YW**CQ**L**	17.7 ± 4.0	93.4 ± 9.2

[a] Each K_d value is the mean of three independent measurements (N.B. = no binding). Critical residues shown in bold.

A surface plasmon resonance (SPR) based competition assay was performed to quantify the interactions between stingins and synthetic $^{25–109}$MDM2 and $^{24–108}$MDMX (or ^{syn}MDM2 and ^{syn}MDMX).[3,10] The quantification technique

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is based on the principle that a surface-immobilized $^{15-29}$p53 peptide accurately measures the concentration of free (unbound) ^{syn}MDM2 or ^{syn}MDMX in an incubation solution that contains a fixed concentration of the protein and varying concentrations of test peptide. PMI and all five stingin peptides competed in a dose-dependent manner with immobilized $^{15-29}$p53 for ^{syn}MDM2 or ^{syn}MDMX binding (see the Supporting Information). Nonlinear regression analyses for PMI yielded a K_d value of (3.2 ± 1.1) nM for ^{syn}MDM2 and (8.5 ± 1.7) nM for ^{syn}MDMX, which are in full agreement with the previously published K_d values of 3.3 and 8.9 nM, respectively, determined by isothermal titration calorimetry.[3] Whereas apamin, as expected, showed no binding to either ^{syn}MDM2 or ^{syn}MDMX, stingin peptides bound ^{syn}MDM2 and ^{syn}MDMX effectively with K_d values ranging from 11.4 to 252 nM (Table 1). Interestingly, stingins 1–3 are MDMX-specific, giving rise to K_d values for ^{syn}MDMX two- to fourfold lower than those for ^{syn}MDM2. By contrast, stingins 4 and 5 are MDM2-specific, as evidenced by a three- to fivefold difference in K_d values between ^{syn}MDM2 and ^{syn}MDMX, in favor of MDM2. Deletion of the C-terminal residue(s) flanking Leu16 in stingins 1 and 2 (Gln17 and His18) slightly weakened MDM2/MDMX binding. Removal of His18 in stingin 4, however, significantly improved binding to both MDM2 and MDMX. Taken together, these results suggest that although the four grafted residues (Phe/Tyr/Trp/Leu) are critical for stingin–MDM2/MDMX interactions, frame shift (from stingins 1–3 to stingins 4 and 5) and modulation of the C-terminal residues flanking Leu16 or Leu17 fine-tune the stingin activity and specificity.

The crystal structure of stingin 5 was determined at 1.48 Å resolution. As shown in Figure 1 A, B, stingin 5 preserves a parental fold of apamin and presents an amphipathic α-helix required for effective MDM2/MDMX binding. Among the four residues (Phe10, Tyr13, Trp14, and Leu17) introduced into the helical region of apamin, only Leu17—the C-terminal residue—is located outside the regular α-helix. The topology of the side chains of the four hydrophobic residues in stingin 5 is such that only minor conformational adjustments are required for its productive binding to the p53-binding cavity of MDM2/MDMX. A superposition of stingin 5 and apamin shows overlapping secondary structural elements and native disulfide bonds, with a root-mean-square deviation (rmsd) between equivalent C^α atoms of 0.6–0.7 Å, thus structurally validating apamin as an ideal template for rational design of miniature protein inhibitors of p53–MDM2/MDMX interactions.

To better understand the molecular recognition between MDM2/MDMX and stingins, we determined the crystal structure at 1.65 Å resolution of ^{syn}MDM2 complexed with stingin 1—a potent antagonist of both MDM2 and MDMX (Figure 1 C, D). As expected, residues 9–17 of stingin 1 adopt an amphipathic α-helical conformation, allowing the side chains of Phe9, Trp13, and Leu16 to bury deep inside the p53-binding pocket of MDM2. These three residues collectively contribute 63% of the total buried surface area (BSA) of stingin 1. Tyr12 stacks against Val93 and forms π–cation interactions with Lys94 and His73 of MDM2, contributing additional 17% of the total BSA.

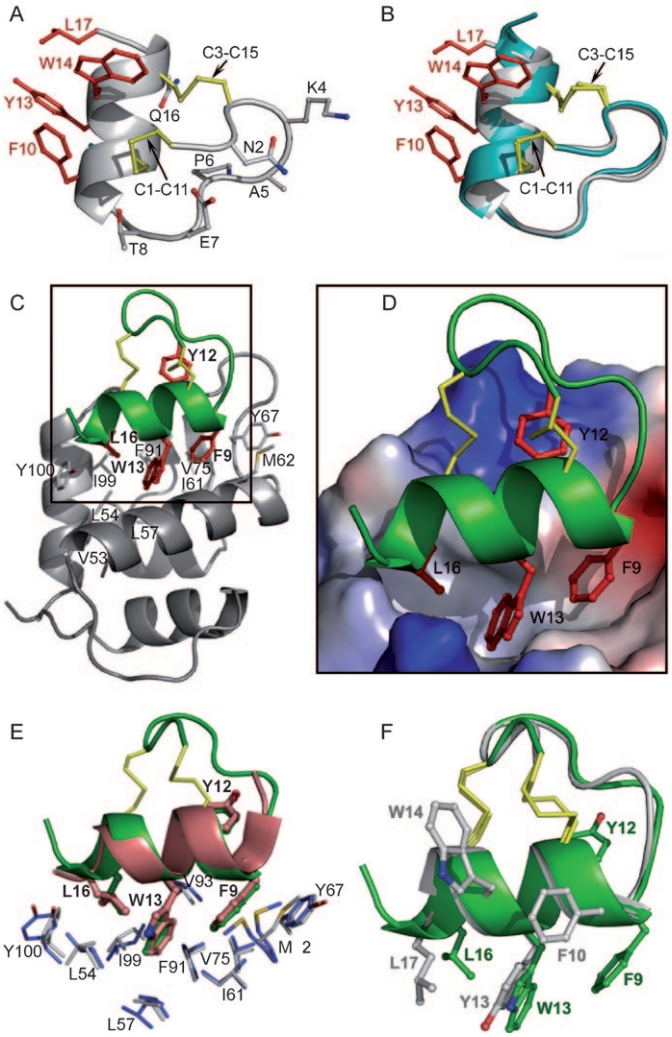

Figure 1. Crystal structures of stingin 5 and of stingin 1 in complex with MDM2. A) Ribbon representation of the overall structure of stingin 5. All side chains are shown in ball-and-stick form. Residues grafted to the apamin sequence are shown in red and disulfide bonds in yellow. B) Superposition of the backbones of stingin 5 (gray) and apamin (cyan). C) The co-crystal structure of synthetic $^{25-109}$MDM2 (gray) and stingin 1 (green). Side chains of Phe9, Tyr12, Trp13, and Leu16 of stingin 1 are colored in red, and residues of MDM2 shaping the stingin-binding pocket shown as gray sticks. D) Close-up view of the interface of the protein–peptide complex. The electrostatic potential at the molecular surface of MDM2 is displayed as negative in red, positive in blue, and apolar in white. E) Stingin 1 (green) and PMI (pink), shown in a ribbon-and-stick representation, from their respective complexes with (superimposed) ^{syn}MDM2. Residues shown as thin sticks line the hydrophobic cavity of MDM2—blue residues from MDM2 complexed with PMI, and gray residues from MDM2 complexed with stingin 1. F) Superimposed structures of stingin 1 (green) and stingin 5 (gray).

Stingin 1 closely resembles PMI and other p53-like peptide ligands with respect to MDM2 binding.[3,5a,11] Shown in Figure 1 E are overlapping stingin 1 and PMI seen in their respective complexes with (superimposed) ^{syn}MDM2. The side chains of Phe9, Tyr12, and Trp13 of stingin 1 and of Phe3, Tyr6, and Trp7 of PMI occupy identical positions in the complexes and make identical contacts with the residues of

MDM2. A notable structural difference between stingin 1 and PMI centers on the C^{α} and C^{β} atoms of Leu16 of stingin 1 and of Leu10 of PMI (C^{α}: 2.1 Å apart; C^{β}: 2.25 Å apart), attributable to an extension, in comparison with PMI, of the α-helix of stingin 1 at the C terminus. The extension of the C-terminal α-helix of stingin 1, supported by the Cys3–Cys15 disulfide bond, allows Leu16 to protrude into the p53-binding pocket earlier than Leu10 of PMI. Concomitantly, an equivalent hydrogen bond seen in the PMI–MDM2 complex (Tyr100 O^{η} to Leu10 O) is lost between the side chain of Tyr100 of MDM2 and the Leu16 O atom of stingin 1.[3] The Tyr100 O^{η} atom in the stingin 1–MDM2 complex forms instead a water-mediated hydrogen bond to the side chain of Gln17 of stingin 1 (see the Supporting Information). Despite these differences, the geometry of the C^{γ}, $C^{\delta1}$, and $C^{\delta2}$ atoms of Leu16 of stingin 1 is nearly identical to that of Leu10 of PMI. Superposition of the FLCYWRCL sequence of stingin 1 and the FAEYWNLL sequence of PMI—the minimally required MDM2/MDMX-binding motif—yields an RMSD value of 0.6 Å between their equivalent C^{α} atoms.

The binding affinity of stingin 5 for MDM2 is 15-fold higher than that of nutlin 3[3]—a *cis*-imidazoline-derived small molecule antagonist of MDM2 that kills tumor cells at low μM concentrations in a p53-dependent manner.[2a,b] However, stingin 5 showed no cytotoxicity at up to 400 μM, presumably because of its inability to permeate the cell membrane. To facilitate cellular uptake of stingin 5, we covalently attached to its N terminus a cluster of nine Arg residues all in D-enantiomeric form ($^{D}R_9$–stingin 5). Unexpectedly, $^{D}R_9$–stingin 5 quantitatively killed both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ at 12.5 μM ($IC_{50} \approx 6$ μM) in a p53- and time-independent fashion, strongly suggesting a necrotic cell death mechanism (see the Supporting Information). p53 peptides conjugated to positively charged cell penetrating peptides (CPPs) have previously been shown to induce necrosis of tumor cells independently of p53 status.[12] The failure of stingin 5 and $^{D}R_9$–stingin 5 to activate the p53 pathway in HCT116 $p53^{+/+}$ cells underscores the importance of the development of viable cellular delivery vehicles for peptide/protein therapeutics in general. For cargo such as p53-like peptides and stingins that contain clustered hydrophobic residues, conjugation of cationic CPPs may lead to a detergent-like molecule indiscriminately toxic against all cell types.

Three major classes of MDM2/MDMX antagonists emulating the activity of the $^{18–26}$p53 peptide are being developed for potential therapeutic applications: low-molecular-weight compounds such as nutlins and MI-209 (an extensively modified spirooxindole compound),[2a,b] various peptidomimetics,[2c,13] and miniature proteins such as avian pancreatic polypeptide,[14] thioredoxin,[15] and scorpion toxin.[10] Miniproteins are genetically deliverable and generally more resistant to proteolysis in vivo than linear peptides. Chen and co-workers recently demonstrated that expression by the adenovirus of thioredoxin displaying the sequence of a phage-optimized peptide inhibitor of MDM2 and MDMX resulted in efficient p53 activation, cell cycle arrest, and apoptosis of tumor cells overexpressing MDM2 and MDMX.[15b] Intratumoral injection of the adenovirus also induced growth suppression of tumor xenografts in mice in a p53-dependent fashion. Compared with other miniprotein activators of p53, stingins prevail in their small size, high potency, and extreme ease of chemical or recombinant production.[16] Stingins are admittedly still less potent than PMI, which is one of the strongest peptide antagonists ever designed for MDM2/MDMX. As disulfide bonding in stingins stabilizes a preformed amphipathic α-helix, thus minimizing an entropic penalty associated with complex formation, further enhancement in binding affinity should be possible through additional sequence optimization at secondary interaction sites.

In conclusion, by grafting four hydrophobic residues, critical for MDM2/MDMX binding, to the C-terminal α-helix of apamin, we successfully converted the bee-venom neurotoxin into a series of potent inhibitors of p53 interactions with MDM2 and MDMX. Structural studies by X-ray crystallography validated the mode of inhibition, that is, apamin-derived stingins directly compete with p53 for MDM2/MDMX binding. Stingins represent a novel class of p53 activators and are superior in many aspects to the existing miniprotein antagonists of MDM2/MDMX. Coupled with a therapeutically viable delivery modality, stingins may have the potential to be used as antitumor agents for clinical use.

Experimental Section

Synthesis of apamin and stingins: All peptides were chemically synthesized on 4-methylbenzhydrylamine (MBHA) resin by activation with *O*-(benzotriazol-l-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and in situ neutralization with diisopropylethylamine (DIEA) according to the protocol developed by Kent and co-workers for Boc chemistry.[17] The oxidative folding is described in the Supporting Information. Peptide quantification was performed by UV spectroscopic measurements at 280 nm using molar extinction coefficients calculated according to a published algorithm.[18]

Surface plasmon resonance based competition binding assay: The K_d values of PMI and stingins for ^{syn}MDM2 and ^{syn}MDMX were determined essentially as described previously [3,10] (see the Supporting Information).

Crystallization, data collection, structure solution, and refinement: Buffer conditions for crystallization are provided in the Supporting Information. The data integration and scaling, as well as structure solution and refinement were performed as described previously.[3] For stingin 5, the crystal structure of wild-type apamin[8] was used as a starting model to determine the initial phase information. For ^{syn}MDM2–stingin 1, the ^{syn}MDM2 coordinates extracted from the ^{syn}MDM2–PMI complex structure[3] were used as a search model for molecular replacement. Data collection and refinement statistics are summarized in Table S1 (Supporting Information). The coordinates and structure factors have been deposited in the PDB with accession code 3IUX. Molecular graphics were generated using Pymol (http://pymol.org).

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