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Functional consequences of retro-inverso isomerization of a miniature protein inhibitor of the p53-MDM2 interaction¹

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Abstract

Peptide retro-inverso isomerization is thought to be functionally neutral and has been widely used as a tool for designing proteolytically stable D-isomers to recapitulate biological activities of their parent L-peptides. Despite success in a wide range of applications, exceptions amply exist that clearly defy this rule of thumb when parent L-peptides adopt an α -helical conformation in their bound state. The detrimental energetic effect of retro-inverso isomerization of an α -helical L-peptide on its target protein binding has been estimated to be 3.0–3.4 kcal/mol. To better understand how the retro-inverso isomer of a structured protein works at the molecular level, we chemically synthesized and functionally characterized the retro-inverso isomer of a rationally designed miniature protein termed stingin of 18 amino acid residues, which adopts an N-terminal loop and a C-terminal α -helix stabilized by two intra-molecular disulfide bridges. Stingin emulated the transactivation peptide of the p53 tumor suppressor protein and bound with high affinity and via its C-terminal α -helix to MDM2 and MDMX – the two negative regulators of p53. We also prepared the retro isomer and D-enantiomer of stingin for comparative functional studies using fluorescence polarization and surface plasmon resonance techniques. We found that retro-inverso isomerization of L-stingin weakened its MDM2 binding by 720 fold (3.9 kcal/mol); while enantiomerization of L-stingin drastically reduced its binding to MDM2 by three orders of magnitude, sequence reversal completely abolished it. Our findings demonstrate the limitation of peptide retro-inverso isomerization in molecular mimicry and reinforce the notion that the strategy works poorly with biologically active α -helical peptides due to inherent differences at the secondary and tertiary structural levels between an L-peptide and its retro-inverso isomer despite their similar side chain topologies at the primary structural levels.

Keywords

Retro-inverso peptide; p53; MDM2; MDMX; D-peptide; Apamin; Stingin

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1. Introduction

The retro-inverso isomer of an L-peptide is composed entirely of D-amino acid residues assembled in reverse order.² When extended, a retro-inverso peptide, also known as a retro-all-D or retro-enantio peptide, assumes a similar side-chain topology to its parent L-molecule with inverted amide peptide bonds.³ Because of this similarity at the primary structural level, peptide retro-inverso isomerization is thought to be functionally neutral.³ A large body of evidence in the literature clearly supports this tenet. However, ample examples of retro-inverso peptides also exist that fail to emulate the modes of action and recapitulate bioactivities of their parent L-counterparts. For example, while success has been achieved in some cases where retro-inverso peptides were used as antigenic mimicry of their parent L-peptides,⁴ failures have been noted in others where retro-inverso isomers failed to elicit cross-reactive antibodies against native immunogens.⁵ This mixed outcome suggests that retro-inverso isomerization has its own limitation and cannot be generalized as a tool for the design of proteolytically stable D-peptides with desired functionality.^{1,6}

Recently we have designed a miniature protein termed stingin of 18 amino acid residues based on the scaffold of the bee-venom neurotoxin apamin⁷ - a highly specific blocker of Ca^{2+} -activated K^{+} channels of small conductance.^{8,9} The amino acid sequences of apamin and stingin are as follows:

CNCKAPETALCARRCQQH (apamin)

CNCKAPETFLCYWRCLQH (stingin)

Both apamin and stingin adopt nearly identical structures where an N-terminal loop and a C-terminal α -helix are stabilized by two disulfide bridges (Cys1–Cys11 and Cys3–Cys15).^{7,10} By grafting the four underlined hydrophobic residues (Phe9, Tyr12, Trp13, and Leu16) to apamin, we succeeded in converting the neurotoxin into a potent inhibitor of the interaction of the p53 tumor suppressor with its negative regulators MDM2 and MDMX.⁷

Functional inhibition of p53 by MDM2 and MDMX, whose genes *MDM2* and *MDMX* are often amplified and/or overexpressed in many tumors harboring wild type *TP53*, directly contributes to tumor development and progression.¹¹⁻¹³ MDM2 is an E3 ubiquitin ligase that specifically targets p53 for proteosomal degradation – a process potentiated by MDM2 interactions with its homolog MDMX.¹⁴⁻¹⁶ Both MDM2 and MDMX can also antagonize p53 transcription activity by sequestering p53 transactivation domain via their N-terminal p53-binding domains. Disrupting the p53-MDM2/MDMX inhibitory complex to rescue p53 function has been validated as a viable therapeutic strategy for cancer treatment.¹⁷⁻²⁰ We have shown that stingin directly competes with p53 for MDM2/MDMX binding,⁷ promising a novel class of miniature protein activators of p53 with antitumor activity.

Various attempts have been made to design proteolysis-resistant peptide and peptidomimetic inhibitors of the p53-MDM2/MDMX for anticancer therapy.^{21,22} Using mirror image phage display^{23,24} coupled with chemical protein synthesis via native chemical ligation,^{25,26} our laboratory has recently identified and designed several potent D-peptide antagonists of MDM2/MDMX that are fully resistant to proteolytic degradation.^{27,28} Crystallographic studies show that all these D-peptide inhibitors adopt, as expected, left-handed α -helical conformations and structurally emulate the binding of a right-handed α -helical p53 peptide to MDM2/MDMX.²⁷⁻²⁹ When encapsulated in liposomes decorated with an integrin-targeting cyclic-RGD peptide, these D-peptide antagonists exert p53-dependent growth inhibitory activity against human glioblastoma in cell cultures by reactivating the p53 pathway, and suppress tumor growth and prolong animal survival as well in nude mouse xenograft models.²⁷

As a potentially powerful designing tool, the retro-inverso isomerization strategy begs the question of whether or not a retro-inverso peptide and its parent L-antagonist can be equally or similarly active against MDM2/MDMX. We have shown unequivocally that retro-inverso isomerization diminishes ¹⁵⁻²⁹p53 peptide binding to MDM2/MDMX by 3.2–3.3 kcal/mol.¹ Similar findings were made with respect to helical peptide ligands of the C-terminal domain of HIV-1 capsid protein (3.0 kcal/mol) and the Src homology 3 domain of Abl tyrosine kinase (3.4 kcal/mol).¹ Contrary to common belief,³⁰ retro-inverso isomerization of a peptide that adopts an α -helical conformation in the complex with its target protein is functionally detrimental. However, much of the published work on peptide retro-inverso isomerization is based on small peptides that do not possess a stable structure alone in solution. The question of how a retro-inverso protein functions at the molecular level remains largely unknown. This has motivated us to use the miniature protein stingin as a structured template to address this important unanswered question by comparatively characterizing MDM2 binding by L-stingin and its retro-inverso isomer. For functional comparison, we also prepared the retro isomer and D-enantiomer of stingin.

2. Experimental Section

2.1. Total chemical synthesis of L-stingin, inverso-stingin (or D-stingin), retro-stingin (or R-stingin), and retro-inverso-stingin (or RI-stingin)

The preparation of L-stingin, D-stingin, R-stingin, and RI-stingin using machine-assisted solid phase peptide synthesis (SPPS) on 4-methylbenzhydrylamine resin and an optimized coupling chemistry developed by Kent and colleagues³¹ was essentially as described.⁷ All synthetic peptides, after purification to homogeneity by reversed phase (RP) HPLC, were ascertained by electrospray ionization mass spectrometry (ESI-MS).

As expected, both L-stingin and D-stingin (at 0.25 mg/ml) underwent spontaneous oxidative folding at room temperature in the presence of 2 M GuHCl, 3 mM reduced and 0.3 mM oxidized glutathione, pH 8.3, yielding a predominant species with a molecular mass of 2209.7 Da. This value is 4 mass units lower than that of unfolded L- or D-stingin, indicating the formation of two disulfide bridges. The crystal structure of L-stingin in complex with synthetic ²⁵⁻¹⁰⁹MDM2 was previously determined,⁷ structurally verifying the native disulfide topology in L-stingin or apamin, i.e., Cys1-Cys11, and Cys3-Cys15.

The oxidative folding of R-stingin and RI-stingin was extremely inefficient and significantly slower than that of L-stingin and D-stingin under identical conditions. To improve their production yield, we synthesized R-stingin and RI-stingin in which Cys3 and Cys15 were orthogonally protected by acetamidomethyl (Acm) group. After HPLC purification, R-stingin and RI-stingin were subjected to air oxidation to form the first disulfide bridge Cys1-Cys11, followed by iodine treatment at acidic pH to spontaneously deprotect Acm-Cys3 and Acm-Cys15 and form the second disulfide bond. This regiospecific orthogonal protection or deprotection strategy for Cys residues has been widely used to correctly fold Cys-rich peptides or proteins.^{32,33}

All folded stingin peptides were quantified by UV absorbance measurements at 280 nm using a molar extinction coefficient of 7240 calculated according to a published algorithm.³⁴

2.2. Circular dichroism (CD) spectroscopy

CD spectra of stingin peptides, 20 μ M in 10 mM phosphate buffer, pH 7.2, were obtained on a Jasco J-810 spectropolarimeter at room temperature using a 0.1-cm path length.

2.3. Fluorescence polarization (FP)

An N-terminally acetylated ^{15–29}p53 peptide was synthesized by Boc-SPPS and purified to homogeneity by RP-HPLC. Succinimidyl ester-activated carboxytetramethylrhodamine (TAMRA-NHS) was covalently conjugated in DMF to N-acetyl-^{15–29}p53 via its Lys24 side chain, and the resultant product TAMRA-^{15–29}p53 was HPLC-purified and lyophilized. The stingin-p53-MDM2 competitive binding experiments were performed in 96-well plates (Corning Life Science, Nonbinding Surface or NBS) on a Tecan Infinite M1000 fluorescence plate reader. Serially diluted stingin solutions were prepared in phosphate buffered saline (pH 7.4) and incubated with 50 nM TAMRA-^{15–29}p53 and 250 nM synthetic ^{25–109}MDM2 in a total volume of 120 μL per well. After a 30-min incubation at room temperature, FP was measured at λ^{ex} = 530 nm and λ^{em} = 580 nm. Nonlinear regression analysis using GraphPad Prism 4.0 was performed to give rise to IC₅₀ values according to the following equation:^{29,35,36}

$$F = F_0 + (F_c - F_0) / \{1 + (IC_{50} / [\text{stingin}])^{-n}\}$$

Where F is measured average FP, F₀ is FP of TAMRA-^{15–29}p53, F_c is FP of the TAMRA-^{15–29}p53-MDM2 complex, [stingin] is stingin concentration, and n is Hill coefficient.

2.4. Surface plasmon resonance (SPR)

SPR-based binding assays were carried out at 25 °C on Biacore T100 instrument using a CM5 sensor chip to which ^{15–29}p53 (17 RU's) is covalently attached. The buffer (HBS-EP) was 10 mM HEPES, 150 mM NaCl, and 0.005% surfactant P20, pH 7.4. Synthetic ^{25–109}MDM2 at 50 nM was incubated for min with varying concentrations of stingin, and injected at a flow rate of 20 μL/min for 2 min, followed by a 4-min dissociation. The concentration of unbound MDM2 in solution was deduced, based on p53-association RU values, from a calibration curve established by RU measurements of different concentrations of MDM2 injected alone. Nonlinear regression analysis was performed using GraphPad Prism 4.0 to give rise to K_d values according to the following equation:

$$K_d = [\text{peptide}] [\text{MDM2}] / [\text{complex}]$$

3. Results

3.1. Characterization of L-stingin, D-stingin, R-stingin, and RI-stingin

We obtained large quantities of highly pure and correctly folded L-stingin, D-stingin, R-stingin, and RI-stingin. Shown in Figure 1 are the four final products characterized by analytical RP-HPLC and ESI-MS. As expected, all four stingin peptides displayed an identical molecular mass (within experimental error). Interestingly, while either pair of enantiomers (L-stingin/D-stingin or R-stingin/RI-stingin) showed similar chromatographic properties as evidenced by nearly identical retention times, sequence reversal of L-stingin (to R-stingin) or D-stingin (to RI-stingin) noticeably shortened retention, likely reflecting a substantial change in their three-dimensional structures. In either L-stingin or D-stingin, the hydrophobic residues critical for MDM2 interactions are located on the solvent-exposed edge of the C-terminal α-helix.⁷ Thus, folded L-stingin and D-stingin are expected to retain strongly on the reversed phase C18 column used. The fact that R-stingin and RI-stingin eluted earlier suggests that some of those hydrophobic residues may be partially solvent-exposed due to structural changes imposed by sequence reversal. The chromatographic data are supported by the mass spectrometric results, where an identical charge state distribution

was observed for either pair of enantiomers, but sequence reversal changed it, that is, the +4 charge state disappeared in both R-stingin and RI-stingin.

The crystal structure of L-stingin in complex with synthetic ²⁵⁻¹⁰⁹MDM2 was previously determined at 1.65 Å resolution.⁷ As shown in Figure 2, residues 9–17 of L-stingin adopt an amphipathic α -helical conformation, burying the side chains of Phe9, Trp13, and Leu16 inside the p53-binding pocket of MDM2. The binding mode of L-stingin is identical to that of p53 transactivation peptide and of other peptide antagonists designed to emulate p53 binding to MDM2.^{37,38}

We further characterized L-stingin, D-stingin, R-stingin, and RI-stingin by CD spectroscopy. As shown in Figure 3, L-stingin is partially α -helical as indicated by double minima at 208 and 222 nm and a strong positive peak at ~195 nm, consistent with its known structural features. The CD spectra of L-stingin and D-stingin are symmetrical about the X-axis, as expected for a pair of enantiomers related to one another by a plane of symmetry. Similarly, R-stingin and RI-stingin are also a mirror image of each other as verified by CD spectroscopy. Importantly, the α -helix present in both L-stingin and D-stingin disappeared in R-stingin and RI-stingin as a result of sequence reversal, confirming a substantial structural change corroborated by the chromatographic and mass spectrometric data.

3.2. Retro-inverso isomerization of L-stingin weakens its MDM2 binding by three orders of magnitude

We first used fluorescence polarization (FP) techniques to quantify the interactions of synthetic ²⁵⁻¹⁰⁹MDM2 with L-stingin, D-stingin, R-stingin, and RI-stingin.^{29,39} FP is a powerful tool to study peptide-protein interactions in solution. FP measurements are based on the principle that a fluorescently labeled peptide ligand tumbles much faster in solution than its much larger complex with a protein, and upon excitation by polarized light, it “scrambles” the polarization of emitted light more efficiently.^{40,41} Thus, complex formation necessarily causes decreased rotational rates and increased polarization of emitted light. When different concentrations of stingin (unlabeled) are added to a preformed complex of ²⁵⁻¹⁰⁹MDM2 and TAMRA-¹⁵⁻²⁹p53, the fluorescently labeled p53 peptide is displaced from the complex as the concentration of stingin increases, resulting in a progressive decrease in FP. A titration curve is generated from measurements of FP, and stingin-MDM2 interactions can be quantitatively evaluated using known mathematical models.^{29,35,36}

Shown in Figure 4 is dose-dependent competition of various stingin peptides for ²⁵⁻¹⁰⁹MDM2 binding with TAMRA-¹⁵⁻²⁹p53 as measured by FP. The IC₅₀ value for L-stingin was determined to be 214 nM. By contrast, RI-stingin was extremely weak in competing with p53 for binding MDM2 under the same experimental conditions. In fact, we could not determine its IC₅₀ value as FP did not drop low enough even at the highest concentration of RI-stingin used.

To measure the binding affinities of various stingin peptides for MDM2, we resorted to the SPR technique as described.^{38,42} Specifically, we immobilized ¹⁵⁻²⁹p53 via its N-terminus or Lys24 to a CM5 biosensor chip, which was then used to capture and quantify free ²⁵⁻¹⁰⁹MDM2 in a buffer solution containing a fixed concentration of MDM2 pre-incubated with varying concentrations of stingin. This highly accurate binding assay has been extensively used to quantify the interaction of MDM2 with various peptide antagonists. As shown in Figure 5, L-stingin bound strongly to MDM2 with a K_d value of 25.1 nM. By contrast, the binding affinity of RI-stingin for MDM2 was merely 18.1 μ M, approximately three orders of magnitude weaker than that of L-stingin.

3.3. Enantiomerization of L-stingin drastically reduces its binding to MDM2 while sequence reversal completely abolishes it

As shown in Figure 4, the IC_{50} value of D-stingin for MDM2 was determined to be 20.3 μ M, roughly 100-fold higher than that of L-stingin, suggesting that enantiomerization of L-stingin drastically weakened its MDM2 binding. Compared with L-stingin, no meaningful competition for MDM2 binding with p53 was observed even at the highest concentration tested for R-stingin, functionally confirming a dramatic structural change imposed by sequence reversal. These findings are supported by K_d measurements using the SPR technique. As shown in Figure 5, the D-enantiomer, D-stingin, bound MDM2 at an affinity of 26.5 μ M, 1000-fold weaker than did L-stingin. The retro isomer of L-stingin, R-stingin, showed no binding at all to MDM2 even at 100 μ M. Collectively, these results confirm that enantiomerization of L-stingin drastically reduces its binding to MDM2 while sequence reversal completely abolishes it.

4. Discussion

It is known that the ability of retro-inverso isomerization to result in D-isomers emulating the modes of action and recapitulating bioactivities of their parent L-peptides is dependent on their intrinsic structure and interaction propensities.⁵ A growing body of evidence indicates that retro-inverso isomerization of α -helical peptide ligands is invariably detrimental at the functional level. In fact, the energetic consequence of retro-inverso isomerization has been shown to be remarkably consistent (3.0-3.4 kcal/mol) with α -helical peptide ligands for four different proteins: MDM2, MDMX, HIV-1 $CTDCA$, and Abl SH3 domain.¹ Similar findings were also reported for the extensively studied S-peptide and S-protein interacting system^{43,44} – a non-covalently associating tight complex termed ribonuclease S formed by a single enzymatic cleavage of ribonuclease A.⁴⁵ Collectively, these reports imply that despite similar side chain topologies at the primary structural level, inherent differences at the secondary and tertiary structural levels between an L-peptide and its retro-inverso isomer are responsible for the detrimental energetic effect of retro-inverso isomerization. This conclusion is clearly validated by comparative structural studies of both L- and D-peptide antagonists complexed with MDM2,^{1,27,28,38} and reinforced by the present study showing that retro-inverso isomerization of the miniature protein stingin impairs its binding to MDM2 by as much as 3.9 kcal/mol. Not surprisingly, L-stingin and RI-stingin differ significantly at the structural level as evidenced by their different CD spectra. Of note, the deleterious effect of 3.9 kcal/mol seen with L-stingin/RI-stingin is larger than 3.2 kcal/mol previously observed for L-¹⁵⁻²⁹p53/RI-¹⁵⁻²⁹p53 with MDM2.¹ The difference may be contributed entropically by the rigidity of the stingin scaffold as opposed to the flexible p53 peptide in both free and bound states.⁷

Perhaps it is equally important to emphasize that retro-inverso isomerization has proven successful to various extent in a variety of biological systems where parent L-peptides and/or their retro-inverso isomers appear to adopt a more extended binding conformation such as β -strands. However, even under those circumstances there is no reason to believe that retro-inverso isomers ought to be functionally equivalent to their parent L-peptides with respect to binding energetics.⁴⁶ This is because a retro-inverso isomer cannot possibly be identical to its parent L-peptide at the level of three-dimensional structure due to the inversion of amide peptide bonds.^{6,47} This viewpoint probably can be best illustrated by studies of two disulfide-cyclized peptide inhibitors of the interaction of IgE with its high affinity receptor, Fc epsilon RI.⁴⁸ An L-peptide inhibitor and its retro-inverso D-isomer were designed to mimic part of the receptor for IgE binding, and exhibited similar binding affinities of 3 and 11 μ M, respectively, for IgE.⁴⁸ NMR spectroscopic studies demonstrated that although both peptides adopted similar β -hairpin structures, they were not structurally identical on their surfaces.⁴⁹ In fact, the parent L-peptide showed, as expected, a right-handed helical twist of

the strands, whereas its retro-inverso D-isomer displayed a more marked left-handed helical twist.⁴⁹

It is generally thought that two mirror image proteins do not recognize a chiral partner in an identical fashion due to steric incompatibility. For example, a chemically synthesized D-HIV-1 protease does not hydrolyze natural substrates of the native viral enzyme, but cleaves their D-enantiomers as efficiently as L-HIV-1 protease does L-peptide substrates.⁵⁰ It is of interest to note that D-stingin also bound to MDM2, albeit three orders of magnitude weaker than its mirror image form, L-stingin. The D-enantiomer of ¹⁵⁻²⁹p53 also bound weakly to MDM2 (unpublished results). Substantially different binding energetics were previously reported for two pairs of enantiomers of human α -defensins HNP1 and HD5 with HIV-1 gp120 and anthrax lethal factor.⁵¹ However, the difference in binding energetics became marginal between the L- and D-forms of human α -defensin HNP4.⁵¹ Thus, although the chiral mode of molecular recognition is a norm in most interacting biological systems such as enzyme-substrate and peptide-protein, stereospecificity may not be stringently maintained in all. In fact, it is known that helix-helix recognition within the membrane can be chirality-independent.⁵²

Finally, it is worth pointing out that sequence reversal of L-stingin resulted in a drastically different isomer at both the structural and functional levels. Despite the fact that L-stingin and R-stingin shared the same disulfide topology, the latter completely lost its MDM2-binding activity. The question of whether or not a retro protein maintains the same structural fold and function as its native counterpart was controversial. An early simulation study using the lattice model approach predicted that the retro isomer of the B domain of *Staphylococcal* protein A could form a well-defined native-like three-helix bundle structure.⁵³ However, subsequent experimental evidence failed to support the foldability of this protein and of the α -spectrin SH3 domain as well.⁵⁴ It was thus concluded that retro proteins and their parent molecules bear no sequence similarity despite their identical amino acid composition and polar/non-polar pattern.⁵⁴ Our findings obviously lent additional support to this premise.

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Figure 1. Stingin peptides characterized by RP-HPLC and ESI-MS. The chromatograms were obtained at 40 °C on a Waters XBridge C₁₈ column (4.6×150 mm, 3.5 μm) running a 30-min gradient of 5%-65% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min.

Figure 2.

Crystal structure of L-stingin in complex with synthetic MDM2. The electrostatic potential at the molecular surface of MDM2 is displayed as negative in red, positive in blue, and apolar in white. The four grafted hydrophobic residues are shown in red ball-and-sticks, and the disulfide bonds in yellow sticks.

Figure 3.
CD spectra of stingin peptides at room temperature.

Figure 4. Interactions between stingin peptides and MDM2 as quantified by fluorescence polarization. The IC_{50} values of L-stingin and D-stingin were determined to be 214 ± 23 nM and 20.3 ± 1.2 μ M, respectively.

Figure 5. The binding affinity of stinging peptides for $^{25-109}$ MDM2 as quantified by SPR. Each curve is the mean of three independent measurements.