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Design and Structure of Stapled Peptides Binding to Estrogen Receptors

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Supporting Information

ABSTRACT: Synthetic peptides that specifically bind nuclear hormone receptors offer an alternative approach to small molecules for the modulation of receptor signaling and subsequent gene expression. Here we describe the design of a series of novel stapled peptides that bind the coactivator peptide site of estrogen receptors. Using a number of biophysical techniques, including crystal structure analysis of receptor−stapled peptide complexes, we describe in detail the molecular interactions and demonstrate that all-hydrocarbon staples modulate molecular recognition events. The findings have implications for the design of stapled peptides in general.

Direct regulation of the expression of specific genes by the nuclear receptor (NR) superfamily of transcription factors controls a wide gamut of physiological processes ranging from homeostasis to differentiation and behavior. Estrogen receptor (ER), a member of the steroid hormone receptor class of NRs, regulates reproduction and additionally plays a critical regulatory role in central nervous system function, maintenance of bone density, and immunity, among others. Thus, ER has been targeted, with much success, as a point of intervention in a number of disease states, principally in breast and endometrial cancers and osteoporosis. Two ER isoforms exist, ERα and ERβ, which share the characteristic domain organization of NRs, namely, a variable N-terminal transactivation (AF1) domain, the well-conserved DNA binding domain, and a C-terminal ligand binding domain (LBD). Estrogens bind to the LBD and induce a conformational change in the receptor that promotes homodimerization and subsequent binding to specific promoter DNA sequences in target genes modulating gene expression. However, an additional group of binding partners exist, the coregulatory proteins, which are recruited to this complex. ER requires the direct binding of a coactivator protein for ligand-dependent signaling to occur (for a review, see ref 1).

A class of ligands that bind the LBD in an estrogen-like manner are the so-called selective estrogen response modulators (SERMs), and these can act selectively on the two isoforms.[2] These ligands bind in the steroid binding site that forms part of the hydrophobic core of the protein.[3] The effect of a specific ligand depends primarily on the induced alterations to the structure, stability, and interactions of the LBD.[4] These conformational changes to the LBD promote or inhibit allosteric binding of coregulatory proteins. The most marked conformational change is in the position of the C-terminal α-helix (H12). In the agonist-bound conformation, a coactivator protein binding groove is formed, whereas in antagonist-bound conformations, this groove is perturbed.[3,4] Structural and functional studies of ER coactivator protein interactions have shown that they are mediated by a short leucine-rich pentapeptide motif (amino acid sequence LXXLL, where X is any residue), which is termed the NR box.[5,6] A number of NR crystal structures with bound coactivator peptides have been solved; they have shown that the peptides form amphipathic α-helices in which the three conserved leucine residues are presented as a hydrophobic face that binds to the coactivator binding groove.[4,7−12] An additional feature of the binding is recognition of the coactivator peptide helix dipole by charged residues on the receptor surface that bind to the N- and C-termini of the helix; this has been termed the "charge clamp". Receptor selectivity is tuned by the peptide sequence adjacent to the NR box.[11,13] The design of selective peptide inhibitors that compete at the NR box binding site of NRs and particularly ERs has been the focus of research aimed at developing a new class of NR-regulating drugs that act allosterically.[14,15]

Stabilized α-helices represent an interesting class of peptidomimetics. Synthetic modification of small linear peptide modulators locked into a helical structure are attractive because they could adopt a biologically active conformation without an associated entropic penalty. Toward this end, a number of scaffolds have been used to modulate the activity of coactivator peptides to target ER. These include intramolecular disulfide bonds linked in an $(i, i + 3)$ fashion to rigidify a 3_{10} helix conformation,[16] while a library of $(i, i + 4)$ lactam-bridged helical peptidomimetics incorporating non-natural amino acids has also been developed.[17] These studies demonstrate that potent and selective compounds for both the ERα and ERβ receptor coactivator binding sites can be derived.

Recently, a new class of stabilized helix peptidomimetic agent, stapled peptides, has received significant attention.[18] Stapled peptides contain an all-hydrocarbon link (the "staple") between successive turns of a peptide α-helix. The key to this approach is

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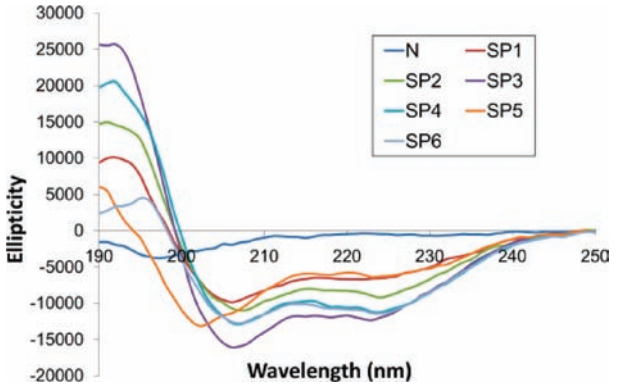

Figure 1. Far-UV CD spectra showing enhanced helical content in stapled peptides.[27] The peptide sequences are shown in Table 1.

the addition of two un-natural amino acids containing olefin-bearing tethers that covalently link the $(i, i+3)$, $(i, i+4)$, or $(i, i+7)$ positions in a peptide (corresponding to amino acids separated by one or two turns of the desired helix).[19] These un-natural amino acids also have a α-methyl group that further stabilizes the α-helix conformation. The earlier work of Grubbs showed a preference for a 3_{10} helix in the absence of this modification.[20] The chemistry is straightforward, and a ruthenium-catalyzed ring-closing metathesis reaction is used to form the staple. Peptides modified in this manner show profound improvements in helicity, stability, protease resistance, potency, and, most significantly, cell permeability.[21] Combined, these properties make stapled peptides pharmacological candidates for the inhibition of intracellular protein−protein interactions, a class of therapeutic targets considered as "undruggable" by traditional small-molecule inhibitors.[22] The number of reports demonstrating this therapeutic potential is growing and includes stapled peptides that inhibit HIV-1 capsid assembly,[23] can directly inhibit the NOTCH transcription factor complex,[24] are able to reactivate the p53 tumor suppressor pathway,[25] and can promote BCL-2-mediated apoptosis.[21]

Here we describe a series of stapled peptides that bind at the coactivator protein binding site of ERs, and we present structures of these complexes and of the peptides in isolation. We describe the use of these data in the design of stapled peptides with improved potency. Additionally, we characterize the interactions biophysically. The presented data highlight significant considerations for the design of stapled peptides for both NRs and protein−protein interaction sites in general.

Using the crystal structure of the NR coactivator peptide 2 bound to ERα (PDB entry 2QGT),[26] we initially designed an 11-mer stapled peptide (**SP1**) with sequence Ac-HS5ILHS5LLQ-DS-NH_2, where *S5* denotes the linked un-natural amino acids at the $(i, i+4)$ positions. To stabilize the helical LXXLL motif, we placed the hydrocarbon link across the interaction site positioned on the solvent-exposed side of the helix in order to conserve the expected protein contacts. Indeed, **SP1** showed a marked increase in helicity, as judged by circular dichroism (CD) spectroscopy (Figure 1), and an ~4-fold gain in binding potency to ERα relative to the equivalent unstapled-sequence peptide (Table 1).

The NMR structure of free **SP1** in aqueous solution (Figure 2A) confirms that **SP1** adopts an α-helical structure that extends well beyond the stapled α-helical turn to the very end of the peptide sequence. The staple and the consensus receptor-contacting

Table 1. Binding Affinities and Kinetics of the Stapled Peptides As Determined by Surface Plasmon Resonance ("IL" and "XL" in the ILXXLL motifs are highlighted)[a]

Peptide Sequence	ERα			ERβ		
	k_a ($\times 10^{-5}$)	k_d	K_D	k_a ($\times 10^{-5}$)	k_d	K_D
N: Ac-HKILHRLLQDS-NH_2	1.39	0.347	2.5	0.792	0.376	5.0
SP1: Ac-HS5ILHS5LLQDS-NH_2	3.80	0.256	0.674	2.55	0.509	1.99
SP4: Ac-HKILHS5LLQS5S-NH_2	n.d.	n.d.	>15	n.d.	n.d.	>15
SP3: Ac-S5KEKS5KILHQLLQDS-NH_2	n.d.	n.d.	8.0	n.d.	n.d.	8.3
SP5: Ac-HKILHQLLQS5SSSS5V-NH_2	n.d.	n.d.	4.0	n.d.	n.d.	2.8
SP2: Ac-HKS5LHQS5LQDS-NH_2	5.48	0.193	0.352	5.36	0.339	0.632
SP6: Ac-EKHKILS5RLLS5DS-NH_2	17.8	0.129	0.075	7.14	0.155	0.155

[a] Abbreviations: k_a, on-rate constant (in $M^{-1}\ s^{-1}$); k_d, off-rate constant (in s^{-1}); K_D, dissociation constant (in μM); n.d., not determined.

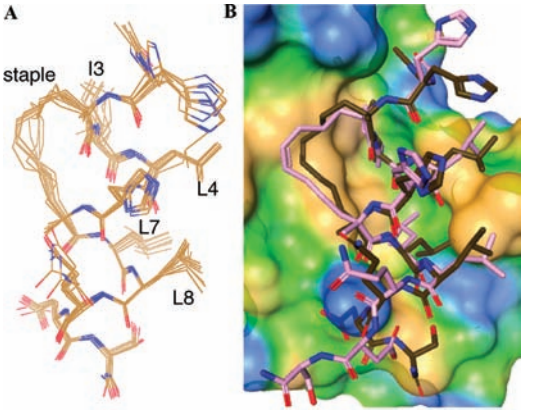

Figure 2. (A) NMR solution structure of unbound **SP1** (ensemble of 10 lowest-energy structures). (B) Average unbound structure of **SP1** (dark-brown) superimposed on the structure of **SP1** bound to ERβ_LBD determined at 1.9 Å resolution (pink). The protein surface is colored by hydrophobicity, with brown indicating the most hydrophobic regions. The all-hydrocarbon staple is shown to bind in a region of the coactivator peptide recognition site.

residues I3, L4, L7, and L8 are already well-ordered in free **SP1** in solution. The 1.9 Å crystal structure of the ternary complex of ERβ_LBD, agonist, and **SP1** (Figure 2B) shows that **SP1** maintains its α-helical structure when bound to the receptor, although terminal residues that are not contacted by the receptor fan out and make space for K314 of ERβ_LBD in the crystal structures. Also, the staple and the side chain of I3 preserve their conformations upon binding. Only the isopropyl group of L7 flips to a different rotamer upon binding.

The ERβ_LBD is in the classical agonist conformation, with H12 ordered to form the coactivator binding site, and **SP1** is shown to bind in the coactivator site. The electron density map is unambiguous, and all of the atoms of the peptide, including the hydrocarbon link, can be modeled. The conserved NR box LXXLL motif does not bind in the groove as expected but instead is perturbed, with the hydrocarbon link making extensive van der Waals contacts with the hydrophobic residues Val307, Ile310, and Leu490 of the coactivator protein binding groove. The

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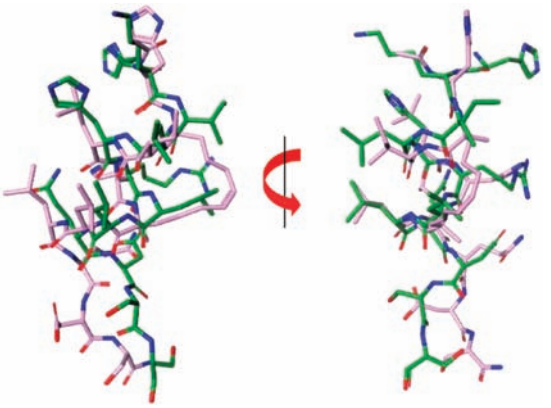

Figure 3. Orthogonal views of the receptor-bound crystal structure for **SP1** crystal (pink) superimposed with the coactivator peptide crystal structure 2QGT (green). A quarter turn of **SP1** with respect to the coactivator peptide occurs, mapping the hydrophobic staple to the position of the recognition site.

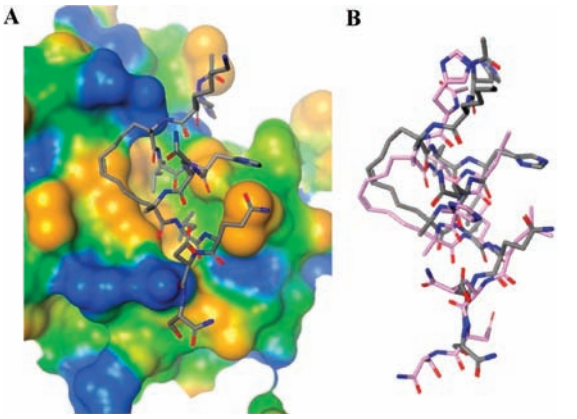

Figure 4. (A) Crystal structure of **SP2** bound to ERα determined at 1.8 Å resolution. The peptide is shown in gray, and the protein surface is colored by hydrophobicity, with brown indicating the most hydrophobic regions. (B) Superposition of the crystal structure of **SP2** (gray) with that of **SP1** (pink). Recognition of the staple is conserved while the sequence register shifts.

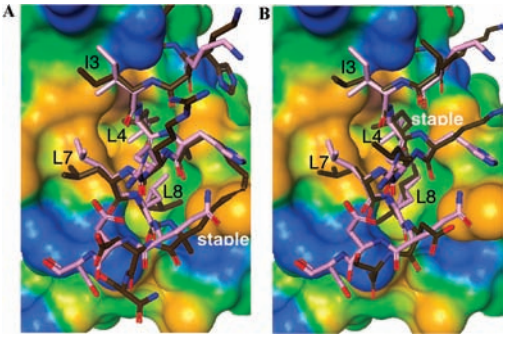

Figure 5. 3D models of **SP6** bound to ERβ_LBD. (A) Superposition of the unbound NMR structure of **SP6** (dark-brown) with the cocrystal of coactivator peptide (pink) and ERβ_LBD (hydrophobic surface representation; 2QGT) in such a way that the IL_LL motifs align. (B) Superposition as (A) in such a way that the staple of **SP6** aligns with L4 and L8 of the coactivator peptide.

well-diffracting crystals indicate that stapled peptides are useful new tools for NR structural biology studies. Comparison with the coactivator protein peptide complex (2QGT) shows that a quarter turn of the helix has occurred, shifting the binding site residues out of register by one position (Figure 3). The recognition motif LXXLL is replaced with the **SP1** motif IXXS5L. A consequence of making stapled peptides based on a natural peptide sequence is that the inclusion of the all-hydrocarbon link significantly alters the physiochemical properties of the peptide, and as this example demonstrates, the hydrophobic staple itself can bind to hydrophobic protein−protein interaction sites.

Moving on from this crystal structure, we explored a series of distinct stapling positions (Table 1). Bridging the staple across the C-terminal leucines of the recognition motif (**SP4**) did not improve the binding affinity relative to the unstapled peptide **N**. Placing the staple away from the recognition motif at the N-terminus (**SP3**) and C-terminus (**SP5**), which was expected to seed α-helicity across the entire sequence, reduced or even abrogated binding to ERα and ERβ (Table 1). The falloff in binding suggests that larger helices are not well accommodated at the coactivator site. This is perhaps unsurprising, as these larger helices could disrupt the residues forming the charge clamp.

Replacing the I and L of the recognition motif by the hydrophobic staple (**SP2**) gave a 2-fold increase in binding potency relative to **SP1**, and **SP2** has a K_D of 352 nM for ERα binding. CD and NMR spectroscopy of unbound **SP2** confirmed that it is α-helical across its entire length (Figures S2 and S3 in the Supporting Information). As observed for **SP1**, the staple and the expected protein contacts L4 and L8 are rigidly prestructured in free **SP2** in solution. A crystal structure of this peptide bound to ERα was solved at high resolution (Figure 4). Again, as in the **SP1** ERβ structure, the staple binds at the hydrophobic groove of the coactivator site. In this case, however, the sequence of the peptide is back in register with the coactivator peptide structure. The remaining two leucines of the NR box have the same interactions as seen with coactivator peptides, with the staple fulfilling the role of the third.

Comparison of the specific interactions of **SP2** and the 2QGT coactivation peptide indicates that the conformations of only two residues of the receptor site have been changed. First, Asp538 in **SP2** has swung in toward the peptide, whereas in the coactivator peptide structure it moves out to accommodate the branched Ile side chain in the peptide's binding motif. Second, Ile358 adopts a different rotamer to pack more tightly with **SP2** than it does with the second leucine of the peptide binding motif. Again bridging the staple over the C-terminal recognition motif, but this time rotating it by 100° toward the other side of the IL__LL protein contacts in comparison with **SP4**, yielded the most potent binder of the ERs, **SP6**, which had K_d values of 75 and 155 nM for ERα and ERβ, respectively. There is a general trend for each peptide to be twice as potent for ERα over ERβ. In all, a 30-fold improvement in K_D was achieved in comparison with the original nonstapled peptide **N** for both ER isoforms. Determination of the binding kinetics by BIAcore also demonstrated a significant difference in the on rate for the stapled and nonstapled peptides. Stapled peptides typically have an order of magnitude higher association rate than the unstapled peptide (for **N**, $k_a = 7.92 \times 10^4$ M^{-1} s^{-1}; for **SP6**, $k_a = 7.14 \times 10^5$ M^{-1} s^{-1}). This is consistent with the peptide being preordered and suggests that a significant fraction of the potency gain achieved is due to a lower entropic penalty on binding. Indeed, the NMR structure of free **SP6** in aqueous buffer confirms the preordering of both the α-helix and the putative protein contacts I5, L6, L9, and L10 as well as the staple (Figure S2B).

In the absence of a cocrystal structure for receptor-bound **SP6**, we analyzed the fit of the unbound NMR structure of **SP6** to the

receptor surface of the coactivator peptide/ERβ complex. Placing the staple of **SP6** in the same geometry as observed in the **SP1**/ERβ_LBD crystal positions the two charged residues R8 and D12 of **SP6** in the conserved hydrophobic pocket of ERβ_LBD, which would be expected to abrogate binding. In contrast, superimposing unbound **SP6** in such a way that the residues of the IL__LL motifs match provides a good fit of **SP6** into the coactivator pocket of ER, provided that the two N-terminal residues of **SP6** are rotated away from the ER surface (Figure 5A).

Alternatively, the staple of **SP6** can be rotated straight into the conserved hydrophobic pocket by superimposing it with L4 and L8 of the coactivator peptide (Figure 5B). The staple again shows an excellent fit to the ERβ_LBD surface, provided that the two N-terminal residues of **SP6** bend away from the protein surface. Taken together, the two binding geometries are consistent with the high-affinity binding measured for **SP6**, indicating that the C8 hydrocarbon staple can substitute for native hydrophobic interactions at various positions of the peptide sequence. As a consequence, staple design can on the one hand benefit from the rigid and prestructured hydrophobic interaction potential of the staple, but on the other hand, one must be cautious about altered binding geometries introduced by the staple. No assessment of the cell permeability or cellular activity of these peptides has been made.

The structural data reported here present significant lessons for the design of stapled peptide inhibitors of NRs and for protein−protein interactions in general. The addition of the all-hydrocarbon staple not only conformationally restrains the peptides but also can affect the interactions of the peptide with hydrophobic protein surfaces. The rigidity of the staple itself, as shown by the solution structures of the peptides in isolation, suggests that there is a shape-complementarity component to stapled peptide binding at these surfaces. Indeed, the recent crystal structure of a stapled peptide inhibitor of the MCL-1 protein, in which the staple was positioned on the noninteraction face of the helix, indicates that the staple makes specific hydrophobic contacts that add additional potency,[28] supporting the finding reported here. In the design of stapled peptide inhibitors of protein−protein interactions, the question of *how the hydrophobic nature of the hydrocarbon linker perturbs the interaction* must be considered.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org. Coordinates and structure factors have been deposited with the PDB under the codes 2YJA, 2YJD, 2LDC, 2LDA, and 2LDD. Chemical shift assignments have been deposited with the BMRB.

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