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# Potent Inhibitors of LXXLL-Based Protein–Protein Interactions

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*Protein–protein interactions between estrogen receptors, ERα and ERβ, and their coactivators (CoAs) are an attractive target for drug intervention. This interaction is mediated by a small pentapeptide motif (LXXLL), termed the NR box. Based on this motif, a variety of cyclic and linear peptides were synthesized in order to gain a better understanding of the association of CoA proteins with the ER isoforms. Utilizing a time-resolved florescence-based coactivator interaction assay, we determined the abilities of these peptides to inhibit this interaction. Using molecular modeling and CD spectroscopy, we have examined the structural basis of their bioactivities with both hormone receptor isoforms. Either homocysteine or penicillamine was utilized as a substitute for cysteine in the disulfide-bridged peptides, while tertiary leucine and neopentyl glycine were used as the surrogates for the NR box leucines. The most potent disufide-bridged peptide ($K_i$ = 70 pM, with ERα) incorporates neopentyl glycine in the NR box, while the most active peptide in this series with ERβ ($K_i$ = 350 pM) incorporates tertiary leucine. Surprisingly, several linear peptides containing a single cysteine residue showed activities with low nanomolar $K_i$ values. Collectively, our results suggest a synthetic approach for designing potent and selective peptidomimetics for ERα and ERβ interactions with CoA proteins effecting estrogen action.*

## Introduction

Nuclear receptors (NRs) are transcription factors that control physiological processes, such as differentiation, development, homeostasis, and behavior, by directly regulating the expression of select target genes.[1] The estrogen receptor (ER) protein, which is a member of the nuclear hormone receptor superfamily plays a primary role in reproduction and regulates a variety of physiological processes associated with the skeletal system, cardiovascular system, and certain nonreproductive centers of the brain.[2] The action of estrogens and their mimics in regulating gene transcription is mediated through two ER isoforms, ERα and ERβ.[1] ER has been implicated in a variety of disease states, including breast and endometrial cancers, cardiovascular disease, osteoporosis, and Alzheimer's disease.[3] Considering breast cancer, most drug-design efforts have focused on developing selective estrogen-receptor modulators or SERMs. These synthetic antiestrogens bind in the ligand-binding domain (LBD) of either ERα or ERβ, and block estrogen action, effectively inhibiting cell growth.[4] SERMs elicit a complex array of tissue-specific effects, and the elaborate pharmacology of ER presents further obstacles to the broader clinical applications of SERMs.

ER pharmacology involves combinatorial collaboration of at least three events.[5] These include ligand-induced alteration of receptor conformation, binding of activated receptor to specific promoter regions within target genes, and the recruitment of coregulatory proteins to the ligand–receptor–gene assembly. Drugs that have been designed to target ER function bind within the ligand-binding pocket and modulate receptor conformation. However, the effectiveness of estrogens in stimulating ER-mediated gene transcription depends on the receptor protein interactions with the coactivator (CoA) proteins.[6] Considering the functional consequences of ER–CoA interactions on hormone-induced gene activation, this crucial protein–protein interaction has been recognized as an attractive target for drug development.[7]

Structural and functional studies have revealed the molecular mechanisms of ligand-dependent interactions between NRs and their CoAs.[8–11] These protein–protein interactions are primarily mediated by a short, conserved, pentapeptide motif

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LXXLL (L is leucine, X is any amino acid), termed the NR box. Crystallographic analyses have shown that this pentapeptide, which is a segment of the CoA protein, binds as a short α-helix to a shallow hydrophobic groove on the surface of the NR LBD. The helical conformation of the NR box allows hydrophobic leucine residues to interact favorably with the shallow solvent-exposed hydrophobic groove. Biochemical studies have demonstrated the feasibility of blocking the interaction between NR and CoAs with the help of small peptides containing the LXXLL interaction motif.[11] Disruption of this helix-mediated protein–protein interaction provides an alternative strategy for regulating the transcriptional activity of NRs. This generalized strategy has been utilized in designing CoA-based peptides targeted toward ER[12–14] and vitamin D,[15] retinoid X,[15] and thyroid receptors.[16,17]

Recently, we reported peptidomimetic estrogen-receptor modulators or PERMs as inhibitors of ER–CoA interactions.[12,13] Disulfide and thioether-bridged side-chain cyclization[18,19] were used as a strategy to induce helicity within the small peptide chains incorporating the LXXLL motif. Here we have advanced the structure–activity relationship (SAR) studies on disulfide-bridged PERMs. The purpose of this study is to understand what factors affect the binding affinity and selectivity of LXXLL-containing peptides. We have studied the effect of change in the configuration of cysteines constituting the disulfide bridge on the bioactivity of these peptides. Using a variety of unnatural cysteine and leucine surrogates, we have obtained more potent and selective analogues of PERMs. We have also designed and synthesized linear analogues of our disulfide-bridged peptides. The study reveals valuable information that can be utilized in designing peptidomimetic or nonpeptidic molecules as the inhibitors of ER-CoA interactions.

## Results

### Cyclic Peptides

We recently reported PERM-1, a cyclic peptide with the sequence H-Lys-cyclo(D-Cys-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-$NH_2$, which is a D-Cys, L-Cys; *i*, *i*+3, disulfide-bridged nonapeptide.[12] The high affinities ($K_i$ values of 25 nM for ERα, and 390 nM for ERβ) observed for PERM-1 have been attributed to the helix-initiating *i*, *i*+3; D-Cys, L-Cys combination for its disulfide bridge. This strategy of side-chain cyclization for helix induction is based on a report by Pelligrini et al.,[22] and, accordingly, PERM-1 displayed partial helical character in our CD studies.[12] However, there have been no systematic studies to assess the potential of the remaining combinations of the cysteine configurations for inducing helicity in the peptide chain.

Hence, we initially synthesized three *i*, *i*+3 disulfide-bridged peptides (Table 1) that incorporated L-Cys, L-Cys (**1**); L-Cys, D-Cys (**2**); and D-Cys, D-Cys (**3**). The bioactivity profile of these configurational variants showed the order of D-Cys, L-Cys (PERM-1) > L-Cys, L-Cys (**1**) > D-Cys, D-Cys (**3**) > L-Cys, D-Cys (**2**). Thus, none of the configurational variants showed higher affinity binding to either ERα or ERβ than PERM-1 (Table 1). Circular dichroism (CD) studies of these peptides were performed in 20% 2,2,2-trifluoroethanol (TFE) to determine their secondary structure. In the case of an α-helix, the spectrum has two typical minima at 208 nm and 222 nm. Although short peptides are not α-helical in aqueous solution, some solvents, such as TFE, induce helical behavior. CD results for the disulfide-bridged peptides **1**, **2**, **3**, and PERM-1 are presented in Figure 1. Although the spectra shown in Figure 1 exhibit minima around 208 and 222 nm, their shapes are not uniform and are distorted from that of an ideal α helix. This is probably due to the presence of a disulfide bridge and variation in the cysteine configuration of these peptides. Surprisingly, PERM-1, with the lowest $K_i$ value (Table 1), did not show the highest helical character. Instead, peptide **1**, which is an all-L-amino acid peptide, exhibited the highest helical content. This finding was expected, as it has been shown that incorporation of a D amino acid residue generally destabilizes the helical peptide by 1 kcal $mol^{-1}$.[23] CD analysis revealed that the helical character observed in 20% aqueous TFE is not the dominant factor determining the binding affinity of these cyclic peptides.

Peptide **4** not only contains the preferred *i*, *i*+3; D-Cys, L-Cys combination, but it also contains two copies of the LXXLL sequence. The N-terminal copy incorporates D-cysteine and isoleucine as the intervening XX residues, while the C-terminal copy has cysteine and arginine as the intervening residues. Coactivator proteins often contain multiple copies of LXXLL motifs; however, they are typically separated by ~50 amino acids; this might allow cooperative binding of LXXLL motifs to dimeric LBDs.[24] Thus, it was not unexpected that peptide **4** showed no further increase in affinity relative to PERM-1 since the LXXLL motifs were overlapping and did not contain the requisite amino acid spacer (Table 1). In fact, these alterations actually caused a decrease in affinity for both ERα and ERβ.

It is believed that in an *i*, *i*+3; D-Cys, L-Cys strategy, both the cysteine side chains are oriented toward the same side of the peptide chain. This presumably compensates for the destabilization caused by the presence of a D amino acid and induces helical conformation favorable for the association of the leucines with the hydrophobic groove. But, other than *i*, *i*+3 side-chain cyclization, we[12] and others[16,17,25] have reported a *i*, *i*+4 side-chain-cyclization strategy using lactam bridges. In our report,[12] *i*, *i*+3 disulfide cyclization showed significantly better association with the CoA binding groove than an *i*, *i*+4 lactam analogue. In the case of peptide **5**, we also attempted an *i*, *i*+4 disulfide cyclization, but this peptide also showed much lower potency than that of PERM-1 (Table 1). This series of peptides involving different combinations of cysteine configurations and ring sizes confirmed that *i*, *i*+3; D-Cys L-Cys combination indeed produces the most favorable receptor association and that higher helicity does not necessarily translate into lower $K_i$ values of PERMs. Utilizing this *i*, *i*+3; D-Cys L-Cys configuration, we further explored the effect of two unnatural cysteine surrogates, homocysteine and penicillamine, on the binding affinities of PERMs.

We previously reported PERM-2, which has the sequence H-Arg-cyclo(D-Cys-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-$NH_2$.[13] PERM-2 is an N-terminal arginine analogue of PERM-1. This peptide displayed higher affinities than those of PERM-1 (Table 1). Using

  

**Table 1.** Peptide sequences and biological activities of PERMs.

| No. | Sequence | $K_i \pm$ (SEM) [nM] ERα | ERβ | β/α |
|---|---|---|---|---|
| | PERM-1[12] H-Lys-cyclo(D-Cys-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 25 (2.2) | 390 (83) | 15.6 |
| | PERM-2[13] H-Arg-cyclo(D-Cys-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 11 (1.1) | 77 (18) | 7.0 |
| **1** | H-Lys-cyclo(Cys-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 416 (130) | 1800 (420) | 4.3 |
| **2** | H-Lys-cyclo(Cys-Ile-Leu-D-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 2400 (870) | 7200 (4100) | 3.0 |
| **3** | H-Lys-cyclo(D-Cys-Ile-Leu-D-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 928 (590) | 3900 (390) | 4.2 |
| **4** | H-Lys-Leu-Leu-cyclo(D-Cys-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 398 (90) | 2000 (330) | 5.0 |
| **5** | H-Lys-cyclo(Cys-Ile-Leu-Arg-Cys)-Leu-Leu-Gln-$NH_2$ | 174 (50) | 1160 (240) | 6.7 |
| **6** | H-Arg-cyclo(D-Cys-Ile-Leu-Homo Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 13 (3.4) | 214 (23) | 16.5 |
| **7** | H-Arg-cyclo(D-Homo Cys-Ile-Leu-Cys)- Arg-Leu-Leu-Gln-$NH_2$ | 35 (1.5) | 591 (190) | 16.9 |
| **8** | H-Arg-cyclo(D-Homo Cys-Ile-Leu-Homo Cys)- Arg-Leu-Leu-Gln-$NH_2$ | 12 (0.4) | 21 (1.4) | 1.8 |
| **9** | H-Arg-cyclo(D-Pen-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 168 (10) | 933 (140) | 5.6 |
| **10** | H-Arg-cyclo(D-Cys-Ile-Leu-Pen)-Arg-Leu-Leu-Gln-$NH_2$ | 88 (25) | 1910 (1700) | 21.7 |
| **11** | H-Arg-cyclo(D-Pen-Ile-Leu-Pen)-Arg-Leu-Leu-Gln-$NH_2$ | 78 (53) | 3970 (1800) | 50.9 |
| **12** | H-D-Lys-cyclo(D-Cys-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 220 (35) | 1900 (870) | 8.6 |
| **13** | H-Ser-cyclo(D-Cys-Ile-Leu-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 322 (32) | 1035 (400) | 3.2 |
| **14** | H-Lys-cyclo(Cys-Leu-Ile-D-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 2100 (200) | 17000 (4100) | 8.1 |
| **15** | H-Arg-cyclo(D-Cys-Leu-Ile-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 13 (9.8) | 216 (54) | 16.7 |
| **16** | H-Arg-cyclo(D-Cys-Ile-*t*-Leu-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 0.16 (0.09) | 0.35 (0.6) | 2.2 |
| **17** | H-Arg-cyclo(D-Cys-Ile-Leu-Cys)-Arg-*t*-Leu-Leu-Gln-$NH_2$ | 7 (1.5) | 1.2 (0.4) | 0.2 |
| **18** | H-Arg-cyclo(D-Cys-Ile-Leu-Cys)-Arg-Leu-*t*-Leu-Gln-$NH_2$ | 50.2 (2.9) | > 10000 | – |
| **19** | H-Arg-cyclo(D-Cys-Ile-Npg-Cys)-Arg-Leu-Leu-Gln-$NH_2$ | 0.9 (0.6) | 2.5 (3.4) | 2.8 |
| **20** | H-Arg-cyclo(D-Cys-Ile-Leu-Cys)-Arg-Npg-Leu-Gln-$NH_2$ | 0.07 (0.06) | 1.2 (1.4) | 17.1 |
| **21** | H-Arg-cyclo(D-Cys-Ile-Leu-Cys)-Arg-Leu-Npg-Gln-$NH_2$ | 0.89 (0.7) | 13.2 (5.9) | 14.8 |
| **22** | H-Arg-D-Ser-Ile-Leu-Ser-Arg-Leu-Leu-Gln-$NH_2$ | 60 (28) | 1850 (290) | 30.8 |
| **23** | H-Arg-Ser-Ile-Leu-Ser-Arg-Leu-Leu-Gln-$NH_2$ | 154 (90) | 1660 (870) | 10.8 |
| **24** | H-Arg-D-Ala-Ile-Leu-Ala-Arg-Leu-Leu-Gln-$NH_2$ | 129 (46) | 4870 (2100) | 37.8 |
| **25** | H-Ser-Pro-Ile-Leu-Ser-Arg-Leu-Leu-Gln-$NH_2$ | 1790 (300) | 2750 (2100) | 1.5 |
| **26** | H-Arg-Ile-Leu-Arg-Cys-Leu-Leu-Gln-$NH_2$ | 13 (15) | 184 (220) | 13.5 |
| **27** | H-Arg-Leu-Ile-Arg-Cys-Leu-Leu-Gln-$NH_2$ | 58 (5.5) | 1612 (470) | 27.8 |
| **28** | H-Ser-Arg-Ile-Leu-Arg-Cys-Leu-Leu-Gln-$NH_2$ | 23 (2.1) | 274 (59) | 11.9 |
| **29** | H-Ser-Arg-Leu-Arg-Cys-Leu-Leu-Gln-$NH_2$ | 22 (5.8) | 123 (31) | 5.6 |
| **30** | H-Arg-Ile-Leu-Arg-Ser-Leu-Leu-Gln-$NH_2$ | 438 (140) | 1800 (350) | 4.1 |

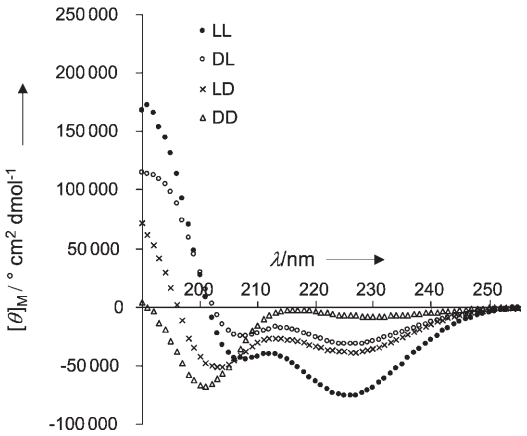


**Figure 1.** Helical character of disulfide-bridged peptides **1** (LL), **2** (LD), and **3** (DD), and PERM-1 (DL) in 20% aqueous TFE.

this peptide as the template, we synthesized its homocysteine and penicillamine analogues. Peptides **6** (D-Cys, L-HomoCys), **7** (D-HomoCys, L-Cys), and **8** (D-HomoCys, L-HomoCys) were synthesized to study the effect of homocysteines, a homologue of cysteine, while peptides **9** (D-Pen, L-Cys), **10** (D-Cys, L-Pen), and **11** (D-Pen, L-Pen) were synthesized to study the effect of penicillamine. An additional methylene unit in homocysteine increases the ring size of the *i*, *i*+3 disulfide-bridged peptides **6** and **7** from 14 atoms to 15 atoms, while a 16-atom disulfide bridge is formed in the bis-homocysteine peptide **8**. This increased ring size presumably adds to the flexibility of a disulfide-constrained peptide chain. Thus, peptides **6**, **7**, and **8** provided a system for studying the effect of disulfide ring size and flexibility on the bioactivity of PERMs. As indicated in Table 1, peptides **6** and **7** maintained low nanomolar $K_i$ values. We found significant binding affinities and a moderate selectivity in a D-Cys, L-HomoCys disulfide combination of peptide **6**. Although a slightly lower binding affinity was observed for peptide **7**, this mixed cysteine–homocysteine peptide maintained selectivity towards ERα. The more flexible peptide **8** showed the lowest $K_i$ value in this series, but this flexibly constrained peptide also showed poor selectivity towards ERα.

Various nuclear receptors and their corresponding coactivators utilize LXXLL-type recognition motifs for transcriptional activation. Hence, ER-subtype selectivity is considered an important aspect of this study as we explore our ability to define the receptor selectivity of LXXLL peptidomimetics. Penicillamine has been routinely used to induce selectivity in disulfide-bridged peptides. The selectivity is gained from the β,β-dimethyl substituents of penicillamine, which constrain the movement of the peptide chain around the disulfide bridge. In the series of penicillamine-containing PERMs, the selectivity gradually increased from peptide **9** to peptide **11**. The D-Pen, L-Pen peptide showed the highest selectivity, as anticipated. Peptide **11**, which is 50-fold more selective towards ERα, is in fact the most selective peptide in our series of cyclic peptides.

From classical site-directed and alanine-scanning mutagenesis studies, it has been reported that sequences N- and C-terminal to the LXXLL motif appear to have the greatest impact on the receptor selectivity and binding affinity.[11,26] These residues are not well conserved among different coactivators and might play a role in determining the specificity of NR-CoA interactions. In this regard, a systematic study was carried out by Ko et al.[27] that revealed that, in the CoA protein, serine at the

  

−3 position allows selective interactions for ERβ versus ERα. In our series of PERMs, Peptides **1**–**11** feature a positively charged residue (lysine or arginine) at the −3 position. Using peptides **12** and **13**, we assessed the contribution of the residue at −3 position toward the receptor selectivity and binding affinity.

Initially we studied the effect of inversion of configuration of the N-terminal lysine residue on PERM-1. Accordingly, we synthesized peptide **12** with D-lysine at the N terminus. $K_i$ values for ERα and ERβ increased significantly (Table 1); this indicates the importance of orientation of this charged residue toward the estrogen receptor. In peptide **13**, we replaced the N-terminal lysine of PERM-1 with serine. This substitution has been utilized previously to improve the selectivity of the LXXLL-containing peptides toward ERβ.[27] In our SAR studies, peptide **13** showed higher $K_i$ values for both ERα and ERβ, but showed about a fivefold lower selectivity toward ERα than PERM-1 (Table 1). As compared to PERM-1, the affinity of peptide **13** for ERα dropped 13-fold, while its affinity for ERβ dropped only threefold. Thus, although substitution of serine at the −3 position assisted in designing ERβ-selective peptides, the single substitution was not sufficient to reverse the selectivity of these peptides from ERα to ERβ.

In further SAR studies, we modified the leucine residues of the NR box motif. To distinguish individual leucine residues, we designated the NR box motif LXXLL as $L^1XXL^2L^3$ starting from the N terminus. Peptide **14** was synthesized as a variant of peptide **2**. In this peptide we changed the core NR box motif sequence from LXXLL to IXXLL. Changing $L^1$ to isoleucine lead to a notable change in bioactivity. Interestingly, peptide **14** showed decreased ERβ affinity without affecting ERα activity. Thus, peptide **14** showed improved ERα selectivity. Similarly, in peptide **15**, we changed the NR box of PERM-2 to IXXLL and noted results similar to peptide **14**. ERα selectivity was improved due to a selective decrease in ERβ affinity.

Utilizing unnatural hydrophobic substitutes for leucines of the NR box, Geistlinger et al.[16,17] and Rodriguez et al.[14] recently reported potent and selective inhibitors of LXXLL-mediated NR-CoA interactions. Through the use of tertiary leucine (*t*-Leu) and neopentyl glycine (Npg), we have adopted a similar approach to investigate the CoA-binding hydrophobic groove. However, we utilized unnatural surrogates that very closely resemble leucine. This strategy not only retains the hydrophobicity of leucine but also only subtly manipulates the NR box. We replaced each leucine of $L^1XXL^2L^3$ with either *t*-Leu or Npg by performing only one substitution at a time (Table 1, peptides **16**–**21**). This positional scanning maps the entire NR box to identify which unnatural leucine surrogate fits the best and at what position.

Peptides **16**, **17**, and **18** have *t*-Leu at positions $L^1$, $L^2$, and $L^3$, respectively. Peptide **16** not only exhibited significantly higher affinity, but it also possessed moderate selectivity towards ERα. Peptide **17** displayed high affinity for both receptors and gained limited ERβ selectivity. Peptide **18** showed a subtle decrease in affinity for ERα, but interestingly lost all detectable binding to ERβ. A similar series of peptides with neopentyl glycine, **19**, **20**, and **21**, was designed and synthesized. Neopentyl glycine substitution at any of the three positions generally increased affinity to both receptors. Peptide **20** showed exceptionally high potency with a $K_i$ value of 70 pM. Collectively, it appears that *t*-Leu at the $L^1$ position, neopentyl glycine at the $L^2$ position, and leucine at the $L^3$ position is the preferred combination for the $L^1XXL^2L^3$ NR box motif to exhibit favorable recognition of estrogen receptors.

### Linear peptides

All the cyclic peptides described in the previous section were based on the idea that some form of conformational constraint, such as side-chain cyclization, is required to induce helicity to a small peptide chain. This because small peptides typically do not adopt well-defined conformations in aqueous solutions; rather they adopt an ensemble of energetically similar conformations. Hence it was assumed that the linear counterparts of these disulfide-bridged peptides would be too flexible to effectively bind to the hydrophobic groove on the ERs. To assess this hypothesis, we attempted to synthesize and purify reduced bis-cysteine counterparts of some of our potent disulfide-bridged peptides. However, synthesizing and assaying such peptides proved rather impractical, as a peptide incorporating two cysteine residues invariably oxidizes at some stage during the synthesis or purification. And even if the linear version is obtained in a pure form, the peptide tends to oxidize (i.e. form a disulfide bridge) when diluted in aqueous solutions for performing assays.

Hence initially, we synthesized peptide **22** in which we replaced cysteines with serines. In this peptide, we incorporated the D-Ser, L-Ser; *i*, *i*+3 combination, analogous to the D-Cys, L-Cys; *i*, *i*+3 disulfide combination. Peptide **22** not only showed a nanomolar $K_i$ value, but also better selectivity than its cyclic counterpart PERM-2. Peptide **23**, an L-serine analogue of peptide **22**, showed lower bioactivity toward both the ER isoforms. Interestingly, peptide **23** showed an almost identical helical content as peptide **22**. The CD spectra of peptides **22** and **23** are given in Figure 2.

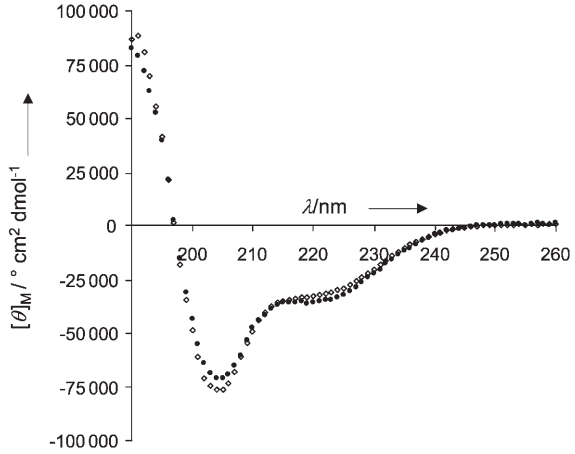


**Figure 2.** Helical character of peptides **22** (◇) and **23** (●) in 20% aqueous TFE.

  

Surprisingly, both peptides showed comparable minima at 208 and 222 nm. We had anticipated that, similarly to disulfide-bridged peptides (Figure 1), peptide **23** with a L-Ser, L-Ser; *i*, *i*+3 combination would show enhanced helicity, while the incorporation of D-serine in peptide **23** (with a combination of D-Ser, L-Ser; *i*, *i*+3) would show a lower helical content. This result contradicts the hypothesis that incorporation of D amino acid significantly destabilizes the helix.[23] We believe that the hydroxyl groups of the serine side chains play an important role in balancing the stability of the helical conformation of peptide **23**. Another peptide (**24**) was synthesized in which the serine residues are replaced with alanines, providing the same combination of configuration. The beneficial effect of incorporating a combination of *i*, *i*+3-spaced D and L amino acids becomes apparent from the results of peptide **24**, which is the alanine counterpart of peptide **22**. Peptide **24**, demonstrated a similar affinity to that of peptide **23**, but also showed the best selectivity in the series of linear peptides.

To compare the SAR patterns established in our disulfide peptides with these non-cysteine linear peptides, we synthesized peptide **25** with serine at the −3 position and proline at the −2 position. This combination is predicted to significantly decrease the selectivity of the peptide toward ERα[11,27] as was indeed the case. SAR studies on this series of peptides established that linear peptides with partial helical character in 20% TFE can effectively inhibit ER–CoA interactions. Our results suggest the receptor is capable of inducing helical character in these peptides, which is required for their biological effects.

We synthesized a series of linear unconstrained LXXLL peptides incorporating a single cysteine residue to further our SAR analysis. This class of monocysteine peptides displayed surprisingly high affinities for ER. Peptide **26** incorporates an LXXLL motif in which arginine and cysteine are the intervening XX residues. This small octapeptide with a molecular weight of about 1000 Da, and with no conformational constraint, showed a $K_i$ of 13 nM for ERα. This affinity is comparable to that of PERM-1 and PERM-2, both of which are D-Cys, L-Cys; *i*, *i*+3 disulfide-bridged peptides. This is surprising because, unlike our previously described cyclic and linear peptides, CD analysis of peptide **26** revealed no characteristics of any specific conformation. In the absence of any helix initiator such as the disulfide bridge, it is unlikely that this octapeptide will adopt a well-defined helix in solution.

We established the credibility of this result by performing additional SAR studies on such monocysteine peptides. First, we wanted to see if the SAR pattern observed in disulfide PERMs could be reproduced in these monocysteine linear peptides. In the case of cyclic PERMs, we had observed that, if the NR box motif is changed from LXXLL (with isoleucine at the −1 position) to IXXLL (with leucine at the −1 position), then there is a noticeable increase in the selectivity of the IXXLL peptide towards ERα. Accordingly, we synthesized peptide **27**, with a sequence the same as that of peptide **26** except that the NR box motif had been changed to IXXLL with leucine at the −1 position, and indeed observed that it was more selective towards ERα than **26** (Table 1). This reproducibility of the SAR pattern from our disulfide-bridged peptides suggests that these monocysteine linear peptides might also be binding to the same CoA-binding hydrophobic groove as our disulfide-bridged peptides.

To provide additional support to this hypothesis, we synthesized peptide **28** with serine at the −3 position. Our previous SAR studies established that serine at the −3 position reduces the selectivity of PERMs toward ERα. Biological data from peptide **28** (Table 1) suggested a similar pattern. We found peptide **28**, with serine at −3, to be less selective ($\beta/\alpha = 11.91$) for ERα than peptide **26** ($\beta/\alpha = 13.53$). Next, in peptide **29**, we removed the hydrophobic isoleucine residue at the −1 position in peptide **28**. Thus, this octapeptide has a positively charged residue (arginine) at −1 position. This change further reduced selectivity of the peptide for ERα as anticipated.[11]

From the above-mentioned SAR pattern of monocysteine linear peptides, it was hypothesized that the presence of a free sulfhydryl group is the significant contributing factor toward the low nM $K_i$ values of these peptides. To confirm this hypothesis, we synthesized peptide **30**, which is the serine counterpart of peptide **26**. This replacement of cysteine with serine resulted in a dramatic change in the bioactivity with peptide **30** showing a more than 30-fold decrease in the affinity relative to peptide **26**. This validated the hypothesis that the free SH group of these monocysteine linear peptides is responsible for the lower $K_i$ values observed.

## Discussion

We have designed and synthesized a series of cyclic and linear peptides to disrupt ER–CoA interactions. The NR box motif incorporated in these peptides was designed specifically to target the ERs. We carried out systematic SAR studies to better understand the association between ER and its CoAs. In the first part of our SAR studies, we confirmed D-Cys, L-Cys; *i*, *i*+3 as the most favorable combination of cysteines. This study also revealed that, unlike in our previous reports,[12,13] higher helical content of the peptide chain in solution (20% aqueous TFE) does not directly correlate with their $K_i$ values.

Based on this D-Cys, L-Cys; *i*, *i*+3 combination, we further synthesized a series of homocysteine- and penicillamine-containing peptides. Penicillamines, by virtue of their β-methyl substituents, provide additional constraint to the disulfide bridge, while homocysteines, because of their extended side chains, make the disulfide bridge flexible. The homocysteine- and penicillamine-containing peptides thus provided a system to study the effect of the extent of conformational constraint on the bioactivity and selectivity of these peptides. While, the bis-penicillamine peptide showed improved selectivity (Table 1) the bis-homocysteine peptide showed lower selectivity than PERM-2. Moreover, the higher bioactivity of a bis-homocysteine peptide underscores the importance of a flexible cyclic system to receptor association. A more flexible disulfide bridge in the bis-homocysteine peptide provides more freedom for the peptide chain to adopt the desired helical conformation in the presence of the receptor. This additional freedom provides favored association with the receptor, albeit at the expense of selectivity. The case of the bis-penicillamine peptide is oppo-

  

site; additional constraints from β methylene units in this peptide make the peptide bind more tightly to the ERα versus ERβ, albeit with a higher $K_i$.

Several reports have utilized strategies of changing the leucines of the NR box or altering the amino acids adjacent to the N and C termini of the NR box for improving selectivity of such peptides towards the NRs. Our series of homocysteine and penicillamines peptides demonstrate that, in addition to the amino acid substitution, the selectivity of NR box-containing peptides can be significantly altered by changing the conformational features of the LXXLL-containing peptide chain.

Recently, we reported the X-ray crystal structure of PERM-1, a helix-stabilized, disulfide-bridged peptide and a potent inhibitor of ER–CoA interactions.[12] The conserved NR box motif of the disulfide-constrained PERM-1 interacts with a shallow CoA-binding groove of ERα. The side chain of $L^1$ is deeply embedded within the groove and forms van der Waals contacts with the side chains of Ile358, Val376, Leu379, Glu380, Leu539, Glu542, and Met543 of the receptor. The side chain of $L^3$ is also buried into the hydrophobic surface and makes van der Waals contacts with the side chains of Ala361, Ile358, Leu372, Gln375, Val376, Leu379, and Lys362 of the receptor. Figure 3 (left) shows the contacts of $L^3$ in a 2.4 Å radius.

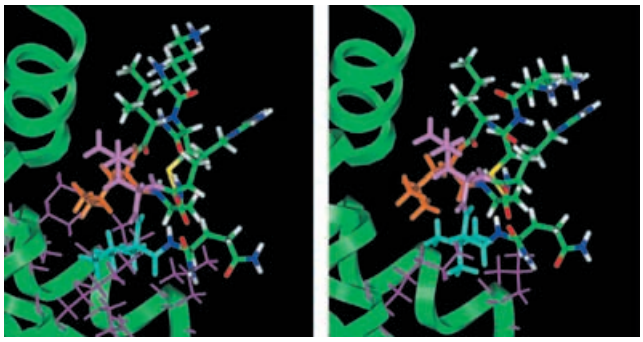

**Figure 3.** Association of PERM-1 (left) and peptide **18** (right) with the CoA-binding hydrophobic surface of the ER (green ribbon) based on the X-ray crystal structure of ERα with PERM-1.[12] $L^1$, $L^2$, and $L^3$ of the NR box $L^1XXL^2L^3$ are shown in orange, pink, and blue, respectively. Left) Five hydrophobic residues (dark pink) of the receptor make vdW contacts with $L^3$ (blue) of PERM-1. Right) This is compared with peptide **18**, here $L^3$ is substituted with t-Leu, which makes hydrophobic contacts with only two amino acids of the ER receptor.

In our SAR studies we observed a substantial decrease in the bioactivity of peptide **18**, in which $L^3$ had been substituted with *t*-Leu. Tertiary leucine has a bulkier but shorter side chain than leucine. Hence, we postulated that the loss of bioactivity is probably due to fewer hydrophobic contacts with the receptor. In our modeling studies, by positioning the *t*-Leu at the $L^3$ position in the crystal structure of PERM-1, we found that three important van der Waals contacts, Val376, Leu379, and Ile358, with the CoA-binding grove of the receptor were eliminated. This is indicated in Figure 3 (right). Elimination of these nonpolar contacts would contribute toward the weaker association and hence the higher $K_i$ of peptide **18**. Similar analysis indicates that substitution of $L^2$ with *t*-Leu does not eliminate any van der Waals contacts and hence peptide **17** is equipotent with PERM-2. Substitution of $L^1$ with tertiary leucine allows an additional contact with Leu539 of the receptor and hence peptide **16** is more potent.

We also synthesized and assayed a similar series using neopentyl glycine as an unnatural leucine surrogate. Neopentyl glycine has a side chain that is the same length as that of leucine but bulkier. As anticipated, we observed substantially greater affinities for the peptides in this series. However the bioactivity of peptide **20** is outstanding. In this peptide with pM potency, $L^2$ of the NR box is substituted with neopentyl glycine. In contrast to the side chains of $L^1$ and $L^3$, the side chain of $L^2$ is only partially associated with the CoA-binding groove and consequently makes fewer hydrophobic contacts. In the crystal structure of PERM-1,[12] the side chain of $L^2$ makes van der Waals contacts with Val355, Ile358, Lys362, and Leu359 of the receptor. And in spite of its weaker association with the receptor, substitution of $L^2$ with neopentyl glycine substantially increases affinity (peptide **20**).

Neopentyl glycine does not have a longer side chain than leucine. Thus, it is difficult to imagine that this substitution would make any additional van der Waals contacts deeper within the CoA binding groove on the receptor. Moreover, as the side chain of $L^2$ is not orientated toward the hydrophobic surface of the CoA-binding groove, it is unlikely that Npg will establish any additional hydrophobic contacts with the receptor. However, in the crystal structure of PERM-1, we observed that the $L^2$ side chain is in proximity to the Ile residue of PERM-1. Isoleucine immediately precedes $L^1$ and, in the helical conformation of PERM-1, it comes into close contact with $L^2$. In PERM-1, isoleucine and $L^2$ are separated by three amino acids. Thus, if Ile is designated the *i*th amino acid, $L^2$ becomes the *i*+4th amino acid. In the helical conformation of PERM-1, these *i* and *i*+4 residues do not make van der Waals contacts. However, from the modeling studies on the crystal structure of PERM-1, we found that substitution of $L^2$ with neopentyl glycine increases the side chain hydrophobic bulk and consequently establishes new van der Waals contacts with the isoleucine residue (Figure 4). This intramolecular hydrophobic contact between *i* and *i*+4 residues is believed to contribute to the stability of the desired helical conformation. In fact, covalent *i*, *i*+4 side-chain cyclization has been routinely used for constraining short peptides in helical conformation.[16,17,24]

The significant improvement in the potency of peptide **20** can be attributed to the observation that neopentyl glycine noncovalently assists the peptide chain in adopting the desired helical conformation by establishing van der Waals contact with side chain of the isoleucine residue. These data taken together indicate that the $L^1$ and $L^3$ residues of the NR box $L^1XXL^2L^3$ are more important for receptor association, while $L^2$ is more important for stabilizing the peptide conformation. Thus, all the three leucine of the NR box are not equally important for receptor association. We believe that the next generation of NR box-based peptidomimetics can be designed by incorporating only $L^1$ and $L^3$ and by substituting $L^2$ with a stable and partially flexible conformational constraint. Further conformational studies on such bicyclic peptidomimetics should

  

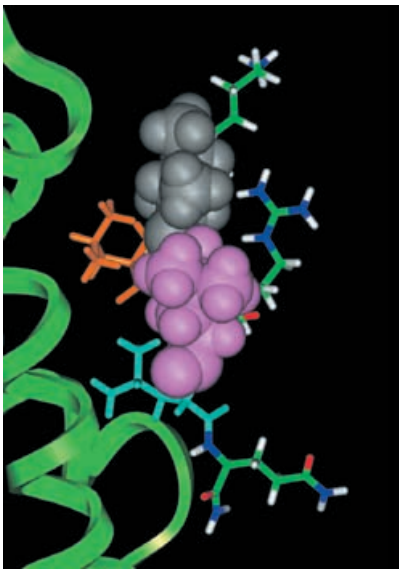

**Figure 4.** Model of association of peptide **20** with the CoA-binding hydrophobic surface of the estrogen receptor (green ribbon). $L^1$ is orange, $L^2$ is pink (space-filled), $L^3$ is blue, and Ile of the peptide chain is gray (space-filled). Npg, when substituted for $L^2$ of PERM-1, establishes a new vdW contact with Ile of the peptide chain. This *i*, *i*+4 noncovalent side-chain interaction between Ile and $L^2$ of the peptide chain (shown with the space filling model) is believed to contribute significantly to the picomolar binding affinity of peptide **20**.

reveal the templates for designing small-molecule inhibitors of this crucial protein–protein interaction.

The linear peptides reported in this paper were designed based on their cyclic counterparts. This series of monocysteine linear peptides showed $K_i$ values that are comparable to those of cyclic peptides. We further investigated the contribution of free sulfhydryl (SH) groups toward the high affinity of these monocysteine peptides. One possibility is that there might be a covalent modification of the receptor by the free thiol group.[28] We explored this possibility by performing an additional coactivator recruitment assay[29] containing 1 mM dithiothreitol (DTT) in the binding buffer. In the presence of a strong reducing agent such as DTT, all the monocysteine peptides showed a substantial decrease in their affinity for the receptor, while all other peptides maintained similar affinities. The assay utilized to characterize the SARs in Table 1 does not contain a reducing agent, and, hence, the disulfide linkage between the receptor and peptide ligand can remain intact. In the presence of a reducing agent, however, the lost affinity of monocysteine peptide ligands reflects reduction of the disulfide linkage. This supports the possibility that the high affinity of monocysteine peptides is due to the covalent derivatization of the receptor. Work by Hegy et al.[30] on the degree of solvent and reagent accessibility of the cysteines in the LBD of the human ER suggests cysteines 381, 417, and 530 to be the likely candidates for such covalent derivatization.

The monocysteine peptides showed $K_i$ values in a narrow range of 15–58 nM and in our SAR studies they showed lower sensitivity to the effect of amino acid substitution than the cyclic peptides. However, the changes in the $K_i$ values of these peptides did follow the trends established from the SAR studies of our cyclic peptides. We believe that a cocrystal structure of a monocysteine peptide bound to the receptor can unambiguously establish the exact position of this covalent derivatization.

Several important concepts have emerged from this SAR study that help us better understand the LXXLL-based protein–protein interaction. We have realized that the hydrophobic surface of the CoA-binding groove is a strong helix inducer. Even the small and unconstrained linear peptides displayed nanomolar affinity. However, the helicity of cyclic as well as linear peptides in solution (20% TFE) does not directly correlate with their bioactivity. This is probably due to the fact that properties other than the helical content of a peptide, such as side-chain packing of the leucine residues, are also factors. Using the unnatural leucine surrogates, tertiary leucine and neopentyl glycine, we found that the three leucines of LXXLL are not equally important. In addition to helicity and side-chain packing, two other important factors influence the affinity and selectivity of these peptide ligands: flexibility of the NR box and the amino acid residues in the immediate proximity of the NR box. A cyclic peptide that strongly constrains the NR box (bis-penicillamine) is the most selective, while a cyclic peptide that has the most flexible NR box (bis-homocysteine) is the least selective—even though it retains high affinity. Thus, cyclization and, hence, the conformational constraint can be exploited for both selectivity and the affinity for ER isoforms. In conclusion, our continued SAR analysis of LXXLL-based peptide inhibitors of ERα–CoA interactions yielded subnanomolar affinity and 40-fold selectivity against ERβ. In addition, the novel and unexpected observations within the SARs mentioned above will aid in the further development of potent and selective therapeutics targeted toward LXXLL-based protein–protein interactions.

## Experimental Section

**Peptide synthesis**: Peptides were synthesized by using Boc solid-phase procedures with an automated peptide synthesizer (Apex 396, Advanced ChemTech, Louisville, KY). 4-Methylbenzhydrylamine resin (MBHA; substitution level of 1.2 mmol $g^{-1}$; Peptides International, Louisville, KY) was shaken overnight with dichloromethane (DCM) and *N*,*N*-diisopropylethylamine (DIEA; 1:9) in an Isolute SPE filtration column (Biotage, Charlottesville, VA). The next day, the resin was washed with DCM (3×), methanol (2×), with *N*,*N*-dimethylformamide (DMF; 3×), and DCM (3×), then dried and stored in airtight vials. Dry treated resin (no more than 50 mg) was placed in a 96-well reaction vessel of the Apex 396. Synthesis was carried out by following the user-generated CHEM-files for the five major steps involved in Boc solid-phase peptide synthesis: 1) swelling of the resin, 2) coupling of the amino acid, 3) washing after coupling, 4) deprotection of the Boc group, and 5) washing after deprotection. These cycles were repeated depending on the number of amino acids in the peptide designed. Almost quantitative yields were observed for the coupling reactions due to the use of excess quantities of reagents (3 equiv), double couplings, high-speed (600 rpm) shaking, and the inert atmosphere. After completion of synthesis, peptide-loaded resin was removed from the reaction vessel, dried, and stored in airtight vials.

  

Peptides were cleaved from the resin by using a hydrogen fluoride cleavage apparatus equipped with a HF-resistant Teflon-Kel-F vacuum line. The required amount of peptide-loaded resin (100 mg–1 g) was placed in Teflon tubes containing a small magnetic stir bar and a sufficient amount of anisole (e.g. 250–500 μL for 500 mg resin). Tubes were fitted to the main apparatus, chilled under acetone and dry ice for 20 min, and then HF (10 mL $g^{-1}$ of the resin) was condensed in the tubes under vacuum. The mixture was cooled by immersing the tubes in ice, and stirring was continued for 1–2 h. HF was evaporated under vacuum to obtain the dry resin and the cleaved peptide in the tubes. After evaporation was complete, soluble peptide was precipitated with cold, anhydrous diethyl ether. The resin was washed three times with ether to remove impurities. The peptide was then solubilized in 20% aqueous acetic acid and lyophilized. All peptides were obtained with 74–78% yields as a white powder. Linear peptides were cyclized as described previously[19] and purified further by using solid-phase extraction columns.[13]

**Time-resolved fluorescence-based coactivator interaction assay**: This assay was performed as previously described.[20] Briefly, white 96-well plates were coated overnight with either full-length recombinant baculovirus-expressed human ERα or ERβ (PanVera, Madison, WI). Protein-coated plates were washed (5×), then blocked for at least 1 h, followed by thorough washing. A NR box peptide–europium conjugate was prepared by incubating the biotin-labeled NR box peptide with Eu-labeled streptavidin on ice. The 96-well plate coated with ER was then incubated for at least 1.5 h with a NR box peptide–Eu conjugate in the presence of 17β-estradiol (Sigma, St. Louis, MO) and the competitor peptide. The NR box peptide used for ERα was the SRC-1 NR box 2 peptide (LTERHKILHRLLQEGSPSD), while the SRC-1 NR box 4 peptide (QAQQKSLLQQLLTE) was used for ERβ experiments. The dissociation constants for both of these CoA peptides for E2-bound ERα or ERβ were determined previously to be $155 \pm 21$ and $261 \pm 72$ nM, respectively. Plates were washed (5×), followed by incubation with gentle shaking in the presence of enhancement solution for 5 min; this allowed release of the bound Eu label. Plates were read in a Wallac Victor II plate reader by using a Europium-label-specific protocol (PerkinElmer Wallac, Inc.). Assays were performed a minimum of three times, and $K_i$ values were determined from the Cheng–Prusoff equation by utilizing GraphPad Prism® software.

**Molecular modeling**: A series of *t*-Leu- and Npg-containing peptides was modeled by using Macromodel 7.0.[21] We utilized a previously published crystal structure[12] in which PERM-1 was used as a template for substituting leucines of the NR box with the leucine surrogates, *t*-Leu and Npg. Van der Waals contacts in a 2.4 Å radius were recorded for peptides **16**–**21** and compared with those of PERM-1.

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**Keywords:** ab initio calculations · coactivator · gene regulation · peptide nucleic acids · steroid receptors · transcription

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