An Inhibitor of the Interaction of Thyroid Hormone Receptor β and Glucocorticoid Interacting Protein 1

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The thyroid hormone receptors (TR's), members of the nuclear receptor (NR) superfamily,[1] are crucial for normal development and for regulation of metabolism in the adult.[2] The TR's integrate multiple input signals including tissue and developmental context and hormonal levels to produce distinct patterns of gene expression.[3] The TR's are attractive therapeutic targets in cardiovascular disease.[4] Herein we report a novel approach to chemical antagonism of thyroid hormone signaling.

The TR's exert their effects by dynamic regulation of the formation of multiprotein complexes that include coactivators, co-repressors, and the basal transcription machinery.[5] The TR conformation adopted upon binding of thyroid hormone (T_3) allows the binding of coactivating proteins, while that adopted by the unliganded receptor prevents coactivator binding and allows for corepressor binding.[5b,6] Genetic analysis of NR coactivator proteins revealed a highly conserved motif (LXXLL), termed the NR box, that mediates the interaction of NR's and coactivators.[6] Coactivators often have multiple NR boxes of differing affinity that can exhibit either cooperative[7,8] or noncooperative[9a] binding. X-ray crystallographic studies of the complex of T_3, the human thyroid receptor β isoform (hTRβ), and the coactivator Glucocorticoid Receptor Interacting Protein 1 (GRIP1) (T_3·hTRβ·GRIP1) revealed that the NR box binds to the receptor as an amphipathic α-helix.[9] Biochemical studies of T_3·hTRβ·GRIP1 complex formation revealed that the second NR box of GRIP1 had the highest affinity for T_3·hTRβ, suggested that binding involved an induced fit of the NR box helix, and indicated that there was no apparent cooperativity in NR box binding.[9a,10,11] We demonstrate below that α-helical peptidomimetics of the second NR box of GRIP1 strongly inhibit T_3·hTRβ·GRIP1 complex formation.

In previous studies, macrolactam constrained peptides have been used to inhibit protein-protein interactions.[12] In this study, a series of macrolactam constrained GRIP 1 NR box peptides (Table 1) were synthesized by solid-phase peptide synthesis by using the Fmoc strategy, with orthogonal protection of the relevant lactam precursor side chains, followed by on-resin formation of the macrolactam.[13] The compounds synthesized included variation in the location, length, and orientation of the lactam ring.

Table 1. Sequence and Observed Ellipticities of Cyclic Peptides

	Compound	$[\Theta]_{197}/-[\Theta]_{222}$	$[\Theta]_{222}/-[\Theta]_{208}$
	Ideal Helix	2.63	1.09
1	NH2-^{685}EKHKILHRLLQDS^{697}-COOH	-1.91	0.47
2	(OregonGreen488)-EKHKILHRLLQDS-COOH	NA	NA
3	Ac-EHHKIKLRLQLDS-COOH	-4.09	0.37
4	c(D^{688}-K^{692})D^{691}-K^{695})Ac-EKHDILDKLLKDS-COOH	-0.53	0.71
5	c(K^{691}-D^{695})Ac-EKHKILKRLLDDS-COOH	-1.81	0.47
6	c(D^{691}-K^{695})Ac-EKHKILDRLLKDS-COOH	0.20	0.47
7	c(D^{688}-K^{692})Ac-EKHDILHKLLQDS-COOH	-0.53	0.71
8	c(K^{688}-D^{692})Ac-EKHKILHDLLQDS-COOH	-0.20	0.81
9	c(E^{691}-K^{695})Ac-EKHKILERLLKDS-COOH	1.41	0.70

The relative numbering scheme for GRIP1 amino acid residues is indicated on peptide **1**. The notation c(X−Y) implies formation of a macrolactam between the side chains at the indicated positions. The NR box leucines are underlined. Linear probe and control peptides are above the bold line; constrained peptides are below the bold line.

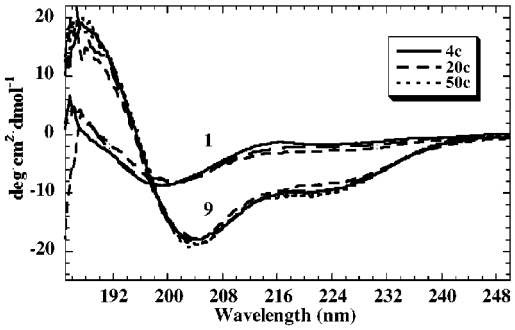

Figure 1. Temperature dependence of circular dichroism spectra of the unconstrained peptide **1** and constrained peptide **9**. Spectra were acquired by scanning solutions of **1** and **9** (50 μM in 20% ACN in 50 mM Tris, pH 8.0) at various temperatures. Mean residue ellipticity (Θ) reported in deg·cm^2·dmol^{-1}.

Solution-phase circular dichroism (CD) analysis of these peptides revealed partial induction of α-helical character in one, **9**, but little to no induction of helical character in the other compounds (Table 1). NOESY NMR experiments with **9** revealed four amide proton resonances that show amide·amide (i, i + 1) cross-peaks characteristic for peptide amides in an α-helical fold with COSY coupling constants less than 5.0 Hz ($J_{HN-HC\alpha}$), well within the range normally exhibited in α-helical segments of proteins. These data imply either that peptide **9** has a helical conformation for 30−40% of its length or that populations of energetically similar conformations are interconverting rapidly. The structures of the CD spectra of unconstrained peptide **1** and constrained peptide **9** were independent of the temperature over the range of 4−50 °C (Figure 1) supporting the former model. The combination of these findings suggests that the constraint in **9** strongly biases conformational equilibria toward an α-helical structure within the portion of the peptide enveloped by the lactam.

The ability of the constrained peptides to displace native GRIP1 peptide from T_3·hTRβ was assessed by using fluorescence polarization (FP) equilibrium competition assays (Figure 2). Control experiments indicated that binding of probe **2** was

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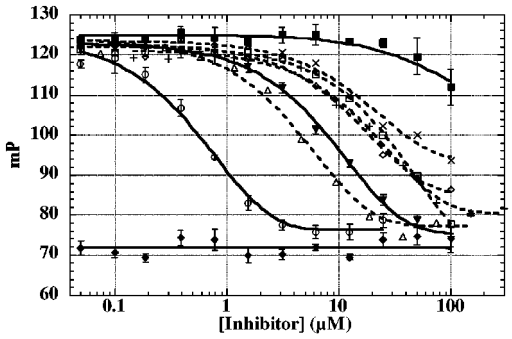

Figure 2. Inhibition of GRIP1 binding to hTRβ by constrained GRIP1. Analogues as determined by fluorescence polarization competition experiments using an Oregon Green 488 labeled GRIP1 peptide **2**; (◆) no T3-probe binding is dependent upon the presence of T_3 ligand; (▼) unconstrained GRIP1 peptide **1** specifically blocks binding of **2**, IC_{50} of 9.6 ± 0.9 μM; (■) control peptide **3** is unable to block binding of **2**; constrained peptides block binding of **2**: (+) **4**, IC_{50} of 21.8 ± 4.0 μM; (△) **5**, IC_{50} of 5.8 ± 0.7 μM; (×) **6**, IC_{50} of 22.1 ± 2.2 μM; (◇) **7**, IC_{50} of 9.1 ± 2.2 μM; (□) **8**, IC_{50} of 20.4 ± 8.6 μM; (○) **9**, IC_{50} of 0.62 ± 0.09 μM. [**2**] was constant at 10 nM, [hTRβ] 1 μM, [T3] 10 μM. Binding buffer: 20 mM Tris HCl at pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM DTT, 0.01% NP-40, and 1 mM EDTA.

dependent upon the presence of T_3 (◆) and that competition for binding to hTRβ was exhibited by unconstrained peptide **1** (▼) but not by control peptide **3,** which has a sequence scrambled NR box (■). While all of the constrained GRIP 1 analogues could successfully compete for binding to hTRβ most did so with affinities worse than or no better than the unconstrained peptide. There were no apparent trends relating the location, length, or orientation of the lactam moiety to relative degree of competitive ability. Strikingly, **9** (○), the single constrained peptide that exhibited substantial helical character, exhibited an IC_{50} 15-fold lower than the unconstrained peptide **1** (▼). The change in inhibitory constant exhibited by **9** indicates that formation of the helix during binding induces a 1.5 kcal/mol cost in free energy of binding. The magnitude of this effect is equivalent with that seen in studies of the yeast transcription factor GCN4 when similar constraints were applied.[14] This study demonstrates that constraint of the NR box to an α-helical structure strongly enhances the affinity of the NR box for the receptor.

The ability of constrained peptide **9** to compete with intact GRIP1 nuclear receptor interaction domain (GRIP1 NID), which contains all three GRIP1 NR boxes, was tested using a semi-quantitative glutathione-*S*-transferase assay. Control experiments indicated that the GRIP1 NID bound to hTRβ in the presence of T_3 (lane 3) and failed to bind in the absence of T_3 (lane 2). This interaction was blocked by unconstrained peptide **1** at high concentration (lane 5) but not at low concentration (lane 4) and was not blocked by control peptide **3** (lane 6). Constrained peptide **9** efficiently blocked the binding of hTRβ to the GRIP1 NID in a dose-dependent manner (lanes 7−10). Although the assay does not allow for exact determination of IC_{50} values, the relative

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Lane	1	2	3	4	5	6		7	8	9	10
^{35}S-hTRβ	+	+	+	+	+	+		+	+	+	+
GST-GRIP1		+	+	+	+	+		+	+	+	+
T3			+	+	+	+		+	+	+	+
1 (μM)				0.01	100						
3 (μM)						100					
9 (μM)								0.01	0.1	1.0	10.0

Figure 3. Inhibition of GRIP1 NID protein binding to hTRβ by **9.** Lanes: (1) ^{35}S-hTRβ alone; (2) no T3 hormone, ^{35}S-hTRβ binding is ligand dependent; (3) no competitor maximal binding of ^{35}S-hTRβ to GRIP 1 NID domain; (4) 0.01 μM **1**; (5) 100 μM peptide **1** will compete for binding to hTRβ; (6) 100μM **3** showed no competition for binding to hTRβ; (7−10) increasing concentration of **9** conformational constraint increases competitive ability for binding to ^{35}S-hTRβ. ^{35}S-hTRβ was in vitro expressed with ^{35}S-methionine. Recombinant fusion of glutathione-*S*-transferase and hGRIP1 (GST-GRIP1$_{(563-767-H6)}$) bound to glutathione agarose beads was exposed to ^{35}S-met-hTRβ in the presence and absence of inhibitor. Binding buffer was 20 mM Tris HCl at pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM DTT, 0.01% NP-40, and 1 mM EDTA.

potency of **9** to **1** was qualitatively in the same range as that observed in the FP studies. Additionally, the interaction was completely blocked by 10 μM **9** whereas the unconstrained peptide never reached this level of saturation, even with 100 μM concentrations. This study indicates that the constrained peptide **9** can block the interaction of intact receptor and coactivator proteins.

Our results suggest that the formation of a Glu-to-Lys macrolactam *c*(E^{691}-K^{695}) in the second NR box of GRIP1 induces a partial α-helical conformation. This conformational constraint allows **9** to compete for the NR box binding site of hTRβ with a 15-fold decrease in IC_{50} relative to GRIP1 thus demonstrating that preforming an α-helical conformation in the NR box leads to stronger interaction between the coactivator and the receptor. The poor competitive abilities of other constrained NR box peptides are most likely due to the lack of α-helicity as indicated by solution CD, thus confirming the requirement for an α-helical conformation in the NR box during binding to the nuclear receptor. The ability to block formation of the T_3·hTRβ·GRIP1 complex should allow functional antagonism of thyroid hormone signaling. Thus, the results of this study suggest that this interface is an appropriate target for the development of protein−protein inhibitors as novel thyroid hormone receptor antagonists.

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Supporting Information Available: Descriptions and results of the synthesis and characterization of the of peptides **1**−**9**, preparation of the allyl protected side chains, circular dichroism, fluorescence polarization, GST pull-down competitions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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