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Novel Selective Inhibitors of the Interaction of Individual Nuclear Hormone Receptors with a Mutually Shared Steroid Receptor Coactivator 2

Timothy R. Geistlinger and R. Kiplin Guy*

Departments of Pharmaceutical Chemistry and Cellular and Molecular Pharmacology, University of California at San Francisco, Genentech Hall, Mission Bay, 600 16th Street 2280, San Francisco, California 94143-2280

Received February 24, 2003; E-mail: rguy@cgl.ucsf.edu

The nuclear hormone receptor transcription factors (NRs) respond directly to small-molecule ligands and control diverse functions in vivo, including development and dynamic homeostasis.[1,2] Both metabolic diseases and cancer have been directly correlated with the misregulation of signaling by the NRs, and they are the target for multiple drugs and drug development programs.[3] Ligand-dependent NR signaling requires direct interaction between NRs and the steroid receptor coactivators (SRCs), effected by a conserved SRC motif (NR box, $L_1XXL_2L_3$).[1] We have previously described potent but nonselective inhibitors of the interaction of NRs and SRCs. Using computational and parallel synthetic techniques, we have produced a library of novel proteomimetics of the second NR box of SRC2 (SRC2-2) that exploit structural differences between the thyroid hormone receptor (TR) and two isoforms of the estrogen receptor (ER) to selectively inhibit the interaction of SRC2-2 with human estrogen receptor α (hERα), human estrogen receptor *β* (hER*β*), and human thyroid hormone receptor *β* (hTR*β*) individually.

Structurally, hERα and hTR*β* interact with the SRC2-2 peptide through similar surfaces, with a shallow hydrophobic groove on the surface binding to an amphipathic α-helical motif on the SRC2-2, burying the three leucines on the hydrophobic face of the NR box helix.[4,5] To explore the possibility of discovering coactivator binding inhibitors selective to particular NR·ligand pairs, we designed a library of α-helical proteomimetics, constrained by a macrolactam at positions E^{691} and K^{695}, that mimic SRC2-2 {c(E^{691}-K^{695})Ac-^{685}EKHKI**L$_1$**ER**L$_2$L$_3$**KDS^{697}-COOH} (**1**{**37,37,37**}, Figure 1A) with non-natural amino acids (Figure 1B) replacing the leucines at positions **L$_1$**, **L$_2$**, and **L$_3$**. The selection of particular non-natural side chains for this library was governed by computational DOCKing (CombiDOCK)[6] methods using hERα·E2·SRC2-2 and hTR*β*·SRC2-2·T_3 X-ray crystal structures. Hydrophobic, non-natural amino acids in the Available Chemical Directory were substituted in silico for each leucine by replacing the Cα and C*β* atom positions of the crystallographic SRC2-2 peptide with those of the introduced side chains. The resulting molecules were screened in silico against the coactivator binding surfaces of the receptors and reduced to a set of 45 diversity elements (Figure 1B) at each position for library synthesis. Computationally, the NRs selected for similar residues at each position, except that hERα tolerated larger and Cα-branched residues (Figure 1B, **24**, **25**, **27**−**34**). Studies were carried out in which each leucine was replaced individually without changing any other amino acids and in which all three leucines were simultaneously varied. As the CombiDOCK studies did not reveal significant cooperativity between the diversity positions, the targeted library was limited to a single substitution in each member.

The designed library was synthesized in parallel on solid support utilizing Fmoc chemistry as previously described for constrained SRC2-2 mimetic **1**{**37,37,37**}.[7] In general, the synthesis proceeded smoothly to yield milligram quantities of each compound after RP-HPLC purification. Many of the Cα-branched compounds proved to be more difficult to synthesize and purify, particularly during macrolactam formation. The final proteomimetic library contained 87 of 111 targeted compounds composed of 25, 30, and 32 individual substitutions, respectively, at positions **L$_1$**, **L$_2$**, and **L$_3$** (Figure 1C). The purity and identity of all compounds were confirmed by LCMS and HR-MALDI-TOF MS, respectively.

The ability of the proteomimetics to compete with SRC2-2 peptide for binding to the NR was assessed using fluorescence polarization (FP) equilibrium competition assays.[7] These studies revealed that the library members exhibited a large range of inhibitory ability for blocking the binding of the SRC2-2 peptide to the NR (Figure 1C). As hypothesized from the DOCKing studies, the majority of phenylglycine and phenylalanine analogues allowed for effective competition, with 71 of 87 compounds giving IC_{50}'s that were equivalent to or better than those of the SRC2-2 peptide with one or more NR. The high level of success in picking compounds with competitive ability validates the use of the CombiDOCK methodology for the design of inhibitors of protein−protein interactions in addition to its conventional use in design of enzyme inhibitors.

The testing of the inhibitors identified the first selective proteomimetics (Figure 1D) that take advantage of differences between the leucine binding pockets of E_2·hERα, E_2·hER*β*, and T_3·hTR*β*. A total of 12 compounds were at least 10-fold selective for binding to hERα in preference to hTR*β* or hER*β* (Figure 1D). Of these, a number were more than 20-fold selective, and one, **1**{**34,37,37**}, was more than 600-fold selective for hERα. Surprisingly, only one compound, **1**{**3,37,37**}, was selective for hTR*β* and one, **1**{**37,14,37**}, for hER*β*. Strikingly, two natural amino acids, tryptophan and isoleucine, provide high levels of selectivity for hERα. Previous studies using genetic selection from random peptide libraries failed to reveal this trend, instead selecting exclusively for leucines within the $L_1XXL_2L_3$ motif while evolving differences in flanking sequence.[8]

The SRC binding pockets of NR have evolved to bind to a simple hydrophobic $L_1XXL_2L_3$ consensus motif while relying upon differences in SRC structure flanking the NR box to convey selectivity in vivo. This study reveals that the SRC binding pockets contain significant differences in shape and electrostatics that allow competitive inhibitors that mimic the NR box to act selectively on one NR. The fact that discrimination between NRs can be achieved solely by manipulation of the side chains inside the small $L_1XXL_2L_3$ motif implies that a suitable small molecule could achieve the same result. Thus, targeting this site may prove to be a general method for producing selective modulators of NR function.

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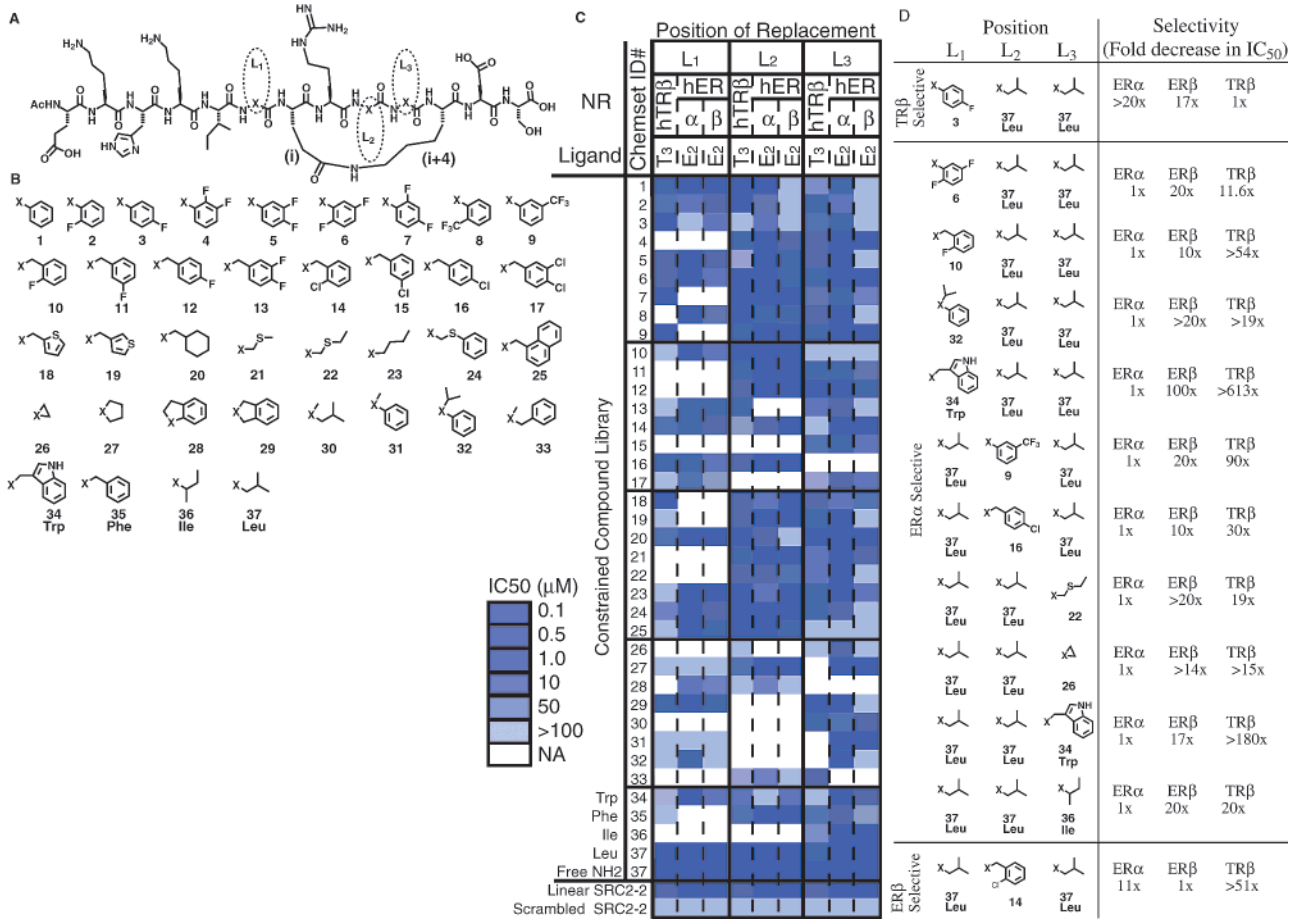

Figure 1. Design, synthesis, and assay of a library of proteomimetics of SRC2-2. (A) The structure of the proteomimetic library scaffold **1** with diversity positions indicated by $\mathbf{L_1}$, $\mathbf{L_2}$, and $\mathbf{L_3}$. **1**{**37,37,37**}: $\mathbf{L_1} = \mathbf{L_2} = \mathbf{L_3}$ = LEU. (B) Targeted diversity elements included in proteomimetic library as chosen by CombiDOCK. The library was produced by substitution of a single $\mathbf{L}_x$ for each member, with other positions being left as leucine. Amino acid side chains shown with the Cα designated as X. Superimposing the X on the scaffold with the X on the side chain reassembles individual library members. (C) Inhibitory activity and selectivity profile of the SRC2-2 proteomimetics. The equilibrium 50% inhibitory concentration (IC_{50}) is given for each library member for competition of the SRC2-2 peptide from each NR (hTRβ, hERα, or hERβ) as determined by in vitro fluorescence polarization assays. Activity is represented as a colorimetric scale, with light blue indicating IC_{50} > 100 μM, dark blue indicating IC_{50} < 100 nM, and gradations of color between the two indicating intermediate IC_{50} values. White boxes denote compounds whose synthesis was not achieved. Individual non-natural amino acids are arrayed on the *Y*-axis and numbered according to Figure 1A. The *X*-axis depicts the position of non-natural amino acid substitution ($\mathbf{L_1}$, $\mathbf{L_2}$, or $\mathbf{L_3}$) and the NR tested. (D) Structures of 12 library members that are specific to one NR due to individual side-chain substitutions at one position. Selectivity among the tested NRs is indicated on the right side, with the fold decrease in the IC_{50} relative to the IC_{50} against the receptor for which the compound is most selective.

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Supporting Information Available: Computational, synthetic, and analytic procedures; table of yields, identity, and purity of library members; examples of purity data and assay data; and table of IC_{50} values (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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