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14. ABSTRACT

The overall objective of this project is to characterize the detailed structural and dynamic response of the estrogen receptor ligand binding domain (ER-LBD) to a variety of ligands ranging from strong estrogens to strong antiestrogens using electron spin labeling. The first two technical aims for this reporting period involved completing preparation of site-directed spin-labeled mutants of the ER-LBD and completing synthesis new spin-labeled ligands for the proposed studies. These tasks have essentially been completed and led to the development of a new fluorescence and EPR-based ER ligand binding assay. Towards the third technical aim of the reporting period, we have completed several initial EPR studies of ligand-dependent dynamics as a function of position in the ER as well as a series of spin-spin distance measurements. The results clearly support our initial hypothesis that the physical response of the ER protein to different ligand types can be resolved and characterized by EPR spin-labeling.

15. SUBJECT TERMS

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2. Final Report

Introduction

This proposal focused on the key first steps in the estrogen response of breast cells, specifically the physical interaction between estrogen-like molecules and the ligand binding domain (LBD) of the ER. Although recent crystal structures of ER-LBD have implicated the C-terminal helix-12 (H12) of the ER in this response, X-ray analysis cannot characterize the dynamic behavior of H12 that is thought to play a major role in the tissue selectivity of the ER. By placing nitroxide spin labels at strategic points on the ER-LBD protein as well as on estrogenic ligands, we have mapped the dynamics and key distances in the complex over the entire range of ligand activities from estrogenic to antiestrogenic. This has afforded the first characterization of the ER response under near physiological conditions, which will significantly aid the design of partially selective estrogen modulators for breast cancer therapies.

Task 1 *Synthesize a novel series of estrogenic probes with a nitroxide reporter group substituted at the 17α position and a short alkyl substituent at the 11β position to control the probe's activity*

11β-substituted estrogen spin labels

Since the Year 2 report, this synthesis was accomplished as summarized in **Figure 1**. Relative binding affinities (vs. estradiol) for the precursor estrogen-linked azide and the final spin label are shown in **Table 2**. The results show that the 11β spin label has a relative minor effect on the binding affinity of the ligand, as predicted. **Figure 2** shows EPR spectra of the 11β spin label in solution (top) and bound to the ER (bottom). The bound compound exhibits broader lines characteristic of a partially immobilized

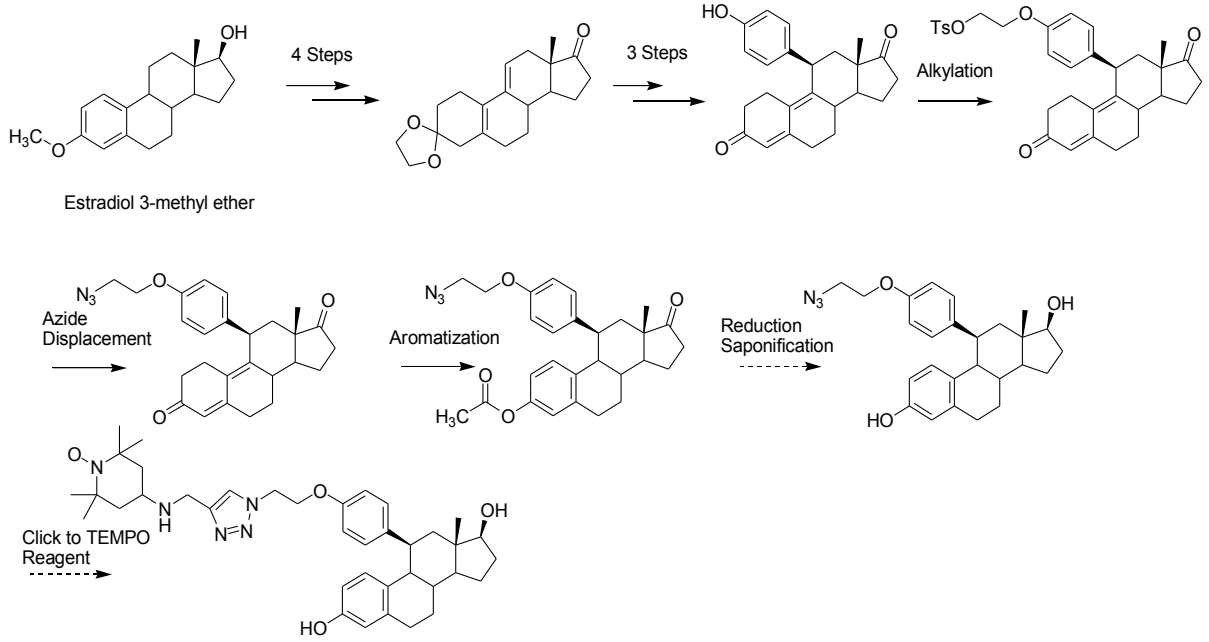
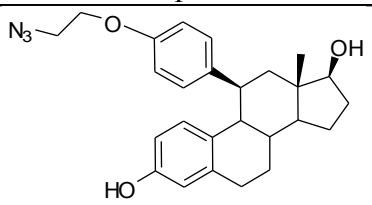
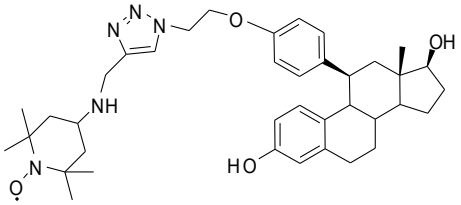


Figure 1: Outline of synthetic scheme towards 11β-substituted nitroxide-labeled estrogens

Table 2 Binding affinities of spin-labeled estradiol (bottom row) and precursor (top)

Compound	RBA ERα	RBA ERβ	β/α
	39.3 ± 9.0	33.7 ± 1.77	0.86
	4.5 ± 1.17	1.9 ± 0.50	0.43

compound, but retains mobility around an axis approximately along the N—O bond of the nitroxide, consistent with the geometry of the tether to the estradiol.

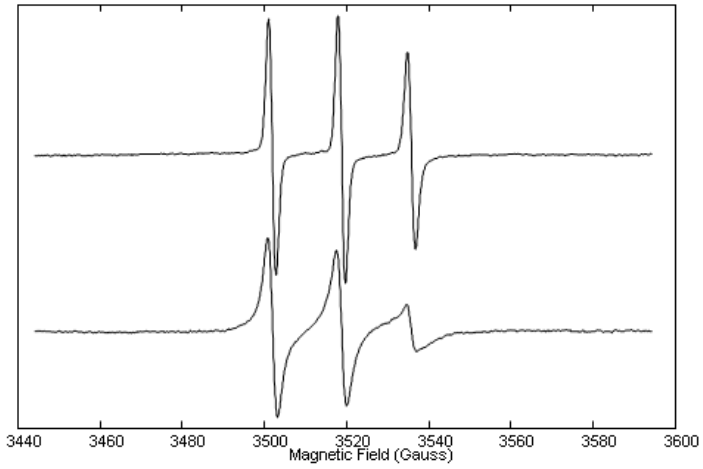


Figure 2 EPR spectra of 11β labeled estrogen in solution (top) and bound to the ER (bottom) showing a difference

Synthesis of 17α-substituted estrogen nitroxides

As noted in the Year 2 report, we observed that the originally proposed 17α-substituted estrogen nitroxides has significantly reduced relative binding affinity (RBA) or the relative stimulatory activity (RSA) with nitrogens in substituents at this position. Since nitrogen unexpectedly switches the compounds from being agonists to being inactive (not strictly antagonists) efforts to make nitroxide substitutions at this site were abandoned. However, the initial compounds did demonstrate unexpected utility for the establishment of a new

EPR-based binding assay for the series of estrogenlike compounds shown in **Figure 3**, reported in the next section.

Binding studies

Figure 4 shows a binding study holding the spin label concentration constant at 50 μM while varying the protein concentration. At higher protein concentrations the narrow three-line spectrum of the unbound probe is replaced by a broad signal of immobilized probe, demonstrating that the probe does bind to the receptor. A binding curve can be constructed by measuring the intensity of a line from the mobile species, as shown in **Figure 5** The dissociation constant K_d of HO2453 appears to be about 20 μM (compared to about 1-10 nM for estradiol).

We have discovered that the nitroxide quenches the fluorescence of two or three tryptophan residues that are in the estrogen binding pocket of the ERα protein. This is a good indication that the label is going into the actual ligand site of the protein, and also provides a very useful new and highly sensitive binding assay as an alternative to the standard radiolabel assay. **Figure 6** shows the fluorescence intensity of the sample as a function of estrogenic nitroxide ligand concentration, which gives a dissociation constant K_d that is quite close to that estimated from the EPR.

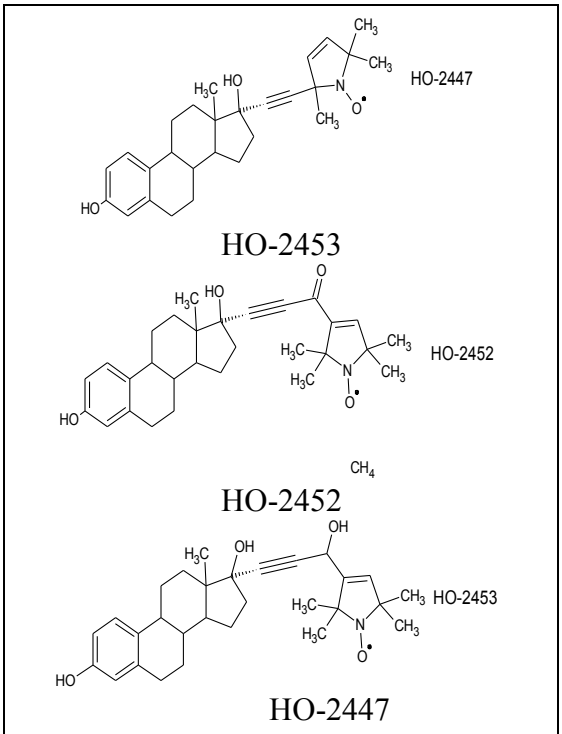


Figure 3. 17α nitroxide-labeled estradiols used in this study.

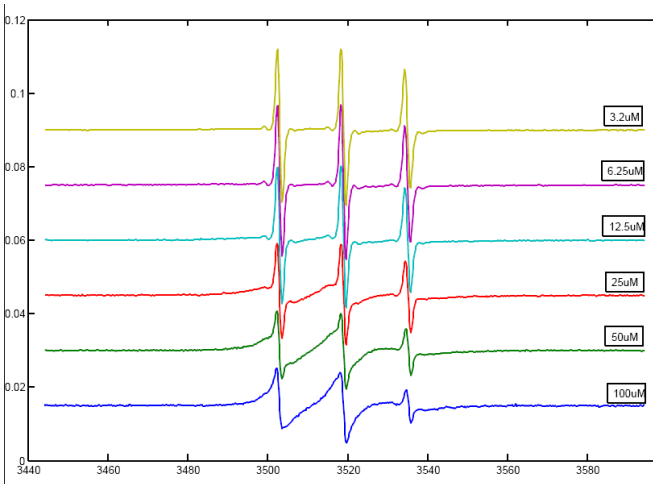


Figure 4: EPR binding study of HO-2453 probe to ERα-LBD. Probe concentration 50μM, protein concentration as indicated.

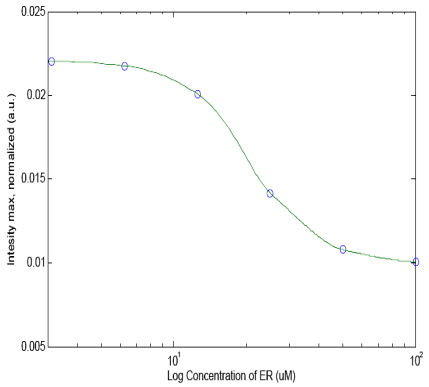


Figure 5: Intensity of low field hyperfine line as a function of ER concentration.

Additional complications were observed with the HO-2452 probe. Mass spectroscopic data indicates that this compound can form covalent attachments to the ERα, most likely via attachment of the alkynyl group to nucleophilic side chains.

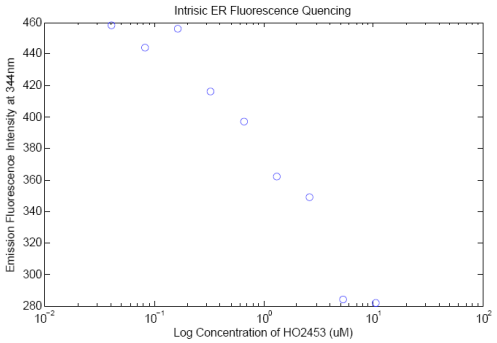


Figure 6: Fluorescence emission of ER at 344nm as a function of HO2453 concentration.

Task 2: *Generate a series of site-directed spin-labeled mutants of the estrogen receptor α isoform (ERα) with labeling sites near the putative flexible Helix 12 region*

As noted in the Year 2 report, the purification protocol for the ER-LBD was developed to afford high yield ER-LBD. Purity and activity were assayed by mass spectroscopy and estrogen binding assays (**Table3, Figure 7**). Teine in the Our mass spectroscopic studies of labeled ER revealed that site 447, a Cys residue buried in the interior of the ER that hat previously been reported to be inaccessible to labels, was in fact occasionally labeled by our nitroxides. We therefore constructed a mutant with this Cys mutated to Ser and repeated many of the spin label studies. Of the range of possible label sites along the H_{12} region of the protein identified in our original proposal, the mutants identified for subsequent studies included mutants M1, C381S/C417S/C447S/C530S/M543C labeled at C530 (in the H_{11}-H_{12} hinge) and M5 (C381S/C417S/C447S/M543C, labeled on both the H_{11}-H_{12} (C530 and C543 respectively) for distance measurements.

Table 3 Characterization of estrogen receptor mutants used in our study

Mutant	Construct	Observed mass (Target mass)	Binding affinity (% activity)
ER-543	C381S,C417S,C530S,M543C	30748.9 Da (30748 Da)	1.5 nM (68 %)
ER-530	C381S,C417S	30792.2 Da (30792 Da)	0.85 nM (62%)

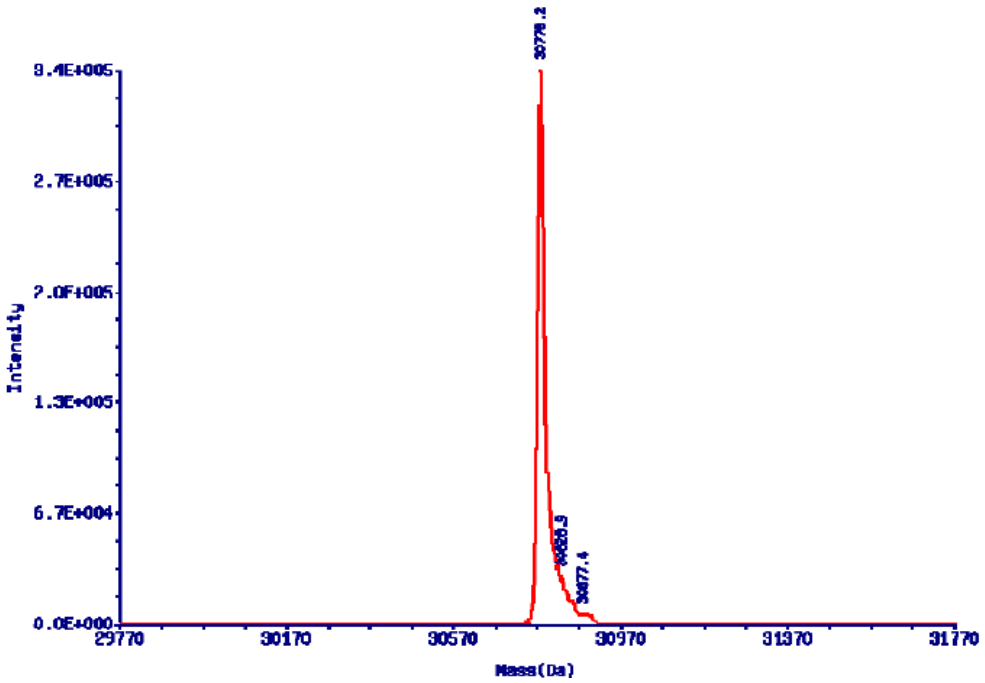


Figure 7 Mass spectrum of ER receptor (wild type) establishing purity of preparation.

Task 3: Characterization of ER structural response to agonists/antagonists by EPR spectroscopy

cw-EPR spectroscopy of singly labeled ER mobility

The specific ligands used in this study are summarized in **Figure 8. Figure 9** shows a completed cw-EPR study of the mobility at one location of the ER-LBD (label site 543) for a series of ligands ranging from estradiol to full antagonists. The splitting and sharpness of the outer peaks in the EPR spectrum reflect the degree of ordering at the label location, whereas the width of the central line ΔH_0 is a inversely related to the probe's mobility. The native agonist estradiol exhibits relatively low probe mobility, indicating a relatively fixed location of the H_{12} helix, whereas the helix becomes progressively more mobile in the presence of antagonists. This is the first experimental demonstration of the enhanced dynamics of this region induced by antagonists which had previously been inferred by X-ray crystallographic studies.

We extended our studies of the hinge region dynamics of the LBD (site 530) reported in Year 2 to include the effects of different coactivator peptide sequences (**Table 4**) bound to the ER. Although investigations of coactivators were not in our initially proposed work plan, they play a major role in determining the downstream effects of ligand interaction with the ER and its tissue specificity. During the course of our investigations we observed significant effects of coactivator binding on the ER dynamics, and were able to observe a dependence of the dynamic behavior of ER on the identitites of both the ligand and coactivator, summarized in **Figure 10**

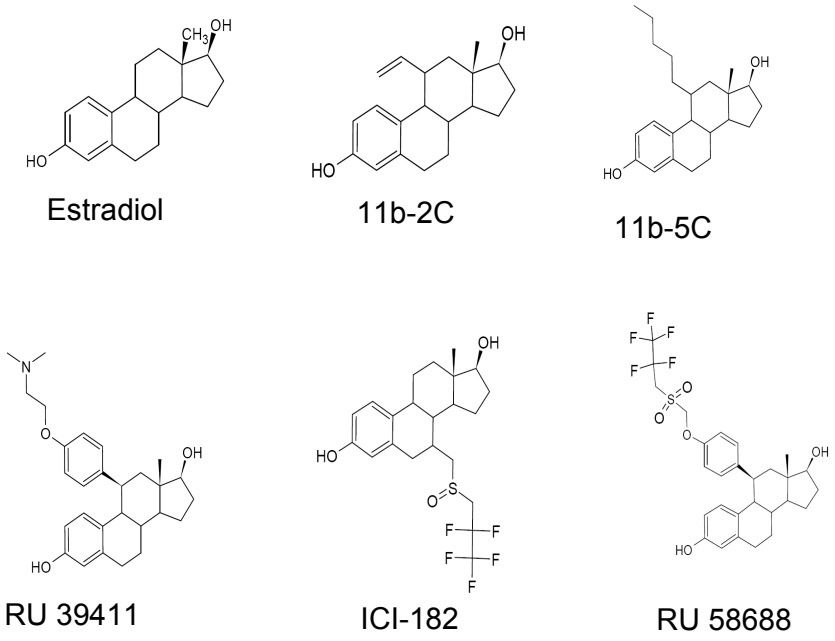


Figure 8 ER ligands used in single-label mobility studies in approximate order from antagonist (top left) to antagonist (bottom right)

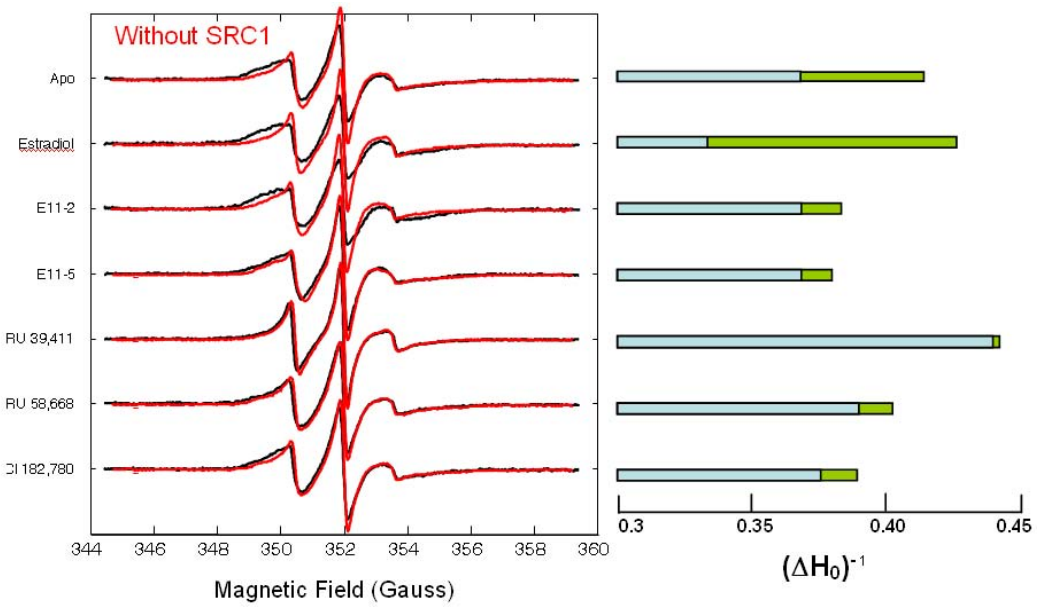


Figure 9: cwEPR study of mobility of Helix 12 for different ER ligands including agonists and antagonists

Table 4 Peptide sequences from coactivators known to bind the ER, with the "NR-box" sequence shown in boldface

SRC-1	L-T-E-R-H-K-I-**L-H-R-L-L**-Q-G
RIP-140	S-F-S-K-N-G-L-**L-S-R-L-L**-R-Q-N-Q-D-S-U
HnCOa-2/TIF2	E-K-H-K-I-**L-H-R-L-L**-Q-D-S
	L-P-Y-E-G-S-L-**L-L-K-L-L**-R-A-P-V-E-E-V

Figure 9 superimposes the series of spectra obtained for ER bound to a ligand series (experiments repeated from Year 2 with Cys 447 removed, shown in red) with a comparable series in the presence of one of the four coactivator sequences investigated. The coactivator produces a pronounced immobilization of the spectrum (shown in black); the degree of difference in the spectrum produced by the coactivator for each ligand is shown in the bar graph at right (blue bars show the reduction in mobility relative to the total length of each bar). Large effects are observed for the agonist ligands, whereas antagonists have a much smaller effect.

The different coactivator sequences are compared in **Figure 10**, which shows the degree of change caused by each coactivator in the presence of the different ligands. The degree of change caused by the coactivator was remarkably similar for all the coactivator sequences in the presence of both agonists and antagonists. However, for the intermediate partial agonist, E11-5, there was a significant variation among the coactivators. These results provide an initial indication that selective estrogen receptor modulators may confer some selectivity on the binding of the coactivator sequences based on the dynamics of the receptor itself.

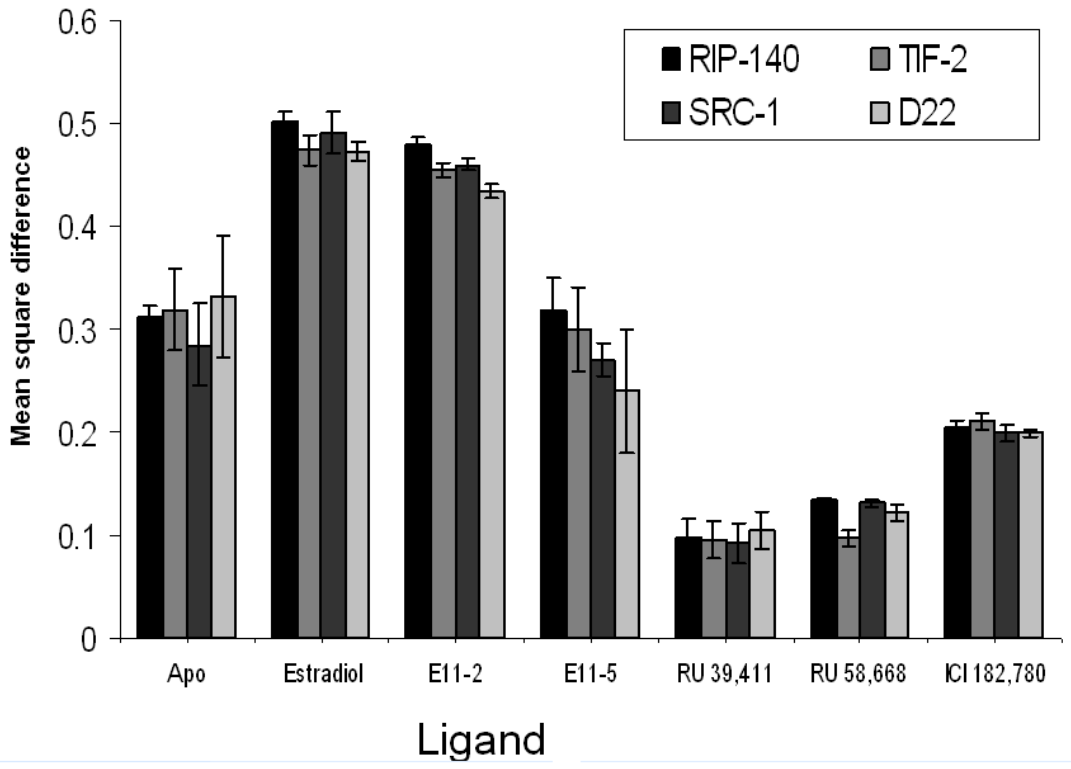


Figure 10 Relative effects of different coactivator peptide sequences (**Table 4**) on the EPR spectrum of ER labeled at residue 530 (H_{11}-H_{12} hinge) for different ligands ranging from agonist to antagonist (**Figure 8**).

Distance measurements in doubly-labeled systems

The initial DEER experiments reported in Year 2 were complicated by the presence of the additional label site 447 mentioned above, which led to multiple distance measurements that could not be uniquely assigned on the basis of dilution studies as predicted in the Year 2 report. To simplify the experiment, we have most recently examined singly labeled ER,

which still gives inter-dimer distances in the DEER. The results are shown in Figure 11. At left are shown the time-domain spectrum, which may be interpreted in terms of a distance distribution, shown in the middle plots. Strikingly, both the ER alone and ER in the presence of the estradiol ligand exhibit a broad distribution of distances around 3 nm, suggesting a loosely held dimer with appreciable heterogeneity. However, upon binding a coactivator peptide, the structure becomes significantly more compact and rigid, as evidenced by the smaller inter-dimer distance (about 2.4 nm) and much narrower distribution of distances (left, bottom). These results suggest that a large scale remodeling of the receptor accompanies binding of the coactivator, and may account for the huge diversity of downstream processes governed by the ER. These DEER studies were carried out in collaboration with the group of Prof. Peter G. Fajer at the National High Magnetic Field Laboratory and the Biology Department of Florida State University.

The leucine zipper interface of the ER dimer

The implication of larger-scale rearrangements in the ER dimer prompted us to take a closer look at the interface between the two halves of the dimer. Primarily responsible for this interaction is the long Helix 11, which associates with itself in a well-known biological structural motif known as a "leucine zipper". To assess the capabilities of EPR for characterizing this interface, we have investigated a model leucine zipper system available to us from a collaborator on a separate project. We have found that the distance distribution provided by DEER affords an accurate measure of the molecular force between the two coils of a leucine zipper. The results are summarized in **Figure 12** and were recently reported in a *JACS Communication* (listed below). We plan to investigate the transmission of molecular signaling between the halves of the Helix 11 interface in the ER-LBD dimer using this method once appropriate mutants are isolated.

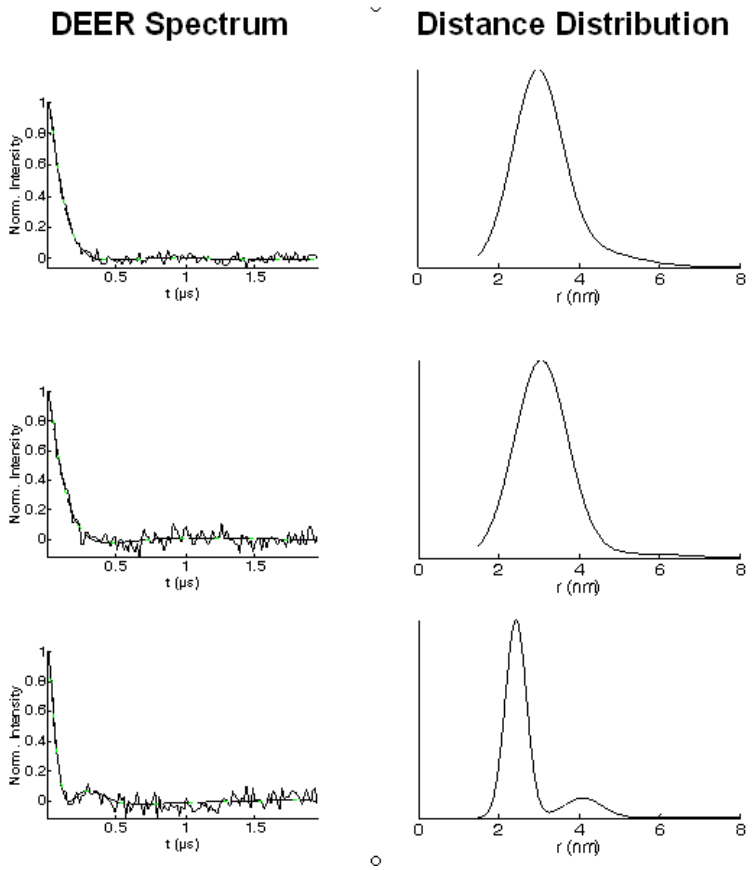


Figure 11. Deer spectrum (left) and inter label distance distribution (right) in dimers of of ER-LBD alone (top), ER-LBD with estradiol (middle), and ER-LBD plus estradiol plus SRC1 coactivator peptide sequence (bottom).

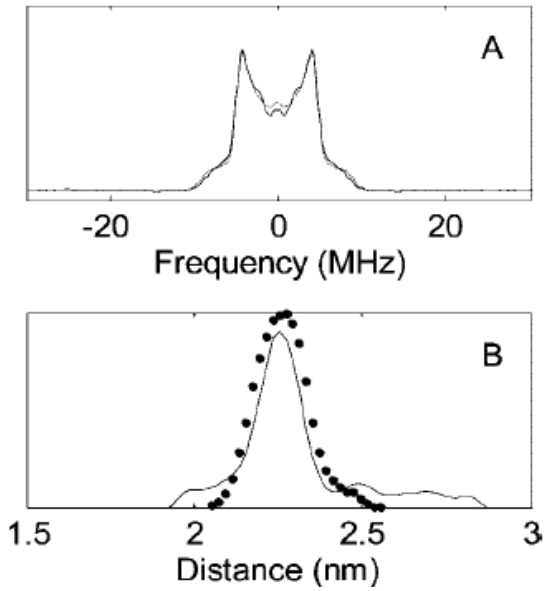


Figure 12 Frequency domain DEER signal of a spin-labeled Leu zipper homodimer based on the sequence of yeast transcriptional activator GCN4 (top) and interlabel distance distribution (bottom). Symbols show calculated distribution based on a molecular model. The first derivative of this curve give the mean potential force holding the dimer together.

3. Key Research Accomplishments

1. Completed synthesis of 17α- labeled estradiols needed for EPR studies.
2. Developed new assay for binding to ER based on EPR and fluorescence of 17α spin-labeled estradiols
3. Completed synthesis of first antagonist 11β-labeled estradiol
4. Optimized expression and purification of all ER-LBD mutants needed for spin-labeling studies, including doubly-labeled mutants, including removal of internal cysteine residues previously thought to be inaccessible to label
5. Quantified significant ligand-dependent dynamic changes in the hinge region between helix 12 and the body of the ER-LBD protein.
6. Identified significant differences in ER-LBD local and global dynamics that depend on both
7. Developed a new EPR-based method for measuring the force transmitted by the H_{11} leucine zipper interface in the ER-LBD dimer
8. Completed initial distance measurements of doubly labeled ER-LBD by DEER spectroscopy demonstrating significant global ligand-dependent changes of the overall ER-LBD complex and in the H_{12} region.

4. Reportable Outcomes

Presentations

1. Stefano V. Gullà, Robert N. Hanson, and David E. Budil, *Site Directed Spin Labeling Study of Ligand Induced Estrogen Receptor Conformations*, 49th Rocky Mountain Conference on Analytical Chemistry, Breckenridge, CO, July 23-27, 2007

2. Stefano V Gullà, Robert N. Hanson, J. Adam Hendricks, Kalman Hideg,[2] and David E. Budil,[1] *New site-directed spin labeling tools for characterizing the dynamic response of the estrogen receptor to therapeutic agents*, 235th National Meeting of the American Chemical Society, New Orleans, LA, April 6-10, 2008

3. Stefano V Gulla[1], Kalman Hideg,[2] David E. Budil, *Characterization of spin labeled estradiol as a probe for Estrogen Receptor binding interactions*, 235th National Meeting of the American Chemical Society, New Orleans, LA, April 6-10, 2008

4. Samantha Rupert, Kelly Barhite, Stefano Gullà, David E. Budil *Spin label studies of interactions between the estrogen receptor and coactivator peptides*, Experimental Biology 2008 meeting, April 5-9, San Diego, CA, 2008

5. Stefano V Gullà, Robert N. Hanson, J. Adam Hendricks, Kalman Hideg,[2] and David E. Budil,[1] *New site-directed spin labeling tools for characterizing the dynamic response of the estrogen receptor to therapeutic agents*, 5th Era of Hope Meeting, June 26-30 Baltimore, MD, 2008

6. Stefano V Gullà, Jean Chamoun, Peter G. Fajer, Kalman Hideg, and David E. Budil, *New site-directed spin labeling tools for characterizing the dynamic response of the estrogen receptor to therapeutic agents*, 50th Rocky Mountain Conference on Analytical Chemistry, Breckenridge, CO, July 25-30, 2008

7. Lisa Ngu, Stefano V Gulla, Robert N. Hanson, and David E. Budil, *Characterization of spin labeled estradiol as a probe for coactivator peptide binding*

*interactions***,** Experimental Biology 2009 meeting, New Orleans LA, April, 2009 (Winner, Best Poster in Drug Discovery and Design category, http://www.asbmb.org/page.aspx?id=146)

Publication

1. Stefano V. Gulla, Gaurav Sharma, Peter Borbat, Jack H. Freed, Harishchandra Ghimire, Monica R. Benedikt, Natasha L. Holt, Gary A. Lorigan, Kaushal Rege, Constantinos Mavroidis, and David E. Budil *Molecular-Scale Force Measurement in a Coiled-Coil Peptide Dimer by Electron Spin Resonance*, *J. Amer. Chem. Soc.* (Communication) **131**, 5374–5375 (2009)

Manuscripts in preparation

1. Stefano V. Gulla, Kalman Hideg, Jean Chamoun, Peter G. Fajer, David E. Budil, *Characterization of novel estrogen-based nitroxide spin probe binding to the estrogen receptor α ligand binding domain*, in preparation for submission to *J. Mol. Endocrinol.*
2. Stefano Gullà, J. Adam Hendricks, Robert N. Hanson, and David E. Budil, *Spin-label study of ligand-dependent receptor dynamics in the ligand-binding region of estrogen receptor α*, in preparation for submission to *Journal of Molecular Biology*.
3. Stefano V. Gullà, Jean Chamoun, Peter G. Fajer, and David E. Budil, S*olution structure of the dimer of the estrogen receptor alpha ligand binding domain by double electron electron resonance (DEER) spectroscopy*", in preparation for submission to *Biophys. J*.
4. J. Adam Hendricks, Stefano V. Gullà, David E. Budil, Robert N. Hanson,*Synthesis of a Spin-Labeled Antiestrogen as a Dynamic Motion Probe for the Estrogen Receptor alpha*, in preparation for submission to the *Journal of the American Chemical Society*.

5. Conclusions

As enumerated in the Key Research Accomplishments above, all of the initially proposed tasks were completed, with the exception of characterization of the spin labeled ER at high EPR fields. In our initial studies, we found that the high field instrument did not have sufficient sensitivity to allow accurate characterization of the ER. However, since the start of this work, our group has been awarded an instrumentation grant from the NSF (award DBI-732001) to construct a high-field DEER spectrometer at 230 GHz. This instrument is nearly on line, and we anticipate that we will be able to apply it to carry out both the high-field characterization of the spin labels and eventually distance measurements by the DEER experiment in house.

We found two new results not anticipated in our original research plan. First, we discovered a significant dependence of the global ER-LBD dynamics on binding of both ligand and a sequence from a coactivator protein. The dynamics depend on both the degree of agonism of the ligand and the peptide binding sequence. Another surprising finding was the discovery of a global response of the ER protein to binding of these factors, suggesting a large scale remodeling of the protein structure that directs downstream effects of the ER. This led to our second unanticipated finding, that the forces in the Helix 11 leucine zipper that hold the ER dimer together may be measurable using EPR spin label methods.
The results obtained at lower field clearly support our initial hypothesis that the physical

response of the ER protein to different ligand types can be resolved and characterized in detail by EPR spin-labeling.

6. References

None.