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The estrogen receptor (ER) is found in the nucleus of several tissues, including breast, bone, liver, the organs of the reproductive system, and the cardiovascular system. The ER binds several types of compounds, including compounds that are quite distinct from its natural ligand. Estrogens bind to and activate the ER, which leads to the stimulation of transcription of genes containing an estrogen responsive element (ERE). Antiestrogens and partial antiestrogens bind tightly to the ER but fail to activate transcription; these compounds are currently in widespread use for the treatment of breast cancer. In addition, a variety of compounds introduced into the environment by human activity have also been found to act as estrogen mimics and alter reproductive function and development. The goals of this project are to understand, on a molecular level, how the ER binds estrogens, anti-estrogens, and estrogen mimics present in the environment, how this binding triggers activity, and how mutations in the ER discovered in breast cancer patients affect ER activity. Towards this end, we have expressed and purified the ligand binding domain of the estrogen receptor, and studied complexes with estradiol, the natura! ligand, and tamoxifen, a partial antiestrogen in use as a breast cancer therapeutic using high-resolution heteronuclear NMR spectrosocpy.

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Annual Report for Breast Cancer Therapeutics, Environmental Estrogens, and the Estrogen Receptor (ER): Characterization of the Diverse Ligand Binding Properties of the ER

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INTRODUCTION

The estrogen receptor (ER) is found in the nucleus of several tissues, including breast, bone, liver, the organs of the reproductive system, and the cardiovascular system. The ER binds several types of compounds, including compounds that are quite distinct from its natural ligand. Estrogens bind to and activate the ER, which leads to the stimulation of transcription of genes containing an estrogen responsive element (ERE). Antiestrogens and partial antiestrogens bind tightly to the ER but fail to activate transcription; these compounds are currently in widespread use for the treatment of breast cancer. In addition, a variety of compounds introduced into the environment as a result of human activity have also been found to act as estrogen mimics and alter reproductive function and development. The estrogenic behavior of these compounds has proven difficult to predict from their structures; at first glance many of these hormone mimics bear little structural resemblance to natural estrogens. The molecular level details of the conformational changes that allow the ER to bind a diverse array of compounds and result in diverse patterns of gene activation are not understood yet are critical to assessing and predicting the behavior of potentially estrogenic compounds. The goals of this project are to develop high resolution strategies to understand, on a molecular level, how the ER binds estrogens, anti-estrogens, and estrogen mimics present in the environment, how this binding triggers activity, and how mutations in the ER discovered in breast cancer patients affect ER activity. A complete structural understanding of how various ligands interact with the ER, and are able to elicit different responses, will assist in identifying compounds with therapeutic benefit for treating breast cancer. In addition, our studies will contribute to the screening of potential environmental estrogens before they are introduced into the environment.

BODY

The large number of compounds that are able to bind and activate the ER necessitates the use of an innovative and rapid approach to effectively screen for estrogenic activity *in vitro*. We are investigating ligand binding to the ER using multidimensional nuclear magnetic resonance spectroscopy (NMR) studies of complexes of these compounds with the ligand binding domain (LBD) of the ER. First, the ER-LBD is uniformly isotopically labeled with ^{13}C and/or ^{15}N. Ligand binding is followed by acquiring heteronuclear single quantum coherance (HSQC) spectra. These finger-print like spectra are exquisitely sensitive to molecular conformation, and will report on not just binding but also the conformational changes that accompany ligand binding. Once this strategy is established, we will study the structural effects of several point mutations isolated from breast cancer cell lines and how ligand binding features may be altered by the mutations. Additionally, we plan to investigate the ligand binding differences between ERα and ERβ, the two natural variants of the ER. These differences may play an important role in the tissue specific activity of partial antiestrogens. Analysis of our NMR data will be significantly aided by the present availability of the crystal structures of the ER bound to both estrogens and antiestrogens. The use of NMR spectroscopy as an efficient tool for screening for compounds with estrogenic or antiestrogenic activity, or with activity specific to either ERα or ERβ will be explored.

Progress in the last year in the first 3 technical objectives has been achieved. Progress towards the tasks in the statement of work are as follows:

Progress in Preparation and Characterization of the ER-LBD: The tasks associated with technical objective 1 were mostly achieved within the first year of the grant, however, for a variety of reasons we have needed to develop more reliable and flexible protein expression and purification strategies. The LBD-ER was prepared by expression in BL21(DE3) *E. coli* by induction with IPTG from a pET plasmid. Complete purification was achieved using an estradiol affinity column that afforded protein of high purity and activity in one step. In addition, the purified protein is highly stable to proteolysis, a problem that rendered protein purified by other strategies inactive within a few days of preparation. A complete buffer screen revealed optiminal buffer conditions that allowed us to prepare samples with protein concentrations of up to 500 μM. Homonuclear NMR data obtained on these samples revealed features consistent with a protein of the anticipated molecular weight. The optimum temperature for data collection was determined to be 30°C. The anticipated molecular weight of the estradiol bound complex was confirmed by light scattering data and shown unambiguously to be a dimer. In the second year of the grant, we extensively re-evaluated the conditions for protein expression. We discovered that our previous protocol did not reliably produce protein, *i.e.*, in certain growths when protein expression was induced at high ODs no protein production was observed. This is presumably due to loss of ampicillin resistance at high bacterial cell concentration. This is not uncommon as the resistance to antibiotic is conferred by a secreted protein. Thus, induction at an OD of 1.0 at 600 nm was discovered to give completely reliable protein production. We modified all of our protocols to accommodate this improvement.

We have also realized that, in order to study the environmental estrogens that bind the ER-LBD much more weakly than the natural ligand estradiol, we will need to produce a recombinant ligand-free form of the ligand binding domain (LBD) of the estrogen receptor. Although our estradiol affinity column provides a very pure ER-LBD, elution from the column requires competition with a ligand of similar affinity (*e.g.* tamoxifen or estradiol itself). For weak binding ligands, this protocol will be very inefficient as most potential ligands are highly hydrophobic and not soluble at the concentrations that are needed to compete the protein from the column. Thus, a stable, ligand-free preparation protocol is needed. Development of this protocol has the added benefit of allowing characterization *in vitro* of the ligand free form by NMR.

Preliminary efforts at preparing the ligand free form of the LBD modeled on a differential detergent solubilization method were initiated at the end of the second year. These efforts were continued in the third year. After expression of the LBD using the standard technique, the pellet is first washed with a buffer containing the detergent octyl glucoside which preferentially solubilizes almost all the other cell proteins. After octyl glucoside extraction, the cell pellet is washed with a buffer containing the detergent Zwittergent 3-12, which then solubilizes the estrogen receptor LBD. We have established that this unusual procedure does indeed yield significant amounts of the ER-LBD. We have determined that, following dialysis of detergent, the LBD refolds and regains the ability to bind estrogen. The circular dichroism spectrum of material prepared by this method reveals the presence of helical structure. However, we could not obtain a concentrated sample of this for NMR analysis. In addition, preliminary attempts at titration of environmental ligands into a sample of the ER-LBD resulted in irreversible precipitation of the protein. Given the unusual nature of this protocol, we could not rule out

residual effects of the detergent or incorrect protein folding as the cause of poor sample solubility. Therefore, we invested a significant effort in developing a more straightforward purification protocol for ligand-free ER-LBD.

We selected a His-tagged strategy for several reasons. First, the addition of a His tag on the protein provides a good handle for purification using a variety of readily commercially available columns and beads. Second, we engineered the His tag such that it could be readily cleaved off with thrombin, thus allowing removal of the tag that might interfere with either activity or the quality of the NMR spectrum. Finally, this strategy broadens the types of approaches that we could use to add in the variety of ligands targeted in this study, as we can investigate ligand addition to both immobilized and free protein.

In order to prepare the His-tagged construct of the ER-LBD, we cloned the ER-LBD sequence (aa 297-554) into a pET 15b plasmid (Novagen) using the restriction enzyme BamH1. The integrity of the subcloning was assured by sequencing of the recombinant plasmid at the University of Colorado DNA sequencing facility. Conditions for protein expression were assessed using test inductions (see Figure 1) and purification was performed using standard protocols for His-tag purifications. After sonicating the cells twice and spinning down cell debris after each sonication, the supernatant was loaded on a Pharmacia chelating column that had been preloaded with nickel ions. The column was washed with a low level of imidazole (30 mM) to remove non-specifically bound proteins, and then the His-tagged ER-LBD was eluted with 500 mM imidazole. The resulting protein is >95% pure by Coomassie-stained SDS-PAGE gel, and stable to long term proteolysis. CD characterization of the ligand free ER-LBD revealed the presence of significant helical structure (Figure 2). The protein is active in ligand binding as it fully binds our estradiol-affinity column. While there are still significant concerns regarding solubility, the His-tagged version of the ER-LBD should allow us to pursue structural studies with weakly binding environmental estrogens. Development of rotocols for introduction of these ligands is currently in progress.

Progress in the Study of Complexes by NMR Growth of uniformly ^{15}N labeled protein needed for Technical Objectives 2 and 3 progressed smoothly based on the expression and purification scheme developed for unlabeled protein using the estradiol affinity column for purification. ^{15}N-^{1}H HSQC NMR data were collected at 500 and 600 MHz. We reported last year that significant improvement of data quality was observed at higher field strengths and with the addition of TROSY pulse sequences. We determined that this project would not be feasible without the TROSY advances. Moreover, the enhancement of signal using TROSY increases as the field strength used for data collection increases. Extensive efforts were aimed during the second year of funding at obtaining triple resonance data for resonance assignments. Pilot triple resonance experiments, including the HNCA and HNCO, have been collected, and every possible parameter has been scrupulously optimized. However, the signal obtained on these experiments is not amenable to full resonance assignment. In fact, only about half of the anticipated resonance signals are observed. This is due to the large size of the ER-LBD, which is a symmetric dimer of molecular weight 56 kDa. Even with our advances using TROSY and deuteration, we have not been able to reduce the T2 relaxation of the complex by a significant enough amount to prevent complete decay during the time of the pulse sequence. Unfortunately, the other triple-resonance experiments that are needed to make assignments, the HNCACO,

HNCACB, CBCACONH etc, are even longer, so the relaxation issues associated with large (*i.e*, slowly tumbling systems) are even worse. Attempts to assign a partial data set such as this would be rather ill conceived, as it would be easy to make a mistake early in the assignment process that would lead to complete misassignment of the resonances. In fact, very few systems larger than 30 kDa have been completely assigned to date, highlighting the challenges associated with working with systems of this size. These studies indicate that further optimization, either through preparation of higher concentration samples or data collection at higher field, are necessary.

Based on our preliminary NMR data collected for assignment, we determined that data should be collected at as high of a field strength as possible. Significant improvement in sensitivity, signal-to-noise, and the TROSY enhancement are all anticipated when moving from 600 to 800 MHZ. To complete a project of this magnitude will require routine access to a high field spectrometer. We are delighted to report that, in the last year, three grant proposals submitted by a consortium of institutions in the Rocky Mountain Region, the University of Colorado, Boulder, the University of Utah Medical School and the University of Colorado Health Sciences Center, were funded to create a shared facility with an 800 MHz spectometer. Funding for this $2.5 million endeavor is provided by a combination of the Keck Foundation, the NIH, the NSF and matching funds from the participating institutions. The three participating institutions will share the time, operating costs and matching costs of the facility equally. Based on the reviews of our proposals, the need of this high field instrument for the ER-LBD project was a compelling component of our application for funding. We are confident that access to high field instrumentation that will be become available with the purchase of our own 800 MHz instrument will significantly enhance the likelihood of obtaining full backbone resonance assignments on this challenging system.

NMR characterization of the ligand-free ER-LBD has been initiated. We obtained one sample of uniformly ^{15}N-labeled protein using the protocol described above. The protein could only be concentrated to 250 μM before precipitation, and the protein stuck non-specifically to all surfaces used in its preparation, both properties associated with unfolded/aggregated material. These features are manifest in the ^{15}N HSQC spectrum (Figure 3). While resonances can clearly be identified, the protein appears to adopt a partially unfolded character. The very strong resonances are likely due to the unstructured His-tag, and provide a reference for the completely unfolded state. The combination of the CD and NMR data on the protein indicate the presence of "molten globule" like state for the ligand-free form of the protein. The CD clearly reveals the the presence of regular secondary structure, in particular a high content of a-helical structure, while the NMR spectrum resembles a partially folded or nascently structured state. These data are consistent with the observation that the ligand-free state of the protein is bound to heat shock proteins *in vivo*. This phenomenon may be needed to confer either affinity or specificity for ligand binding, and will be interesting to further characterize.

New Developments in the Project:

1. ER-LBD Binding to BRCA1: Last year, two exciting advances in the understanding of BRCA1 have been made. First, a region of BRCA1 that interacts with the estrogen receptor has been identified. Second, the structure of the complex of BRCA1 and it's biological partner has been determined. Recent reports indicated that the interaction, surprisingly, is **not** ligand

dependent. Therefore, the protocols we established in this granting period for production of both ligand bound and ligand free ER-LBD will be extremely important to determine the nature of this interaction. To this end, we are collaborating with the laboratory of Rachel Klevit at the University of Washington, Seattle, to determine the structural nature of the interaction. Prof. Klevit's laboratory reported the structure of the domain of BRCA1 predicted to interact with the ER-LBD. We will be providing our highly purified material to conduct high-resolution NMR studies in the presence of BRCA1 to look for protein binding and ligand binding-dependent conformational changes.

2. Investigation of Estrogenic Effects of DDT: During the course of this research, we became interested in the estrogenic properties of the pesticide dichlorodiphenyltrichloroethane or DDT, which was in widespread use in the United States from the 1940s until it was banned in the early 1970s. In both cellular and organismal studies, DDT acts as an estrogenic compound. As a result of these findings, many investigators have asked whether there is a possible connection between DDT exposure and breast cancer. However, DDT itself is a very poor estrogen, and only weakly binds the estrogen receptor *in vitro*.

Many studies over the years have examined the possible connection between environmental pollutants and the development of breast cancer. Recently, the Journal of the National Cancer Institute published the combined analysis of five studies evaluating the association of levels of DDE and PCBs in blood plasma or serum with breast cancer risk. We noticed that , in these studies, DDE, a metabolite of DDT, is used as a marker for DDT exposure. When correlated to DDE levels, the majority of studies published to date do not support the hypothesis that elevated exposure to DDT increases the risk of breast cancer. We wondered, however, whether residual DDE levels are an appropriate marker for determining the estrogenic component of DDT exposures.

A substantial body of research indicates that it is **not** chemicals like DDT or DDE (which contain a chlorinated phenyl ring) that are estrogenic compounds and therefore potentially carcinogenic. Rather, compounds capable of triggering an estrogenic response typically contain a hydroxylated phenyl ring, a configuration that allows the chemical to bind and activate the estrogen receptor. It should be considered, therefore, that it is not DDT or DDE themselves, but hydroxylated metabolites of these compounds (see Figure 4), that are acting as estrogenic compounds and that are then being cleared from the system.

Studies spanning over six decades have definitively established the importance of a hydroxylated phenyl ring for estrogen receptor binding and estrogenic activity. For example, an extensive analysis of structure-activity relationships (SAR) for estrogen receptor ligands published in 1999 concluded that, "A phenolic hydroxy group, which mimics the 3-OH on the A-ring of estradiol (and at the corresponding position of nonsteroidal estrogen ligands), appears repeatedly as the most important factor in receptor-ligand interactions.".

The importance of a hydroxylated phenyl ring for estrogenic activity is reinforced by the crystal structures of the ligand binding domains of both estrogen receptor α (ERα) and estrogen receptor β (ERβ) published in recent years. All of the structures indicate that the key phenolic hydroxyl of the bound ligands is hydrogen bonded to a highly conserved arginine and a glutamic

acid in the ligand-binding domain (*e.g.* arginine 394 and glutamic acid 353 in ERα). This hydrogen bonding interaction acts as a clamp on the phenolic hydroxyl and it is unlikely that the chlorinated phenyl ring of DDT or DDE is able to fulfill this interaction.

We also note that, for a given exposure, an individual with a more active hydroxylation response (*e.g.* through the cytochrome P450 system) would have a **greater** exposure to hydroxylated (and presumably, therefore, estrogenic) metabolic products, but a **lower** residual level of DDE. For all of these reasons, we have questioned whether using residual levels of DDE is an accurate marker to use when assessing the estrogenic consequences of DDT exposure.

We are quite surprised that this point has not been brought out in the literature to date, and have initially pointed this out in a letter to the editor, which has been accepted at the Journal of the National Cancer Institute. We are in the progress in preparing a more complete description of these concerns as a review article, targeted for submission in August of 2002.

We have not yet begun work on Technical Objective 4. This objective was essentially to repeat the above studies, which are conducted on the ligand binding domain from ERα on ERβ. However, until the conditions for the ERα studies are well established, branching out to studies with ERβ is not prudent. As our work has proceeded, we have devoted some effort to the DDT-breast cancer question due to its immediate relevance to epidemiological studies and on-going risk assessments. With respect to our NMR chemical shift mapping studies, we have requested and received a 1 year extension on this grant and will continue to pursue high field NMR and other structural studies of the ER-LBD.

KEY RESEARCH ACCOMPLISHMENTS

- Two new protocols for the preparation of ligand-free ER have been explored and evaluated.
- High-resolution data on the ligand free form of theER-LBD has been acquired.
- Analysis of the differences between ^{15}N-^{1}H HSQC NMR data on the ligand-free form of the ER-LBD and the estradiol and tamoxifen complexes has been initiated.
- Funding for a $2.5 million 800 MHz instrument was obtained. Access to this instrumentation will significantly enhance our ability to analyze ligand binding by the ER-LBD.
- A collaboration to investigate the BRCA1 interaction with the ligand free and ligand bound ER-LBD has been initiated
- Observations regarding the possibility of hydroxylated metabolites of DDT as hormone mimics and relationship to of breast cancer have been made and published.

REPORTABLE OUTCOMES

Papers published:

L. W. Glustrom, R. M. Mitton-Fry and D. S. Wuttke, "Environmental Estrogens and Breast Cancer: The Importance of Hydroxylated Intermediates," reviewed letter to editor, *J. Natl. Cancer Institute*, in press.

L. W. Glustrom and D. S. Wuttke, "DDT as an Environmental Estrogen: The Need to Consider Hydroxylated Metabolites," to be submitted August, 2002.

Posters presented: The work described in this progress report has been presented at several meetings as either a talk or poster. These include (1) The University of Colorado Annual Retreat (Winter Park, Colorado) and (2) The University of Colorado Biophysics Supergroup Meeting. A full report of this work will be presented at the Era of Hope 2002 Department of Defense Breast Cancer Research Meeting in Orlando, Florida, September of 2002.

Graduate Student Training: Two graduate students, Dana Warn and Aaron Miller, have been trained on this research project. Dana Warn was initially responsible for protein expression and purification protocols, and NMR spectroscopy. Her stipend and tuition was provided through an EPA-STAR fellowship. During the second year of funding, Dana Warn obtained a Master's Thesis in Chemistry and Biochemistry from the University of Colorado entitled "Environmental Estrogens and Breast Cancer Therapeutics: Development of a Technique to Characterize the Diverse Ligand Binding Characteristics of the Estrogen Receptor," awarded December, 2000. Aaron Miller was a first year graduate student who worked on the development of a preparation protocol for the ligand-free estrogen receptor ligand binding domain.

Undergraduate Training: Andrea Wismann, an undergraduate at the University of Colorado, Boulder, conducted independent research on this project during the summer of 2000. Through this research experience, she learned protein expression and purification strategies, as well as circular dichroism spectropolarimetry. Bryn Weaver, an undergraduate Biochemistry and MCDB major, has joined the project. She has been investigating the properties of the ligand-free ER-LBD. In particular, she has expressed and purified the ligand-free ER-LBD and determined the circular dichroic properties of this state of the protein. Her work in the Summer of 2002 was supported by a UROP grant from the University of Colorado for undergraduate research education.

Professional Research Associate Training: The majority of the protein expression, purification and characterization has been performed by Leslie Glustrom, a professional research associate in the laboratory who is currently being trained on this project.

CONCLUSIONS

In the third year of funding, progress has been made towards the stated goals. In addition, with further insights derived from our data and the published literature, we have explored new directions in the project. We have developed new protocols that have allowed us to obtain estradiol-free ER-LBD. This protein will be essential for studying weakly bound compounds. In addition, the availability of this protein has allowed us to enter a collaboration exploring the interaction of the ER-LBD with BRCA1 in both the ligand bound and ligand free states. Since our pilot experiments indicated that we will likely not be able to obtain NMR resonance assignments at the presently available field strength, we actively pursued other access to high end instrumentation. Given the NMR time required to obtain data suitable for resonance assignment, we cannot accomplish this through collaboration. However, in the last year we obtained both private and public funds that will be used to install an 800 MHz NMR spectrometer in the Rocky Mountain region. We are confident that, with access to this instrumentation that we will be able to rapidly screen both a variety of ligands and medically relevant mutants of the protein. Finally, we are pursing an observation that when considering possible correlation between DDT exposure and breast cancer risk, it is important to consider the hydroxylated byproducts of DDT metabolism. These compounds fit the SAR profile of a good estrogen, and can be readily reconciled with the data available from crystal structures of the ER-LBD.

REFERENCES

No references are included in this report.

APPENDICES

Figures referred to in the text are appended below.

Figure 1: SDS-PAGE gel showing induction/purification of His-tagged ligand free ER-LBD.
Figure 2: CD spectrum of ligand free ER-LBD.
Figure 3: ^{1}H-^{15}N HSQC spectrum obtained at 600 MHz of the ligand free ER-LBD.
Figure 4: Chemical structures of key compounds for assessing the estrogenicity of DDT.

Figure 1

Test Inductions and Purified His ER-LBD

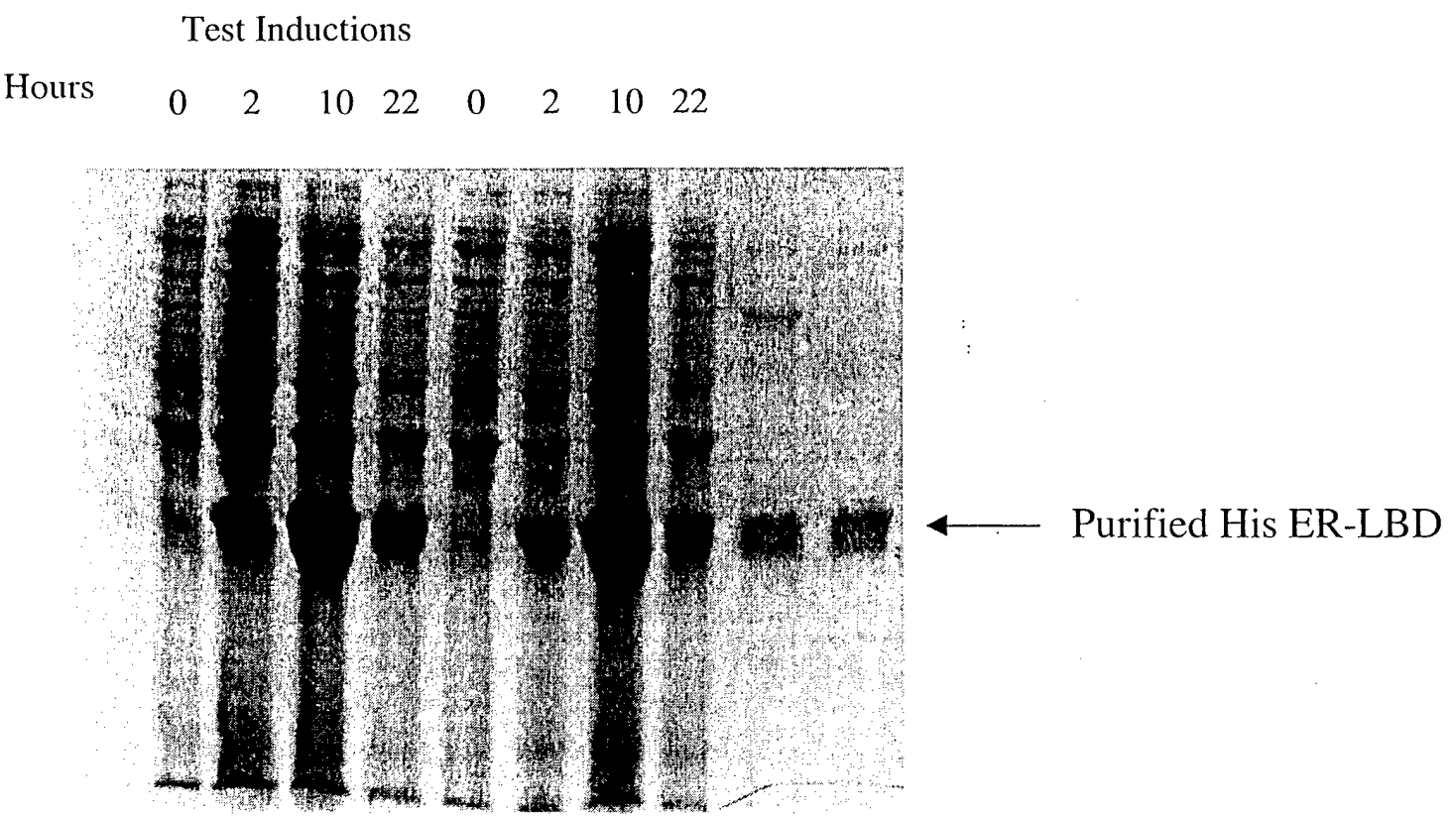
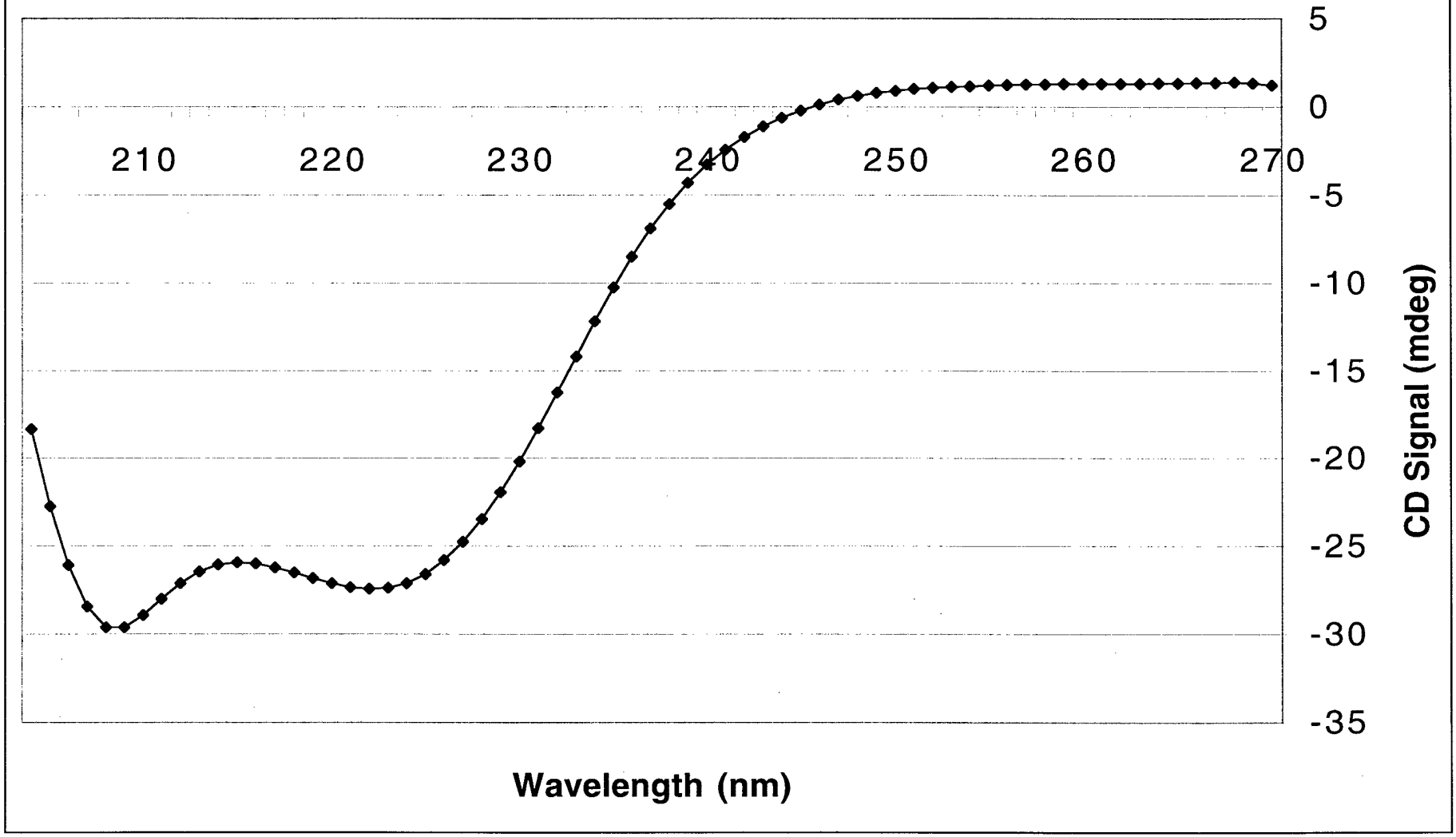
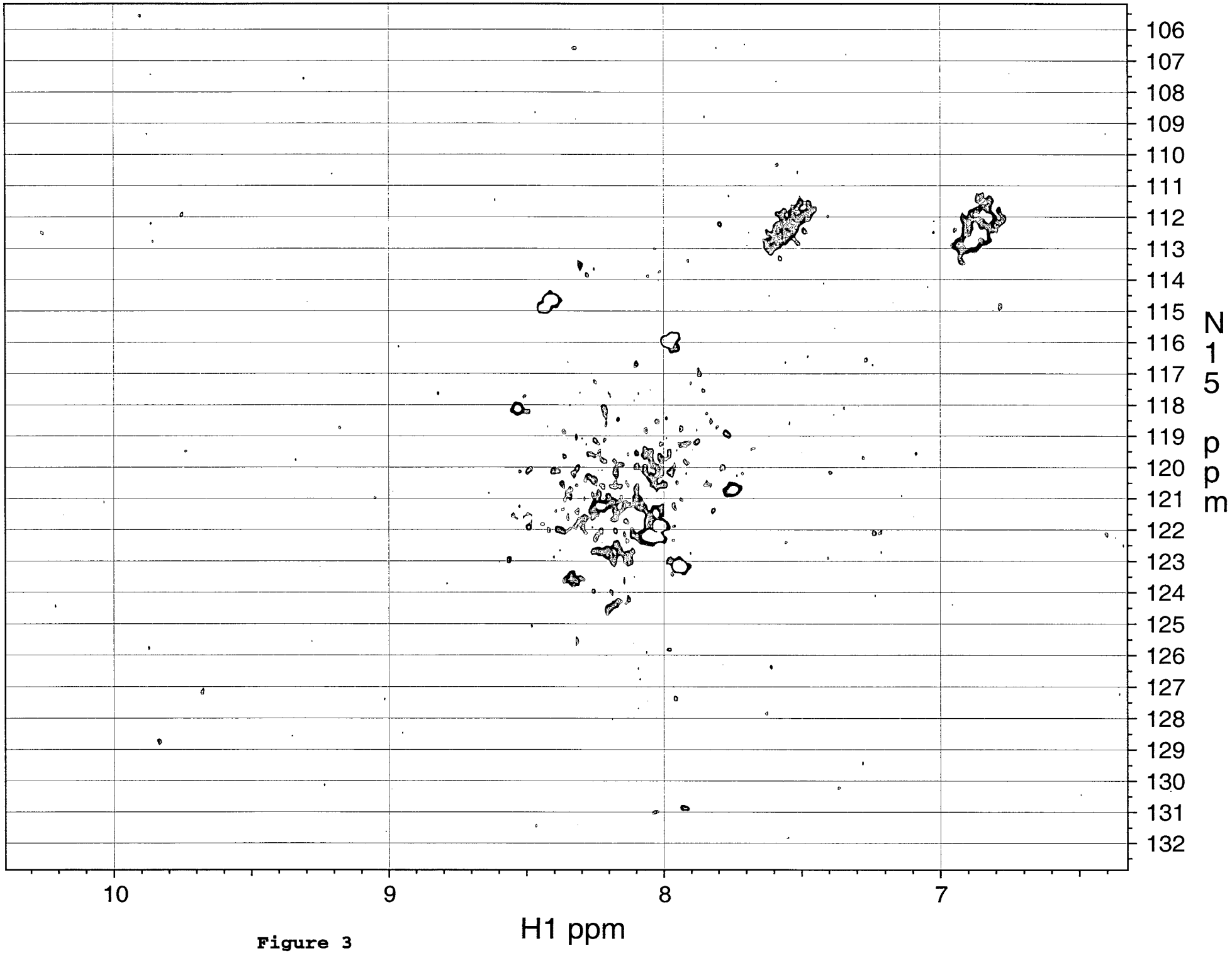


Figure 2
CD Spectra of Ligand Free His ER-LBD

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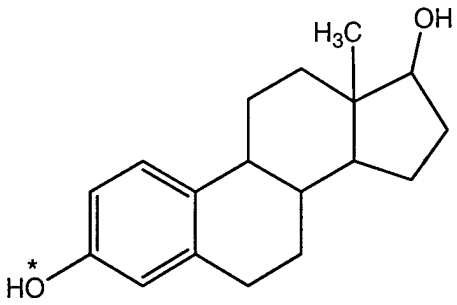
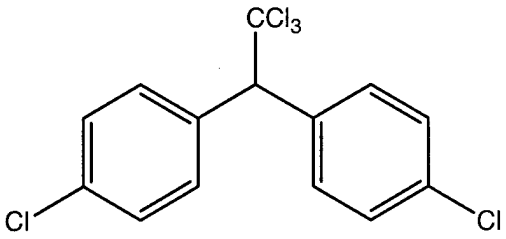
H1 ppm

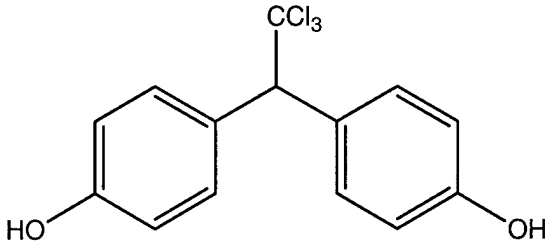
Figure 3



Estradiol (Estrogen)



DDT



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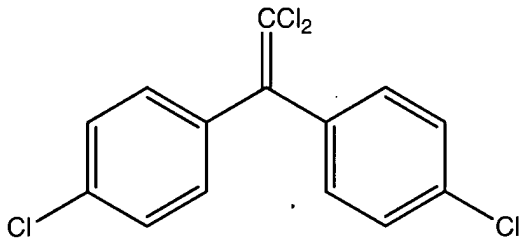

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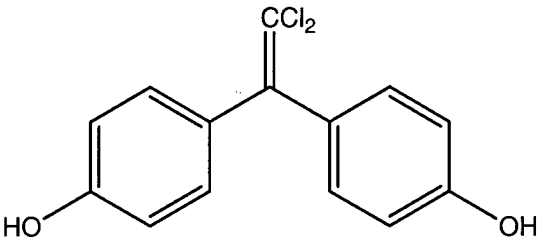

Dihydroxy-DDE

Figure 4. Structures of Key Compounds. The phenolic OH in estradiol (*) is key for receptor binding and estrogenic activity. Compounds like DDT and DDE have a chlorine in this position whereas hydroxylated metabolites like HPTE and dihydroxy-DDE have the key phenolic group and display both increased estrogen receptor binding and estrogenic activity. See text for details.