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Computational Characterization and Prediction of Estrogen Receptor Coactivator Binding Site Inhibitors

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14. ABSTRACT

Many carcinogens have been shown to cause tissue specific tumors in animal models. The mechanism for this specificity has not been fully elucidated and is usually attributed to differences in organ metabolism. For heterocyclic amines, potent carcinogens that are formed in well-done meat, the ability to either bind to the estrogen receptor and activate or inhibit an estrogenic response will have a major impact on carcinogenicity. Here we describe our work with the human estrogen receptor alpha (hERa) and the mutagenic/carcinogenic heterocyclic amines PhIP, MeIQx, IFP, and the hydroxylated metabolite of PhIP, N2-hydroxy-PhIP. We found that PhIP, in contrast to the other heterocyclic amines, increased cell-proliferation in MCF-7 human breast cancer cells and activated the hERa receptor. We show mechanistic data supporting this activation both computationally by homology modeling and docking, and by NMR confirmation that PhIP binds with the ligand binding domain (LBD). This binding competes with estradiol (E2) in the native E2 binding cavity of the receptor. We also find that other heterocyclic amines and N2-hydroxy-PhIP inhibit ER activation presumably by binding into another cavity on the LBD. Moreover, molecular dynamics simulations of inhibitory heterocyclic amines reveal a disruption of the surface of the receptor protein involved with protein-protein signaling. We therefore propose that the mechanism for the tissue specific carcinogenicity seen in the rat breast tumors and the presumptive human breast cancer associated with the consumption of well-done meat maybe mediated by this receptor activation.

15. SUBJECT TERMS Estrogen Receptor, Ligand, xenoestrogens, co-activator binding inhibitor, breast cancer, carcinogen

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Introduction

In an effort to treat breast cancer, selective estrogen receptor modulators (SERMs) have been used to modulate the estrogen-signaling pathway with mixed results [1]. A classic example of a SERM is tamoxifen. When used as a therapeutic for a limited time, tamoxifen is effective in disrupting the estrogen-signaling pathway. Unfortunately, with prolonged use, breast tumor cells become resistant to tamoxifen and are able to use the bioactivated metabolite of tamoxifen to interact with coactivators that activate the estrogen-signaling pathway, reversing its original role [1]. An alternative therapeutic approach is to target the binding site of the coactivator protein. Recent studies have shown that some small molecules may bind in sites (e.g. coactivator site) other than the estradiol binding site [2] and still disrupt the estrogen-signaling pathway. By binding in the coactivator site while estradiol is bound in the estrogen receptor (ER) ligand binding domain (LBD), these small molecules act as coactivator binding inhibitors (CBIs) because the coactivator proteins can no longer bind; thus, gene transcription is inhibited. Potentially, these CBIs can act as a new therapeutics against environmental or natural agonists of ER. Quantitative structure-activity relationship (QSAR) studies have been used to develop therapeutics that will compete and bind in the estradiol binding site of the ER LBD [3-5]. Because these studies have focused on the estradiol binding site, new potential ER disruptors that bind in the coactivator site have been missed. We propose to develop a new computational approach to predict therapeutically useful ER disruptors by investigating CBIs binding to the coactivator site in conjunction with estrogenic compounds bound in the estradiol site

Body

Task 1: Computationally predict the relative binding of CBIs in the coactivator site and the CBI binding properties.

Three estrogenic compounds were chosen as positive controls, estradiol (E2), 1-methyl-2-amino-6-phenylimadazo[4,5b]pyridine (PhIP), and diethylstilbestrol (DES). These three compounds have been shown to be capable of activating the estrogen receptor and producing an estrogen specific response (see Appendix A and more **Korach 1985**). Other compounds that have been shown to inhibit the function of ER (Appendix A) were chosen as possible CBI's. These compounds are aromatic heterocyclic amines and known mutagens (**refs**).

The entire ER-LDB was used in the docking calculations by including it in all the atom interaction grids. As shown in appendix A and **figure 1**, estradiol docking recovered the crystallographic binding mode with a probability of binding in the native binding cavity of ~99%. Striking however, was the discovery of a second binding site in the interior of the protein (**Task 1.1**). This site was found because the whole protein was included in the docking calculations and has only been discussed twice before in the literature, once in the paper describing the original crystal structure (**Brzozovski 1997**) where it was defined only as a solvent channel. A second mention of this cavity in the literature was an attempt to explain unusual kinetics experiments (**van Hoorn, 2002**). In our ranking, we found that compounds, which inhibited the activation of the ER, showed a preference in binding to the alternative site (**Figure 2 and activiation figure**) (**Task 1.2**).

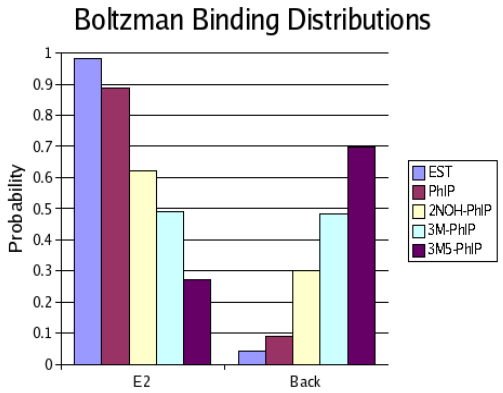


Figure 1: The Boltzman binding distribution of E2 and other heterocyclic amines on the ER-LDB at the native binding cavity and the back cavity. Details of the calculations and compounds are described in appendix A.

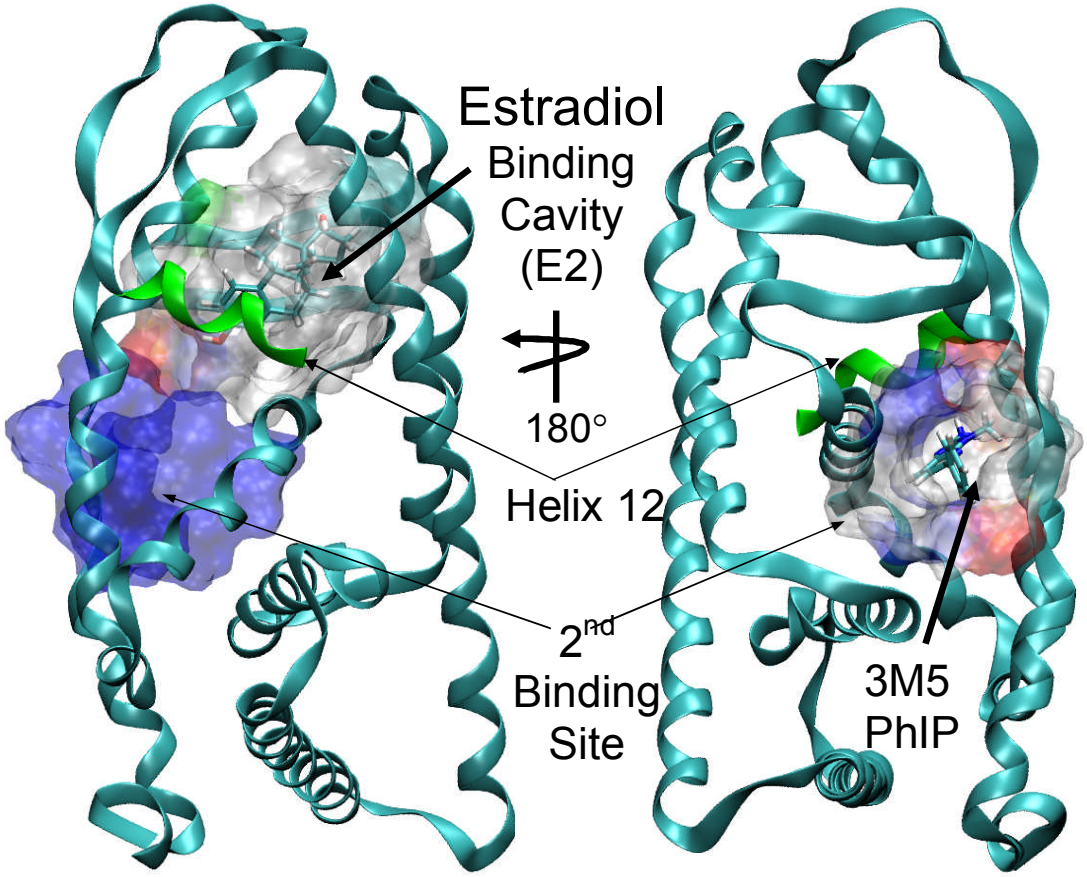


Figure 2: ER-LDB shown with the backbone in ribbons and binding cavities in surface representation colored by residue type (red, negative charge; blue, positive charge; green, polar; white non-polar). The protein is rotated 180° to show the opposite positions of the cavities on the surface. E2 is shown in stick representation inside the crystallographic binding site, while the PhIP congener 3-methyl-5phenyl-PhIP is

shown in its predicted binding cavity. The blue surface on the protein at the left defines the region of residues critical for binding the co-activator protein.

The end of the alternative binding site adjoins residues important for binding the co-activator protein (**Figure 3**). We hypothesized that compounds bound in this alternate cavity could affect the dynamics of residues on the surface of the protein. Molecular dynamics simulations of highest ranking CBI (3M5-PhIP) showed that significant distortion of the protein surface was possible when the alternate cavity contains small molecules (**Task 1.3**). In our simulations of 3M5-PhIP bound to ER-LDB we noticed that Met 357 was very dynamic, the sidechain would move significantly during the 2ns simulation. This sidechain motion affected the surface of the protein, especially in the region required for co-activator binding (**Figure 4**). Two separate simulations had similar results. Control simulations of E2 and the Tif-2 peptide from the co-activator protein under underway. Comparison of the control simulations against simulations of the CBI bound in the alternate cavity will allow for better quantification of the surface distortions.

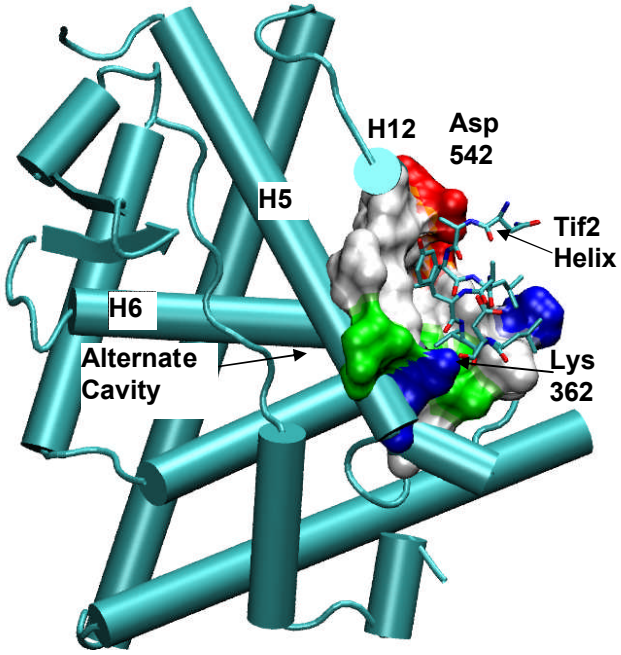


Figure 3: The ER-LDB with a bound co-activator peptide NR2 from TIF2. The ER protein backbone is colored in cyan and shown in cartoon with cylinders and arrows signifying α-helices and β-strands, respectively. The peptide is drawn in stick representation. Surface residues of the ER protein that interact with the peptide are shown as surfaces colored by residue type as described in figure 2. Arrows show positions of the charge clamp residues, Asp 542 and Lys 362.

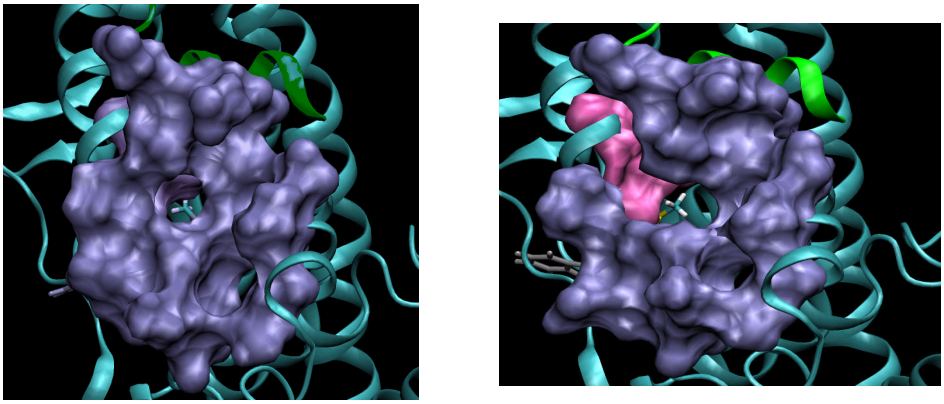


Figure 4: The ER-LDB before molecular dynamics and after 2ns of simulation time. The protein backbone is shown in ribbon representation and colored in cyan. Methionine 357 is shown in stick representation. Surface residues that interact with the helical peptide of the co-activator protein are in blue. Residues that become accessible to solvent after simulation are shown in magenta. The difference in exposed surface area of the protein is about 200 A2. Before the simulation is started the sidechain of Methionine 357 points in towards the alternate cavity were 3M5-PhIP is present. Early in the simulation, the sidechain switched positions to point out towards the solvent accessible surface. This disrupts at least one small hydrophobic pocket that is required for optimal co-activator binding.

(**Task 1.4**) The resulting combination of CBIs and estrogenic compounds are currently being re-ranked based on the MD simulations. We also received a 40,000 compound library to screen using our methods developed in this project and this work is continuing.

Task 2: Biologically assay the effect of the CBI on the estrogen receptor activation (Months 1-12)
Order and obtain the CBIs and estrogenic compounds. (Month 1)
Measure estrogen receptor activation by transfecting a reporter plasmid that contains 3 vitellogenin of estrogen response elements upstream of a luciferase reporter gene in MCF-7 human breast cancer cells. Receptor activity will be measured in the presence estradiol and each individual CBI. (Month 2-3)
Compare the effect of the CBIs on estradiol to their effect on the other estrogenic compounds analyzed in the docking program. (Months 4-6)

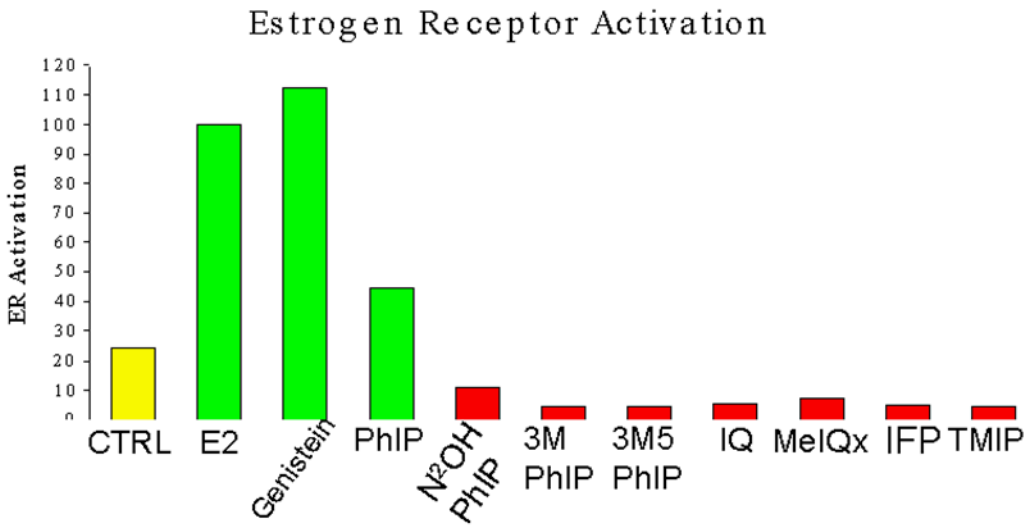
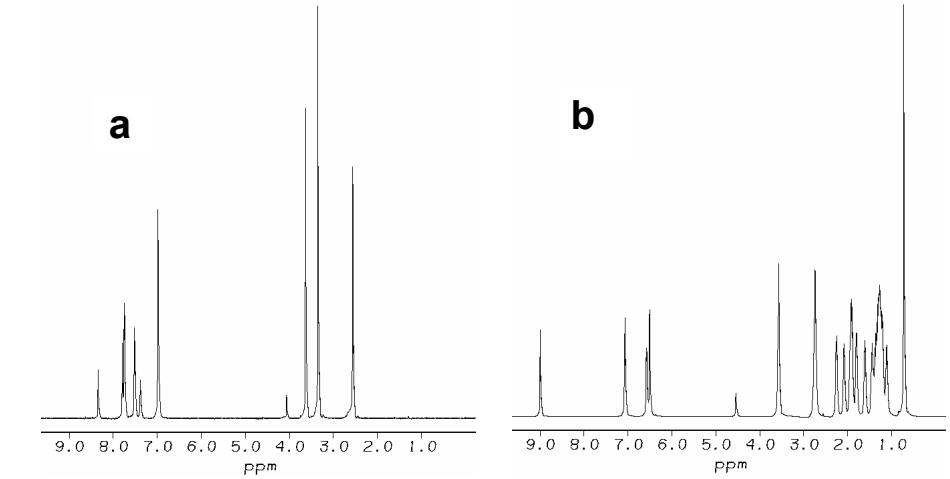

Estrogen Receptor Activation
ER Activation
120
110
100
90
80
70
60
50
40
30
20
10
0
CTRL
E2
Genistein
PhIP
N2OH PhIP
3M PhIP
3M5 PhIP
IQ
MeIQx
IFP
TMIP

Figure XXX

Task 3: Spectroscopically measure the binding of the CBI to the ER LBD

Full length, recombinant human estrogen receptor α (hERα) is commercially available through Sigma Aldrich, Inc. It is supplied as 1.5 μM purified protein (750 pmoles in 500 μl of 50 mM tris-HCl, pH 8.0, 500 mM KCl, 2 mM EDTA, 1 mM sodium orthovanadate and 10% glycerol). Recombinant hERα is produced as an active, soluble 66.4 kDa protein by a baculovirus expression system, and thus possesses post-translational modification. For NMR binding studies, it was first necessary to determine alternative buffer conditions in which the ER protein remained stable. Both tris and glycerol contribute large proton signals in the NMR data that interfere with the detection of submicromolar quantities of sample. In addition, high salt concentrations can result in protein precipitation when small volumes of compounds, such as PhIP and E2, which are only soluble in organic solvents such as DMSO, are added. After numerous trials, we had determined that the protein was able to remain stable for a few days after being exchanged after extensive dialysis (Spectrapor 7, MWCO 1000 dialysis tubing) into 10mM Na_2HPO_4, pH 8.0, 120mM NaCl, 2.7mM KCl (PBS) buffer (**Task 3.1**).

Various NMR spectroscopic techniques can be used to screen for interactions between proteins and ligands (reviewed in (Coles et al., 2003 and Meyer and Peters 2003)). Transferred NOESY (trNOESY) experiments are routinely used to detect ligand binding to a target protein under conditions of fast exchange (ligands that bind with mM to mM dissociation constants). The intensity of each intra-ligand NOE crosspeak is governed by the population-weighted cross-relaxation rate. A strong negative NOE crosspeak is observed for binders (black peaks), as opposed to weakly positive (red peaks) or zero NOE crosspeaks for non-binders or in the absence of protein. Thus, the sign flip of the NOE cross peak between the free versus bound states acts as a simple binary filter to distinguish binders from nonbinders.



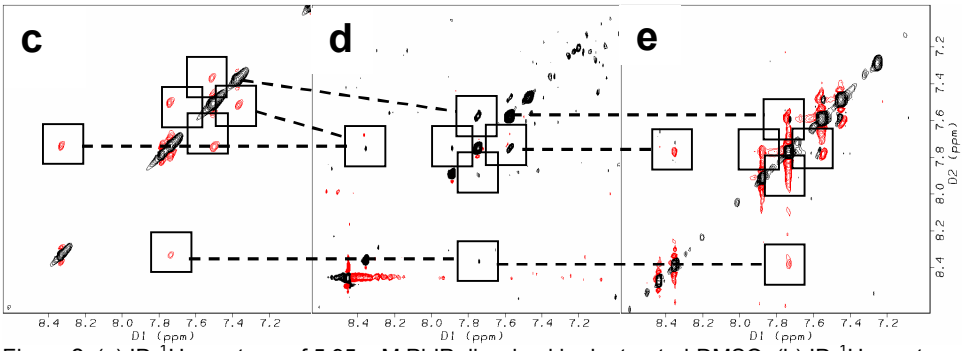


Figure ?: (a) ID-^{1}H spectrum of 5.95 mM PhIP dissolved in deuterated DMSO. (b) ID-^{1}H spectrum of 132 mM estradiol dissolved in deuterated DMSO.(c) The expanded region of a 900 ms mixing time NOESY spectrum of 5.95 mM PhIP in DMSO exhibits weak positive NOE crosspeaks (boxed in red). (d) The expanded region of a 300 ms mixing time NOESY spectrum of 3 μM ER and 120 μM PhIP indicates that PhIP binds as evidenced by the strong, negative crosspeaks (boxed in black).Concentration of PhIP is 50X greater in (c) than in (d). Spectrum in (d) is plotted at 2 X lower intensity level relative to spectrum in (c) for presentation purposes. (e) The expanded 300 ms mixing time NOESY spectrum after addition of 1.5 μM estradiol to the ERα+PhIP sample shows that PhIP does not bind (red peaks). Spectrum (e) is plotted at 2X lower intensity level relative to spectrum in (d). This result suggests that PhIP has been displaced from binding to ERα by estradiol, and that both ligands may be binding in the same site. Note that the chemical shifts of PhIP change depending on solvent (DMSO in spectrum (c) versus aqueous buffer in spectra (d) and (e)).

We observed a sign flip in NOE crosspeaks of PhIP from positive (red) to negative (black) in the trNOESY spectra when PhIP was added to ERα, confirming experimentally that PhIP does indeed bind to ER (**Figure ?c and d**) (**Task 3.2**). Since estradiol is known to bind with high affinity to ERα (K_d ~ 10^{-8}-10^{-9} M), we could not use the trNOESY method to detect its binding to ERα. However, since the estradiol's binding site on ERα is known, it is possible to use this information to design a trNOESY competition experiment that can provide information about where PhIP is binding. If PhIP is binding in a different site than estradiol, than we should be able to still observe negative (black) peaks because PhIP and estradiol are binding in two different sites, and estradiol is not displacing PhIP binding. In contrast, if PhIP is binding in the same site as estradiol, then adding estradiol to the PhIP/ERα mixture should competitively

displace it since estradiol binds much more strongly to ERα. In this case, we would observe another sign flip of the PhIP peaks from black (binding) to red (not binding). Addition of estradiol to the PhIP/ERα mixture resulted in PhIP cross peaks flipping sign from black to red **(Figure ?d and e)** and thus suggesting that both PhIP and estradiol are binding in the same site on Erα (**Task 3.3**). This result is consistent with the results shown by us and others that PhIP exposure increases MCF-7 cell proliferation and ERα activation. It also agrees with our computational model and with the idea that PhIP directly binding to ERα is responsible for the effects observed. However, we can not completely rule out the possibility that PhIP is binding in another site, and that estradiol prevents PhIP from binding because of a conformation change in the protein.

Thus in order to further clarify the identity of the ligand binding site, we initiated the expression of ^{15}N-labeled ERα ligand binding domain (LBD) (**Task 3.4**). Dr. Myles Brown (Harvard University) has generously provided us with the expression vector for the hERα-LBD). The GST-LBD fusion protein exhibits binding affinity to estradiol (K_D ~ 0.1 to 3.3 nM) that is comparable with reported values measured for wild-type MCF-7 ERα expressed *in vivo* (Halachmi et al., 1994).

The hERα-LBD is expressed as a GST fusion protein in BL21(DE3) cells. Cell growth is carried out for 16 hours at 37 $^{\circ}$C with shaking at 300 rpm (INOVA shaker) using either an autoinduction method, as introduced by Studier (Studier, 2005) or with a modified version of the autoinduction method described specifically for NMR studies (Studier, 2005; Tyler et al., 2005). Cells are harvested by centrifugation and lysed by sonication on ice in a buffer containing 50mM Tris-Cl, pH 7.5, 100mM NaCl, 20mM β-mercaptoethanol, 0.5% NP-40, followed by centrifigation at 30,000g at 4 $^{\circ}$C for 30min to remove cell debris.

Although the expression yields are very high for the GST-ERα-LBD protein, as can be seen in **Figure ??**, the protein is insoluble. Currently, we are in the process of modifying the expression protocol (addition of glycerol, tween and other detergents, as well as lower temperature) to increase the solubility of the protein. Once this is accomplished, the resulting supernatant will then be diluted and bound to a glutathione sepharose 4B column (Amersham) (Fabbro et al., 1999). After several washes with PBS buffer, the hERα-LBD protein will be cleaved from GST and eluted from the sepharose-GST column by treatment with thrombin (Amersham). Yields will be determined by the Bradford protein assay (BioRad). The purified protein will then be concentrated into PBS buffer, pH 7.4 by centrifugation using either a Centricon YM-3 or Ultra-4 concentrators (Amicon) (Tyler et al., 2005).

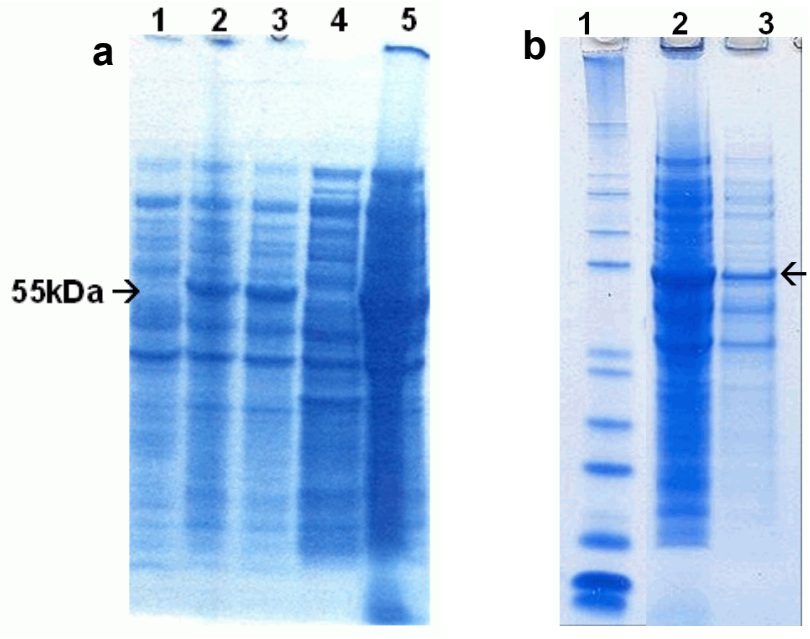


Figure ??: Polyacrylamide gel electrophoresis (4-12%) results showing high expression levels of GST ERα-LBD. (a) Most of the protein is in the insoluble fraction (lane 1: GST-ERα-LBD uninduced; lane 2: induced 1hr. @ 37C; lane 3: induced 2hrs. @ 37C; lane 4. clear lysate (soluble fraction); lane 5. insoluble fraction. (b) ER expression in autoinducing minimal media, (lane 1: molecular weight markers; lane 2: Grew ~16hrs, 37C, 300rpm, cell lysate; lane 3: 10 times dilution).

The availability of ^{15}N-labeled ERα-LBD protein will allow us to carry out Structure Activity Relationship by NMR (SAR-by-NMR) studies (Hajduk et al., 1997) that will clearly define the binding site and orientation of not only PhIP, but other CBIs and HAs.

Task 4: Submit manuscript for publication.

A large portion of the work detailed above in Tasks 1-3 has been submitted and accepted for publication in the American Chemical Society journal, Chemical Research in Toxicology. At the time of this report, the projected publication date is October 5th 2005. We anticipate submission of a second manuscript describing the characterization of the co-activator binding site interactions with ligands by ^{15}N NMR and molecular dynamics simulations.

Key Research Accomplishments

Posters and Accepted Abstracts:

- 19th National Meeting of the Protein Society San Diego CA, August 2004.
- AACR Anaheim CA, March 2005.
- DOD BCRP Meeting "Era of Hope" Philadelphia PA, June 2005.
- 36th Meeting of The Environmental Mutagen Society San Francisco CA, September 2005.

Manuscripts:

- "PhIP Carcinogenicity in Breast Cancer:Computational and Experimental Evidence for Competitive Interactions with Human Estrogen Receptor " in press Chemical Research in Toxicology, Oct 2005.

Reportable Outcomes

Presentations:

- Biosciences Directorate Symposium LLNL, November 2004.
- Biosciences Directorate Postdoctoral Symposium LLNL, July 2005.

Invited Talks-Lectures:

- University of California-Davis Cancer Center, Sacremento CA, January 2005.
- Chemistry Department University of the Pacific, Stockton CA, February 2005.
- Bio-engineering Department University of the Pacific, Stockton CA, March 2005.
- Edward Teller Education Center, Lawrence Livermore Natl. Lab., July 2005.

NIH R01 grant entitled "Dietary exposure to multiple heterocyclic amines may cause fewer breast tumors than exposure to single carcinogens" to be submitted October 1, 2005.

Conclusions

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JOURNAL OF MEDICINAL CHEMISTRY 45 (3): 584-589 JAN 31 2002

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Appendix A

PhIP Carcinogenicity in Breast Cancer: Computational and Experimental Evidence for Competitive Interactions with Human Estrogen Receptor

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Running Title: Heterocyclic amines in breast cancer
Keywords: PhIP, Heterocyclic amines, Breast Cancer, Molecular Modeling, Estrogen Receptor Activation
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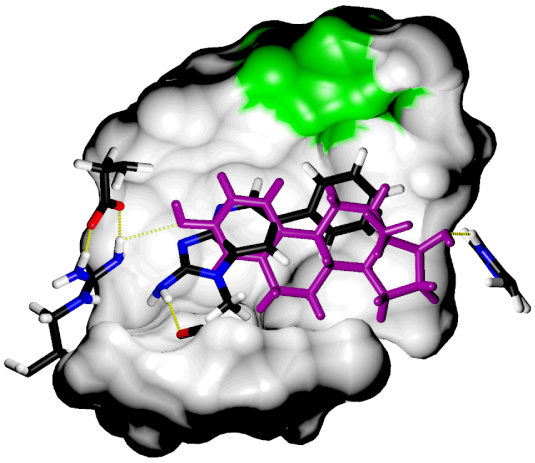
Manuscript Information: 18 text, 5 figure pages
Character Count: 32417 Word Count: 5016
Abstract: 201 words
Classification: Cell and Tumor Biology
Abbreviations:
ERα human estrogen receptor alpha
LBD ligand binding domain
E2 estradiol
TrNOESY transferred nuclear Overhauser effect spectroscopy
PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine)
MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline)
IFP (2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine)
N^2-hydroxy-PhIP (2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine)



Abstract

Many carcinogens have been shown to cause tissue specific tumors in animal models. The mechanism for this specificity has not been fully elucidated and is usually attributed to differences in organ metabolism. For heterocyclic amines, potent carcinogens that are formed in well-done meat, the ability to either bind to the estrogen receptor and activate or inhibit an estrogenic response will have a major impact on carcinogenicity. Here we describe our work with the human estrogen receptor alpha (ERα) and the mutagenic/carcinogenic heterocyclic amines PhIP, MeIQx, IFP, and the hydroxylated metabolite of PhIP, N^2-hydroxy-PhIP. We demonstrate that PhIP binds with the ligand binding domain (LBD) both by computational docking and NMR analysis. This binding competes with estradiol (E2) in the native E2 binding cavity of the receptor. In *in vitro* assays, we find that PhIP, in contrast to the other heterocyclic amines, increases cell-proliferation in MCF-7 human breast cancer cells and activates the ERα receptor. We also find that other heterocyclic amines and N^2-hydroxy-PhIP inhibit ERα activation. We propose that the mechanism for the tissue specific carcinogenicity seen in the rat breast tumors and the presumptive human breast cancer associated with the consumption of well-done meat maybe mediated by this receptor activation.

Introduction

Changes in breast cancer incidence among immigrant populations suggest that lifestyle factors, including diet, may be an important cause of the disease and a potent clue for treatment. One dietary modification that is frequently found among immigrants from eastern to western countries is an increase in the consumption of cooked muscle meats. Well-done cooked muscle meats are known to contain potent mutagens and mammary carcinogens (e.g. in rodents) belonging to the heterocyclic amine (HA) class of chemical compounds (Felton et al., 2004). Three of the heterocyclic amines commonly found in meats cooked under household conditions are: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and 2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine, (IFP). The relative amounts of these compounds formed during cooking depend on both meat type and cooking conditions (Knize et al., 1998). PhIP is frequently the most mass abundant heterocyclic amine produced during the cooking of beef, pork, and chicken, (Keating et al., 2000; Knize et al., 1998; Norrish et al., 1999; Pais et al., 2000; Sinha et al., 1995; Skog et al., 1997; Wakabayashi et al., 1992). Humans are routinely exposed to varying amounts of these food-derived compounds, and there are studies supporting their role in human carcinogenesis (Knize and Felton, 2005).

When given in the diets of rats, PhIP has been shown to cause the formation of hormone-dependent mammary tumors (Ito et al., 1991). A powerful liver carcinogen, MeIQx also causes breast tumors in Sprague-Dawley rats but is a much less potent mammary carcinogen than PhIP (Snyderwine, 2002; Wakabayashi et al., 1992). IFP has been shown to be a potent mutagen, but has not yet been tested for carcinogenicity in an animal model. Although the mechanism of carcinogenesis for these compounds

has not been fully elucidated, metabolic activation and subsequent formation of DNA adducts is believed to be critical (Snyderwine, 2002; Snyderwine et al., 2003). The metabolic activation of PhIP, a two-phase process, is representative of the activation pathways of the other HAs. During Phase I metabolism, PhIP is oxidized via cytochrome P4501A2 (CYP1A2) to a hydroxylated intermediate, 2-hydroxyamino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (N^2-hydroxy-PhIP). N^2-Hydroxy-PhIP is then converted to a more biologically reactive form via Phase II metabolizing enzymes, primarily the acetyltransferases or sulfotransferases. This esterification generates electrophilic *O*-sulfonyl and *O*-acetyl esters, which have the capacity to bind DNA and cellular proteins.

In addition to diet, hormonal factors also play a role in mammary gland tumorigenesis. Estrogen receptor α (ERα), a member of the nuclear receptor family, regulates estrogen-responsive gene expression upon ligand binding. Structurally, the ERα protein contains four major domains: activation, DNA binding, hinge, and ligand binding domains (LBD). Crystal structures of LBD-ERα with agonist and antagonists bound have been published (Eiler et al., 2001; Henke et al., 2002; Pike et al., 1999; Pike et al., 2001; Renaud et al., 2003; Ruff, 1999; Shiau et al., 1998; Shiau et al., 2002; Warnmark et al., 2001). Ligand dependent ERα activation begins with 17-β-estradiol (E2) binding the ERα monomer and releasing protein chaperones from the receptor (Pratt and Toft, 1997). With E2 bound, ERα monomers then dimerize (Kumar and Chambon, 1988) and interact with co-activator and co-repressor proteins (Moras and Gronemeyer, 1998). Transcription begins when all factors have been recruited and ERα binds to the estrogen response element (ERE) of the specific gene.

Because exposure to PhIP alone is sufficient to cause tumors in rodent models, PhIP may be important in mammary tumor development beyond general DNA adduction and tumor initiation. Exposure to PhIP has been shown to increase cell proliferation in mammary gland terminal end buds, potential sites of tumor development, suggesting that PhIP promotes tumorigenesis by causing the further replication of PhIP-DNA adducts in target cells (Snyderwine, 1999). This potential role in promotion is strengthened by the findings of Pfau et al. and Gooderham et al. demonstrating that PhIP exposure increases human breast cancer cell proliferation and activates ERα (Gooderham et al., 2002; Pfau et al., 2000) at concentrations that are less than those required for mutagenic activity. Gooderham and coworkers have also shown that the pure anti-estrogen ICI 182,780 abolishes PhIP transcriptional activity, further suggesting that PhIP binds specifically to ERα (Lauber et al., 2004). However, in studies on ovarectomized Sprague-Dawley rats, PhIP did not produce any estrogenic response (Kawamori et al., 2001), suggesting that PhIP is not a strong estrogen mimic in this assay system. Other studies propose that PhIP promotes carcinogenesis by retarding mammary gland development (Snyderwine et al., 1998), increasing serum prolactin levels (Venugopal et al., 1999b) and inhibiting apoptosis (Venugopal et al., 1999a).

Here, we are the first to report that (1) PhIP binds directly to the ERα-ligand binding domain and competes with estradiol in the native binding cavity, and (2) the PhIP phase I metabolite, N^2-hydroxy-PhIP, and two heterocyclic amines, MeIQx and IFP, inhibit ERα activation. We also confirm the proliferative effect of PhIP on MCF-7 cells and the estrogen receptor activation shown by Lauber et al. (Lauber et al., 2004).

Taken together, these results suggest that PhIP activates the estrogenic response via direct binding to ERα, thereby explaining its tumor specificity.

Methods

Ab initio calculations

Each ligand (E2, PhIP, N^2-hydroxy-PhIP, IFP, and MeIQx) was geometry optimized at the RHF 6-31G* level with Gaussian 98, revision A.11.4 (Frisch M. J. et al., 2002) (Fig. 1A). Default settings for convergence criteria were satisfied for each ligand. Atomic partial charges for the ligands were determined by electrostatic potential fitting (Wang et al., 2000).

Homology Modeling

A high-resolution crystal structure of the ERα ligand binding domain (1GWR, (Warnmark et al., 2002)) was chosen from the Protein Data Bank to model protein-ligand interactions in docking. There were residues in two loops and other atoms in the structure where electron density was not present in chain A (Warnmark et al., 2002). These loops and missing atoms were modeled by aligning 1GWR to another ER structure (PDB ID 1G50), using an in-house alignment program, LGA (Zemla, 2003). The primary sequences of the modeled loops were identical between structures. Appropriate hydrogen atoms were added to the structure using the CHARMM 27 force field (MacKerell et al., 1998).

Docking

The estrogen receptor protein (1GWR homology model) and ligands (E2, PhIP, N^2-hydroxy-PhIP, and MeIQx) were prepared for the docking studies as described in the Autodock manual (Morris et al., 1998). The original source code was modified to allow a grid cube size of 128 points with a 0.375 Å grid spacing. The resulting grid covered ~80% of the ligand binding domain. Using the lamarkian genetic algorithm option, the total number of docking steps was set to 200 and cluster analysis was activated. Each ligand pose was then grouped according to its position on the protein. A Boltzmann probability, *Ki*, was then derived from the calculated free energy ΔG_i, gas constant *R*, and temperature, *T* (30° C) of each pose, *i*.

$$K_i = e^{(-\Delta G_i / RT)} \qquad \text{Equ. 1}$$

Docking poses in each grouping, *s*, were then weighted according to Equ. 2.

$$p_i = \frac{\sum_{i,s} K_{i,s}}{\sum_i K_i} \qquad \text{Equ. 2}$$

Here, p_i, is the weighted probability of finding pose, *i*, in grouping, *s*. These probabilities were then summed for each category.

Transferred Nuclear Overhauser Effect Spectroscopy (trNOESY)

All spectra were recorded at 30° C using a Varian Inova 600 MHz spectrometer. ^{1}H-1D and phase sensitive 2D-nuclear Overhauser effect spectroscopy (NOESY) experiments (900 ms mixing time) were carried out on 1.0 mg of PhIP (5.94 mM) or 27 mg of E2

(132.1 mM) dissolved in 750 μL deuterated DMSO) in the absence of ERα protein (Fig. 1B, C). Human, recombinant ERα protein was purchased from Sigma-Aldrich as 750 pmols of purified protein in 500 μL 50 mM Tris-HCL, pH 8.0, 500 mM KCL, 2 mM DTT, 1mM EDTA, 1 mM sodium orthovanadate and 10% gylcerol. We first determined alternative buffer conditions in which the ERα protein remained stable. Both Tris and glycerol contribute large proton signals in the NMR data that interfere with the detection of micromolar quantities of PhIP and E2. In addition, high salt concentrations are not compatible with adding small volumes of PhIP and E2, which are only soluble in organic solvents such as DMSO, and results in precipitation of the protein. We determined that ERα remained stable for several days after being exchanged (using dialysis, MWCO=10,000) into 50 mM Na_2PO_4, pH 8.0, 200 mM KCL, 1% β-mercaptoethanol. Transferred NOESY (trNOESY) experiments (300 ms mixing time) were used to detect whether binding occurred upon addition of 40 molar excess of PhIP (120 μM) to a 3 μM solution of ER in NMR buffer. TrNOESY is useful in detecting binding when the ligand is in fast exchange between the free and bound states (10^{-2}-10^{-6} M range for the dissociation constant). Adding molar excess of E2 to the solution of ER and PhIP results in precipitation. This is most likely due to the fact that E2 strongly binds ERα. To determine if E2 could displace PhIP from the binding pocket, 0.25 molar ratio of E2 (1.5 μM) was added to the 6 μM ERα protein-240 μM PhIP solution. 300 increments were collected in t_1, each with 128 or 256 scans and 1024 complex data points collected in t_2. The total amount of DMSO in the protein/ligand samples did not exceed 2%. NMR data was processed using VNMR software (Varian Inc., Palo Alto CA), and the 2D frequency domain matrices were analyzed using FELIX (version 97, Accelrys, San Diego, CA).

Cell Culture

MCF-7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were grown in DMEM with 5% FBS, 1% non-essential amino acids, 10 µg/ml insulin, 2 mM L-glutamine, and 1% penicillin/streptomycin. All tissue culture supplies, with the exception of the charcoal stripped fetal bovine serum, were obtained from Invitrogen (Carlsbad, CA). Cells were maintained at 37°C with 5% humidity. For proliferation assays and estrogen responsive reporter assays, Opti-MEM (phenol red-free medium) was used with 5% charcoal stripped fetal bovine serum (Clontech, Palo Alto CA) to minimize estrogen-like activity contributed by the phenol red and serum (Soto et al., 1995; Zacharewski, 1998).

Cell proliferation assay

For cell proliferation assays 3 x 10^3 cells were seeded into each well of a 96-well tissue culture plate in Opti-MEM medium. Twenty-four hours after plating, the medium was replaced with medium supplemented with increasing concentrations of E2 (Sigma, St. Louis MO), PhIP (Toronto Research Chemicals, Downsview, Ontario), MeIQx (Toronto Research Chemicals, Downsview, Ontario) and IFP, treated with the appropriate concentration of vehicle or left untreated. IFP is a natural product isolated from a heated mixture of creatine, glutamic acid and sucrose (Pais et al., 2000). Heterocyclic amines were initially dissolved in DMSO and then serially diluted in medium. DMSO concentration was never greater than 0.5%. Ethanol was used to

dissolve E2. The effect of the heterocyclic amines was determined by comparing cell growth to the appropriate vehicle-treated cells and untreated cells.

The effect of test compounds on cell growth was evaluated 48 and 72 hours after the initiation of treatment. Cell growth was quantified using an Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison WI) with absorbance of the wells measured in a standard multi-well plate reader at 490nm. Briefly, this colormetric assay measures the bioreduction of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) to a formazan product. The conversion of MTS is directly proportional to the number of living cells in culture. Each experiment was performed at least three individual times with 4 – 6 replicates per experiment. Effect of chemical was determined by comparing cell growth to the appropriate vehicle-treated cells and untreated cells. Error is presented as the standard error of the mean. Statistical significance was assessed using Wilcoxon Rank Sum Test.

- *Estrogen Responsive Reporter Assay*

Heterocyclic amines were tested for estrogenic and anti-estrogenic activity using a standard estrogen responsive reporter plasmid containing three vitellogenin estrogen responsive elements (EREs) upstream of the luciferase reporter gene (EREx3-Luc). The EREx3-Luc was a kind gift of Diane Klotz, NIEHS. EREx3-Luc was co-transfected with a commercially available control renilla reporter plasmid (pRL-TK, Promega, Madison WI) to control for transfection efficiency and for the normalization of the results. The reporter plasmids were transfected into MCF-7 cells (Klotz et al., 1996; Nodland et

al., 1997; Reel et al., 1996; Safe et al., 1998), which constitutively express ERα and have well-characterized responses to estrogenic stimuli. The ability of the compounds to induce luciferase reporter gene activity was compared to 10^{-9} M E2 and appropriate negative controls, as previously described (Charles et al., 2002).

MCF-7 cells were plated in 96-well plates, designed specifically for use in a luminometer (Perkin Elmer, Wellesley, MA) at a density of $1X10^5$ cells/well in Opti-MEM medium. The cells were left untransfected or were co-transfected with 200 ng each of luciferase reporter and renilla control plasmids using lipofectAMINE™ 2000 (Invitrogen, Carlsbad, CA) optimized for 96-well plate experiments according to directions. After a 5-hour transfection, the untransfected cells were incubated with just culture medium to quantify background levels. The transfected cells were incubated with the same culture media alone or supplemented with heterocyclic amines, E2 or both. Control cells were incubated with a corresponding amount of compound solvent at the highest concentration.

The cells were incubated at 37°C for 18 hours and then assayed for luciferase and renilla activity using a dual luciferase/renilla reporter assay kit (Promega, Madison, WI) according to directions. Each experimental condition was duplicated six to eight times in the 96-well plate and each plate was read in the luminometer twice. Luciferase activity was normalized to the renilla controls and compared to 10^{-9} M E2, standard in estrogen responsive luciferase assays. Estrogen receptor-mediated activation was determined by comparisons to negative and E2 controls. All experiments were repeated more than 3 times and all data were normalized to E2 (10^{-9}M) activation. For competition assays, HAs (alone or in combinations) and/or E2 were added

simultaneously. Error is presented as the standard error of the mean. Statistical significance was assessed using Wilcoxon Rank Sum Test.

Results

Docking Studies

Initial docking of E2 to the ERα LBD as a control calculation reveal the ligand pose as seen in the 1GWR crystal structure, with the initial position of the ligand at least 15 Å outside of the native binding cavity. PhIP and N^2-hydroxy-PhIP were manually docked in the same orientation as E2 to match the crystal structure (Brzozowski et al., 1997), where the exocyclic amine and imidazole ring were overlaid on the E2 hydroxyl group and 'A-ring', respectively. These initial structures started the docking trials of PhIP, N^2-hydroxy-PhIP, IFP, and MeIQx, using the same grid of the protein as for E2. Eleven groupings contain 99% of all docking poses for each HA. For E2 and PhIP, the E2 binding cavity accounts for ~98% and ~89% of the total binding probability, respectively. PhIP binds favorably into the E2 binding site although the calculated probability of binding is lower when compared to E2 (Fig. 2A). Several poses of PhIP are observed in the E2 binding site. One pose is rotated by 180° with the phenyl ring pointing toward residues Glu353 and Arg394. However, the most favorable pose places the exocyclic amine proximal to the hydrogen bonds between Glu353 and Arg394, similar to the initial docking position and E2 pose in the co-crystal (Brzozowski et al., 1997) (Fig. 2B). Side-chains from Leu387, Leu391, and Phe404 make hydrophobic contacts with each face of the imidazo ring. A possible hydrogen bond between the exocyclic primary amine and the carbonyl of Leu387 is also observed. The donor-H-

acceptor angle is greater than 150°, and the hydrogen bond distance is less than 2.6 Å (Fig. 2B). The phenyl ring of PhIP is further stabilized by hydrophobic contacts with Leu346 on one face and by Met343 and Met421 around the edge of the ring. The face of the phenyl ring opposite Leu346 is open to solvent. Furthermore, the phenyl ring is turned relative to the rest of the molecule, which allows for a better fit to the binding cavity wall and is consistent with the geometry-optimized structure (Fig. 2B).

Two significant groupings of N^2-hydroxy-PhIP, IFP, and MeIQx are observed, one in the native E2 binding site and another 11 Å away, close to helix 5. The lowest energy pose of N^2-hydroxy-PhIP is in the E2 binding site and is in the same orientation as PhIP (i.e. all hydrophobic contacts were the same). However, the hydroxyl group does not form any hydrogen bonds with protein side-chains or backbone. Instead of forming any obvious hydrogen bonds, the hydroxyl group was further buried into a solvent channel under the Glu353•••Arg394 hydrogen bond bridge.

The planar and rigid IFP molecule assumes the same basic orientation of PhIP and N^2-hydroxy-PhIP; the exocyclic amine of IFP is pointed toward the Glu353•••Arg394 hydrogen bond bridge. A hydrogen bond is also observed between the primary amine of IFP and the carbonyl oxygen of Leu387. The binding orientation for MeIQx in the E2 binding cavity is the opposite that of PhIP and N^2-hydroxy-PhIP because the exocyclic amine points toward His524, similar to the orientation of the d-ring of E2 in the crystal structure (1GWR). We observe a hydrogen bond with the side-chain hydroxyl group of Thr347 and the exocyclic amine from MeIQx. In both IFP and MeIQx, the fused rings do not appear to make optimal hydrophobic interactions in comparison with PhIP and N^2-hydroxy-PhIP.

NMR Ligand Binding Studies

We performed transferred NOESY (trNOESY) experiments (Clore et al., 1982; Gronenborn and Clore, 1982; Roberts, 1999) to detect PhIP binding to ERα protein. An observed sign flip in NOE crosspeaks of PhIP from positive (red) to negative (black) in the TrNOESY spectra (Fig. 3A, B), is an indication of binding to ERα protein. Adding E2 to the PhIP/ ERα mixture causes the sign flip in the NOE crosspeaks to revert back to positive (red), indicating that PhIP is no longer bound to ERα (Fig. 3C).

MCF-7 Human breast cancer cell assays

Incubating ATCC MCF-7 cells with$1x10^{-9}$M to$1x10^{-7}$M E2 for 48 hours produces a slight increase in cell proliferation (7-12%) that is significantly different from vehicle-treated cells. By 72 hours the increase in cell number is 20% above control cells and by 96 hours the increase has risen to 40% (data not shown). This increase in cell number is consistent with other reports of proliferation response for this MCF-7 cell line (Rasmussen and Nielsen, 2002). Adding increasing doses of PhIP for 48 hours also causes a 7-10% increase in proliferation that is significantly different from control cells, but not significantly different from E2 at similar concentrations (Fig. 4). By 72 hours the increase in proliferation reaches 20% for $4x10^{-8}$M PhIP, which is not significantly different from the increase in cell number caused by a similar concentration of E2 (data not shown) cells treated with N^2-hydroxy-PhIP show a slight, non-significant, increase in cell growth at low doses ($4x10^{-9}$M). However at higher concentrations ($4x10^{-6}$M), cell

growth is inhibited. Treating cells with concentrations of MeIQx and IFP higher than $4x10^{-8}$M for 48 hours causes a significant decrease in cell growth (Fig. 4).

A standard luciferase reporter assay was used to measure the effect of HAs on ERα-dependent transcription activity. This assay relies on the ERα normally expressed by the MCF-7 cells. Active ERβ is not expressed in MCF-7 cells (Bardin et al., 2004; Cestac et al., 2005; Paruthiyil et al., 2004; St-Laurent et al., 2005), and therefore ERβ interactions cannot contribute to the results. As shown in figure 5A, increasing amounts of PhIP causes a dose-dependent increase in ERα activity that is significantly different than untreated cells. In contrast, increasing amounts of N^2-hydroxy-PhIP and the other HAs do not show this activation. Competition assays were used to determine the effect of simultaneous incubation of HAs ($4x10^{-7}$M) and 10^{-9} M E2 on ERα activation (Fig. 5B). Treating MCF-7 cells with PhIP and E2 produces a response that is 13% less than E2 alone. N^2-Hydroxy-PhIP inhibits the E2-mediated activation of ERα by 40%, and MeIQx and IFP inhibit ERα activation to levels that are significantly lower than untreated cells. Cell viability was measured under identical conditions and is not affected by incubation with HAs separately or in combination with E2 at these concentrations (data not shown).

Effect of HA combinations on ERα activity

Competition assays were performed with PhIP, N^2-hydroxy-PhIP, and MeIQx to determine the effect of simultaneous exposure to $4x10^{-7}$M PhIP and increasing amounts of the other compounds (Fig. 5C). Treating MCF-7 cells with either a 1:1 or 1:3 molar ratio of PhIP to other compounds significantly inhibits ERα transcription activity to levels that are lower than cells exposed to PhIP alone.

Discussion

Heterocyclic amines are known to be potent mutagens and rodent carcinogens, but the complete mechanism of carcinogenicity for these compounds has not been elucidated. This report is the first to show that PhIP binds directly to the ERα-ligand binding domain and competes with estradiol in the native binding cavity. We also demonstrate the novel finding that N^2-hydroxy-PhIP, the PhIP phase I metabolite, and two heterocyclic amines, MeIQx and IFP, inhibit ERα activation. Among the HAs tested, activation of the receptor is limited to PhIP, and other HAs can act as potential anti-estrogens. We also show that PhIP increases MCF-7 cell proliferation and activates ERα dependent transcription, in agreement with results published by Lauber et al. (Lauber et al., 2004).

PhIP Binding to Erα

The docking of E2 to the ERα LBD validates our model of ERα LBD and the docking algorithm for our system because the most stable E2 pose found was the same orientation as the co-crystal E2 conformation. The most favorable binding pose of PhIP was also found in the E2 binding site, even though the probability of PhIP binding is lower than E2 binding to the E2 site. This lower binding probability is consistent with the known high binding affinity of E2 to ERα ($K_d \sim 10^{-9}$-10^{-10} M) and our NMR results show E2 competing out PhIP (see below).

We designed an NMR competition experiment implementing trNOESY techniques that provides information about the PhIP binding site on the ERα protein. If PhIP binds the same site as E2, then adding E2 to the PhIP/ERα mixture should displace PhIP since E2 binds more strongly to ERα than PhIP (as predicted computationally). If PhIP is binding in a different site than E2, then the negative (black) PhIP-bound peaks should remain. When E2 is added to the PhIP/ ERα mixture, a reversion in the NOE crosspeaks from the negative (black) back to positive (red) is observed, indicating that PhIP is no longer bound to ERα. These data highly suggest that PhIP is binding to the ligand-binding domain. However, another possibility is that PhIP is binding in another site, and E2 is preventing PhIP from binding because of a conformation change in the protein. Although this possibility may exist, the computational prediction of PhIP binding in the E2 binding site and our *in vitro* data on stimulated cell proliferation (see below) leads us to the conclusion that PhIP is binding in the E2 binding site competitively.

The effect of the HAs on the proliferative activity of whole cells was tested using a modified E-SCREEN assay (Rasmussen and Nielsen, 2002). This assay is based on the ability of estrogen-responsive MCF-7 cells to proliferate in the presence of compounds that mimic estrogen. Although a standard assay for screening for estrogenicity, the proliferative response of the MCF-7 cells varies depending on the MCF-7 subline employed. In these studies we used ATCC wild-type MCF-7 cells, which have been shown to vary in their response to estrogen incubation (Rasmussen and Nielsen, 2002). Using these cells, we found that micromolar concentrations of PhIP were able to stimulate proliferation up to 40% above vehicle-treated cells over a 72-hour

incubation. Lauber et. al. determined that the same increase in cell proliferation could be caused by only nanomolar concentrations of PhIP (Lauber et al., 2004). These authors used MCF-7 cells obtained from the European Collection of Cell Cultures (ECACC) in their assays; presumably this MCF-7 cell variant is more sensitive to estrogen or estrogen-like compounds. Nevertheless, both studies demonstrated increased cell proliferation when incubated with PhIP.

After much discussion regarding the potential estrogenicity of PhIP and its relevance to tumor progression (Gooderham et al., 2002; Lauber et al., 2000; Pfau et al., 2000), we and others (Lauber et al., 2004) now confirm that PhIP activates ER and stimulates cell growth. Both E2 and PhIP are able to activate ERα-dependent transcription in MCF-7 cells transfected with a standard estrogen responsive reporter plasmid containing three vitellogenin EREs upstream of the luciferase reporter gene. In other words, PhIP is able to use the endogenous ERα present in MCF-7 cells to effect transcription. Thirty minute pre-incubation with the complete anti-estrogen ICI 182,780 inhibited this increased activity (data not shown) (Lauber et al., 2004), further suggesting that PhIP is effecting transcription through direct interaction with ERα. These results and the fold-activation of ERα transcription activity (compared to E2) in the presence of micromolar concentrations of PhIP are in agreement with previous work (Lauber et al., 2004).

The *in vitro* data, without the supporting NMR and computational data, could also be explained by PhIP acting on the ERα pathway at points upstream or downstream from the receptor protein. For the present studies, the trNOESY results, together with the *in vitro* data and docking results strongly support the idea that PhIP and E2 compete

for the same binding cavity within the ERα LBD. Taken together, these results demonstrate that PhIP initiates an estrogenic response in target cells by binding directly with the ERα.

N^2-Hydroxy-PhIP, MeIQx and IFP Inhibition of ERα

Close examination of our docking data shows that PhIP makes a hydrogen bond between the exocyclic-amine and the carbonyl-oxygen of Leu387 (Fig. 2B). *N^2*-Hydroxy-PhIP shares all the same hydrophobic contacts as PhIP. However, because of the non-optimal placement of the hydroxy group, no hydrogen bonds are made with the protein, decreasing its probability of binding in the E2 cavity (Fig. 2A). These data suggest that the driving force in binding in the E2 cavity is hydrophobic and that electrostatic interactions such as hydrogen bonds play only a secondary role.

In the cellular assays, N^2-hydroxy-PhIP inhibited the ability of E2 to activate ERα. When compared to PhIP, the docking data predicts that the binding of N^2-hydroxy-PhIP should be weaker. The cell assays confirm these predictions. When N^2-hydroxy-PhIP is co-incubated with physiological concentrations of E2, much of the ER activation remains, showing that N^2-hydroxy-PhIP is not a particulary effective inhibitor.

Like PhIP and N^2-hydroxy-PhIP, MeIQx and IFP both contain an exocyclic amine with a methyl group at the adjacent carbon. However, MeIQx and IFP contain three fused-heterocyclic rings, which limits hydrogen bonding and hydrophobic interactions compared to PhIP. The low barrier rotation of the phenyl ring in PhIP optimizes non-bonded contacts, which is not possible in MeIQx and IFP. Our docking data suggest that MeIQx and IFP do not bind favorably in the E2 binding cavity because the fixed

conformation of the fused-rings does not allow optimization of hydrophobic contacts. The lack of a favorable binding conformation is confirmed by the inability of these to compounds to stimulate reporter gene transcription in our in vitro cell assay (Fig. 5).

When MeIQx and IFP are co-incubated with physiological concentrations of E2, ERα activity is significantly inhibited. This inhibition is identical to results obtained when the compounds are added alone. Compared to N^2-hydroxy-PhIP, MeIQx and IFP are much more effective inhibitors so that E2 activity remains below control levels. These results suggest that the mechanism of binding for MeIQX and IFP are different than PhIP and N^2-hydroxy-PhIP; MeIQx and IFP may even bind in other regions of the ERα protein.

Implications of Diet and Metabolism

The estrogenic potency of PhIP is comparable to other environmental estrogens found in the diet. Genistein, a known phytoestrogen found in soy products, was compared to PhIP in ERα activation assays (data not shown). At low concentrations ($4X10^{-7}M$) there was no statistical difference in the activation of the ERα by either compound, but higher concentrations of genistein elicited a much greater ERα response than higher concentrations of PhIP. When investigating compounds from food, it is difficult to predict exposure levels for individual target cells. The concentrations of PhIP examined here are orders of magnitude higher than what a single cell may be exposed to after a meal of cooked meat. However, chronic exposure to PhIP over a lifetime may add to the total estrogenic burden of the body.

In addition, it is not clear what the biological consequence of being exposed to xeno-estrogens may be. Although exposure to environmental estrogens may increase

the activity of endogenous ERα, exposure to other compounds may inhibit the activation (anti-xeno-estrogens), effectively canceling out the effect of the xeno-estrogens. In fact, although exposure to PhIP may be stimulating ERα activity, simultaneous consumption of the other HAs, MeIQx and IFP, may diminish that effect. Our previous studies have shown that when meat is cooked, the ratios of formation of PhIP:MeIQx and PhIP:IFP varies according to cooking conditions (Knize et al., 1998). In figure 5C, 1:1 ratios of PhIP:MeIQx and PhIP:IFP mixtures significantly inhibit the ability of PhIP to activate ERα. If the ratios are increased to 1:3, both MeIQx and IFP completely prevent any stimulatory action by PhIP. When cells are exposed to combinations of HAs and metabolites, as would happen during dietary consumption of cooked meat, the PhIP-mediated activation of ERα can be abolished. Thus, the biological consequences of exposure to HAs may depend upon many complex factors, including the ratio of HAs formed in the meat, how well each compound is absorbed from the digestive tract, and the concentration of the compounds at the target cells.

Metabolism of the compounds will also contribute to the complexity of the overall human exposure. MCF- 7 cells contain active P450 metabolism; however most of the activity is CYP1A1. Our previous investigations have shown that these cells do not significantly metabolize PhIP (K. S. Kulp, unpublished results), suggesting that metabolites of the HAs are not confounding our measurements of the effects of the compounds on cell proliferation and ERα activity. However, in humans consuming cooked meats, hepatic and extra-hepatic metabolism of PhIP to N^2-hydroxy-PhIP is extensive. In fact, only a small percentage of an ingested and absorbed dose of PhIP is excreted as the parent compound; the rest is metabolites (Malfatti et al., 1999). Our

results show that co-incubation of PhIP and N^2-hydroxy-PhIP inhibits the stimulatory effect of PhIP on the ERα and that the amount of inhibition depends upon the amount of N^2-hydroxy-PhIP present in the incubation. This suggests that the estrogenic potential of PhIP may depend in large part on the extent of Phase I activation of the compound, either in the liver or at the level of the target cell.

Conclusions

Here we show by experiment and computational methods that the food mutagen PhIP can activate human ERα by binding to the LBD (Figs. 2, 3), and that PhIP stimulates MCF-7 breast cancer cell proliferation, suggesting a mechanism for tissue-specific carcinogenesis of this carcinogen. We also show that IFP, MeIQx, and the primary hydroxylated metabolite of PhIP do not stimulate breast cancer cell proliferation (Fig. 4). Moreover, MeIQx, IFP and N^2-hydroxy-PhIP inhibit estrogen receptor activation (Fig. 5). Together, these results imply that these dietary constituents may play an active role in both activating and inhibiting hormone sensitive cancers. More importantly, understanding the mechanisms of action for the inhibitory HAs may lead to potent therapeutics against breast cancer.

Acknowledgments

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Figure Legend

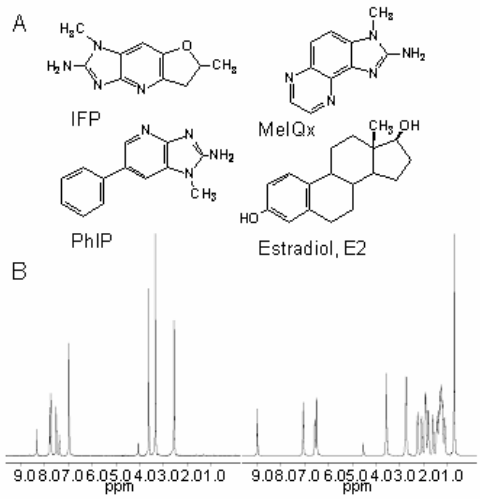
Figure 1. Structures of heterocyclic amines and E2 (A) focused on in this study and 1D-^{1}H spectra of 5.95 mM PhIP (B) and 132 mM E2 (C) dissolved in deuterated DMSO (100%) showing the relative position of the NMR peaks for each compound.

Figure 2. Boltzmann probability distributions for each ligand in the E2 binding cavity (A). Cut away view of the ligand-binding cavity of ERα (B) with lowest energy docking poses of E2 (purple) and PhIP (colored-sticks). The surface is colored according to residue type; nonpolar (white), polar uncharged (green). Key hydrogen bonding residues are shown in colored-stick representations (Glu 353, Arg 394, Leu 387, His 524). Figure was made with VMD (Humphrey et al., 1996) and rendered with Raster3d (Merritt and Bacon, 1997).

Figure 3. The expanded region of a 900 ms mixing time NOESY spectrum (A) of 5.95 mM PhIP in DMSO (<2%) exhibits weak positive NOE crosspeaks (boxed in red). The expanded regions of 300 ms mixing time NOESY spectra of ERα with (B) addition of 40 fold molar excess PhIP indicating that PhIP binds, as evidenced by the flip in sign to negative crosspeaks (boxed in black), and (C) after addition of 0.25 molar ratio of E2 to a 1:40 ERα + PhIP sample showing that PhIP does not bind (red peaks) when E2 is present.

Figure 4. Effect of PhIP, N^2-Hydroxy-PhIP, MeIQx, IFP and E2 on MCF-7 cell proliferation. Cells were treated with compounds for 48 hours. Error is presented as standard error of the mean. * = significantly different from control, $p<0.01$. For PhIP treatments only, * = significantly different from control, $p<0.01$ and not significantly different from estradiol treatment at a corresponding molar concentration. X Estradiol ◆ PhIP, ■ N^2-hydroxy-PhIP, ▲ MeIQx and ● IFP.

Figure 5. The effect of heterocyclic amines on estrogen receptor activation. Cells were treated with compounds as described in Methods. Error is presented as standard error of the mean. Compound concentrations are molar and E2 concentration is 10^{-9}M in all experiments. Increasing concentrations of Has (A). N^2-Hydroxy- PhIP, MeIQx and IFP are significantly different from PhIP-treated cells at the same concentrations ($p<0.01$). All compound treatments are significantly different from control and estradiol ($p<0.01$). Competition assays with estradiol (B). Cells were treated with $4x10^{-7}$M compound alone or compound with 10^{-9}M E2 (hatched bars). Effect of combinations of heterocyclic amines on estrogen receptor activation (C). Simultaneous addition of PhIP and N^2-hydroxy- PhIP, or PhIP and MeIQx are significantly different from PhIP alone (*) ($p<0.01$) All compound treatments are significantly different from control and estradiol ($p<0.01$).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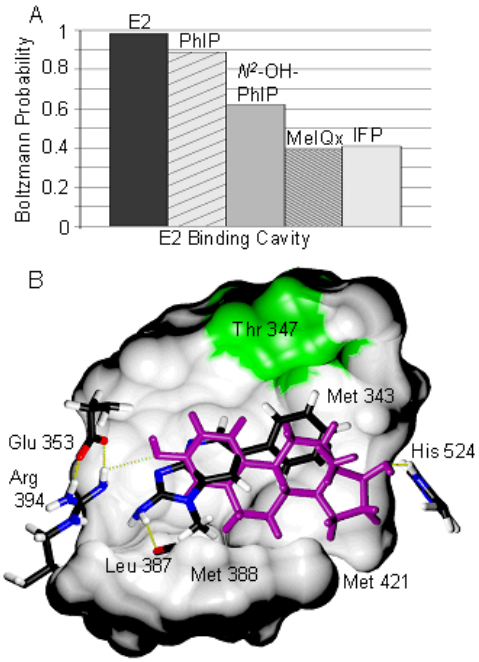
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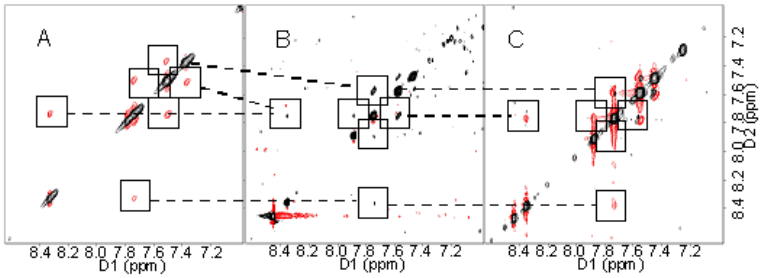
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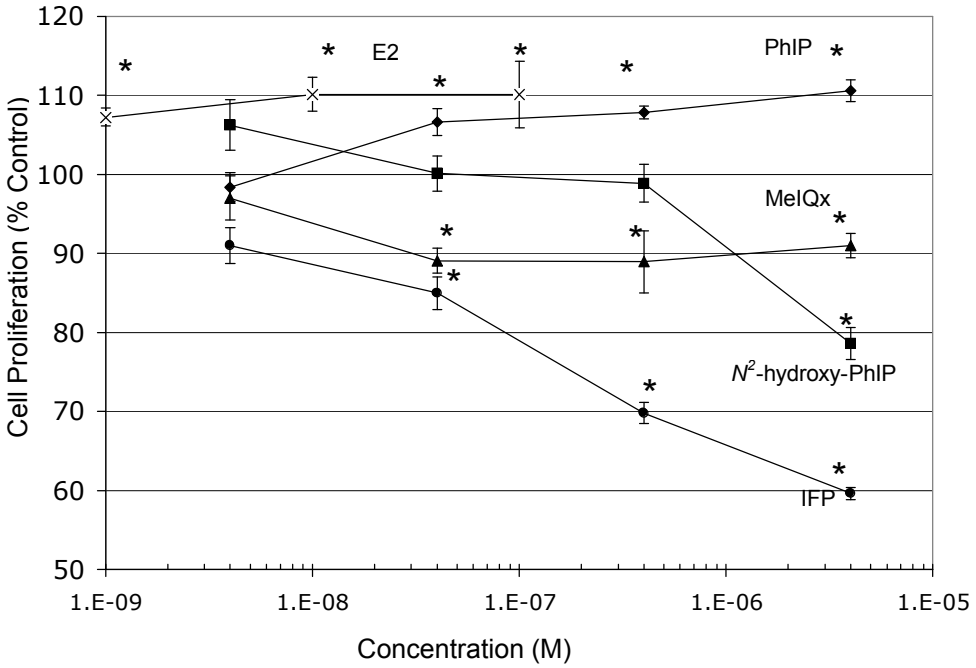
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Cell Proliferation (% Control)
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1.E-05
Concentration (M)
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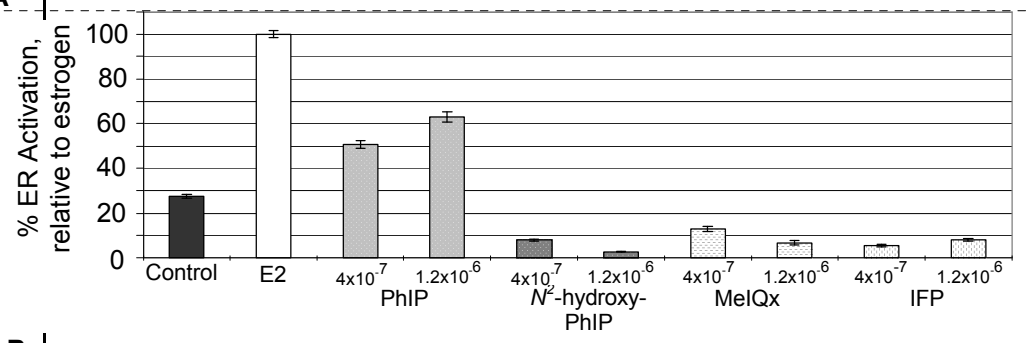
MeIQx
N^2-hydroxy-PhIP
IFP

A



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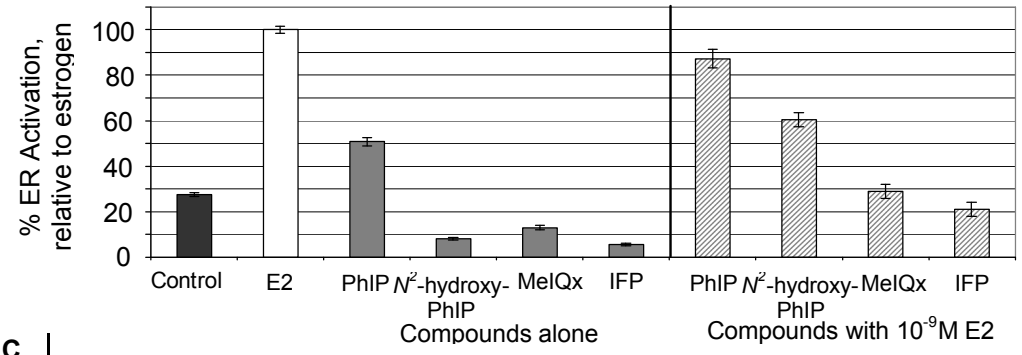

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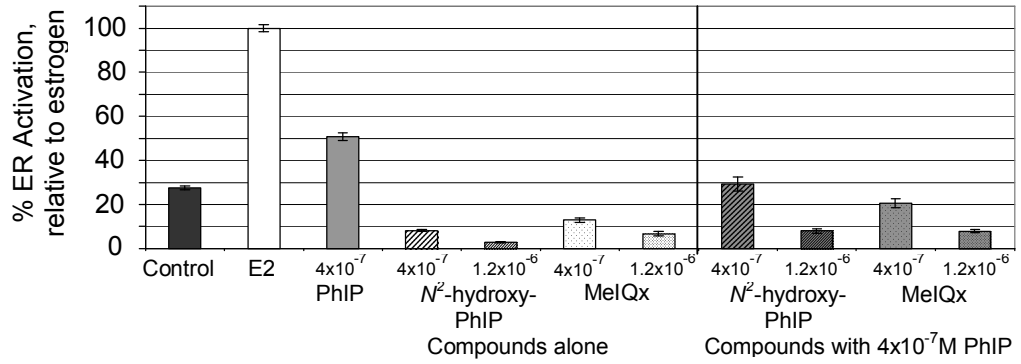

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Appendix B

Statement of Work
Computational Characterization and Prediction of Estrogen Receptor Coactivator Binding Site Inhibitors

Task 1: Computationally predict the relative binding of CBIs in the coactivator site and the CBI binding properties. (Months 1-12)
(1.1) A minimum of three CBIs and a minimum of three estrogenic compounds will be docked into the coactivator site and the estradiol site, respectively, using AutoDock. (Months 1-2)
(1.2) The resulting combination of CBIs and estrogenic compounds will be rank ordered by effective binding. (Months 3-4)
(1.3) From the resulting docked compounds, molecular dynamics (MD) simulations will be run on the energetically favorably bound CBIs to investigate their effects on protein binding and stability. MD simulations will be carried out using NAMD and each simulation will be run for 2 ns. (Months 4-11)
(1.4) The resulting combination of CBIs and estrogenic compounds will be re-ranked based on the MD simulations. (Month 12)
Task 2: Biologically assay the effect of the CBI on the estrogen receptor activation (Months 1-12)
(2.1) Order and obtain the CBIs and estrogenic compounds. (Month 1)
(2.2) Measure estrogen receptor activation by transfecting a reporter plasmid that contains 3 vitellogenin of estrogen response elements upstream of a luciferase reporter gene in MCF-7 human breast cancer cells. Receptor activity will be measured in the presence estradiol and each individual CBI. (Month 2-3)
(2.3) Compare the effect of the CBIs on estradiol to their effect on the other estrogenic compounds analyzed in the docking program. (Months 4-6)
Task 3: Spectroscopically measure the binding of the CBI to the ER LBD.
(3.1) Obtain the compounds used in the biological assay. (Month 1)
Order and obtain a pure sample of the human ERα (Month 1)
(3.2) If the CBIs demonstrate the ability to modulate estrogen receptor activation in the cells assays described in Task 2, they will be assayed for binding to Erα by themselves, using a transferred nuclear Overhauser effect spectroscopy (trNOESY) assay. (Months 4-6)
(3.3) CBIs that bind ER and demonstrate activity in Task 2 will be assayed against estradiol in a competition NMR assay to determine if they bind in the presence of estradiol. If they bind in the presence of estradiol, then the CBIs are binding to another site. (Months 6-8)
(3.4) To rank order the binding of CBIs, CBIs will be added individually to ERα, using an NMR assay (Months 8-10)
Task 4: The results will be put into a draft of a paper to be submitted later (Month 12)